Biologicals xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Biologicals

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

Development of a relative potency test using ELISA for human rabies vaccines

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ARTICLE INFO

Keywords:
ELISA
NIH potency test
Rabies vaccines
Prophylaxis

ABSTRACT

The NIH potency test for human rabies vaccines has disadvantages for use, especially in developing countries where rabies is endemic and prophylaxis needs ample, rapid, and reliable vaccine supplies. In China, 60–75 million doses of human rabies vaccines are administered each year. Vaccine quality control is of paramount importance, as is the release of potency-validated vaccines. We intended to design an alternative to the NIH *in vivo* method, and developed a relative potency test using an ELISA. Using Pearson's correlation analysis, we found a close relationship between the rabies vaccine glycoprotein content *in vitro* and the potency values *in vivo*. We suggest the relative potency test developed here as a simplified method for human rabies vaccine quality control in China and a possible alternative to the NIH method.

1. Introduction

China has the second highest reports of human rabies cases in the world, with an average of over 2000 deaths annually [1]. According to the China National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), 60–75 million doses of human rabies vaccines are administered annually for rabies postexposure prophylaxis. China has the potential to become the world's largest market for human rabies vaccines. It is estimated that the cost of production and administration of human rabies vaccines is more than 10 billion yuan (~1.6 billion US dollars) each year. Following regulations in China, each lot of human rabies vaccine must be tested first by the manufacturer, then by NICPBP, before released for use. Therefore, it is vital to develop a simple, rapid, and reliable method for evaluating the quality of newly produced rabies vaccines.

The NIH test is currently the classical method for potency testing of human rabies vaccines, and often poses difficulty in vaccine quality control. The NIH *in vivo* method has been criticized for its complexity, high data variability, and ethical concerns related to the use of laboratory animals [2–6]. It becomes even more challenging in developing countries where rabies is endemic and the need for proper prophylaxis is critical [7,8]. For both practical and ethical reasons, it would be valuable to have an alternative to the NIH test by developing a rapid and reliable *in vitro* method. One key component in the alternative *in vitro* methods is to measure rabies virus glycoprotein, which is responsible for inducing rabies viral neutralizing antibodies and protection of animals against virus challenge [9]. The World Health Organization (WHO) also endorses the use and validation of *in vitro* testing, especially quantification of glycoprotein content as an alternative for determining adequate rabies vaccine potency [7,8,10,11]. Several *in vitro* assays have been developed in an attempt to quantify the glycoproteins in rabies vaccines. These assays include: the antibody-binding test [12], single radial immunodiffusion (SRD) test [13], and enzyme-linked immunosorbent assay (ELISA) [3,4,12,14,15]. The ELISA appears to be the best quantitative method, and it typically uses specific monoclonal antibodies (mAbs) against rabies virus glycoprotein for capture or detection [2,6,15,16]. The ELISA method is relatively easy to perform, has a short turnaround time, and is capable of quantifying antigens [17]. Previous investigations demonstrated a positive correlation between the ELISA and the NIH test [3–6,15,17–19]. In this study, we described an immunocapture ELISA using specific mAbs against rabies virus nucleoprotein/glycoprotein as captors, or detectors. By comparing the ELISA results of a test vaccine to that of a reference vaccine, we developed a relative potency test, and demonstrated a close correlation between the rabies vaccine glycoprotein content *in vitro* and the potency *in vivo*. The relative potency test is a simplified method,

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https://doi.org/10.1016/j.biologicals.2018.06.003
Received 26 February 2018; Received in revised form 14 June 2018; Accepted 26 June 2018

with the potential to be applied for human rabies vaccine quality control in China.

2. Material and methods

2.1. The vaccine strain

CTN-1 is an early rabies vaccine strain attenuated in mice and developed as a human rabies vaccine in China. The original virus was isolated from a human rabies patient in Shandong in 1956 [20]. During the virus passage and vaccine development, the CTN was renamed by various series numbers. In this study, we described a highly Vero cells adapted CTN-181, also called CTN-1.

2.2. The test samples in the manufacturing process

The test samples were provided by the Rabies Vaccine Department of Wuhan Institute of Biological Products Co. Ltd, Wuhan, China. These samples included uninfected Vero cell supernatants, rabies virus working seed stock, harvested virus, concentrated virus, inactive virus, purified virus, and human rabies vaccines. In brief, rabies virus CTN-1, a vaccine strain, was grown in Vero cells, harvested and clarified by microfiltration, concentrated by ultrafiltration, inactivated using beta-propiolactone, purified by gel filtration chromatography, and lyophilized according to China Pharmacopeia.

2.3. International reference, in-house internal reference rabies vaccines, and test vaccines

The 5th international standard for rabies vaccine was obtained from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, UK). Each ampoule was assigned a unitage of 16 International Units (IU) or 10 ELISA International Units (EU) of rabies virus Pitman-Moore (PM) glycoprotein. An in-house internal reference rabies vaccine was prepared using rabies virus CTN-1 for comparison to the international standard, and used as a control in our ELISA. The 33 test vaccines were provided by the Rabies Vaccine Department of Wuhan Institute of Biological Products Co. Ltd, Wuhan, China.

2.4. Monoclonal antibodies

We used a mixture of five murine mAbs as captors. The murine mAbs $2A_3$, $2D_{10}$ and $5G_7$ were used against rabies virus nucleoprotein, and $4C_1$ and $5C_2$ were against rabies virus glycoprotein [21]. The mAbs $4C_1$ and $5C_2$ are also used as detecting antibodies. The indirect immunofluorescence titers for $4C_1$ and $5C_2$ were 1:21870; and 1:160000 for $2A_3$, $2D_{10}$ and $5G_7$ [22]. The 4C1 and $5C_2$ were characterized to be the IgG2a subclass, and the $2A_3$, $2C_{12}$, $2D_{10}$ and $5G_7$ the IgG1 subclass [22]. Unfortunately, the hybridoma $2C_{12}$ was lost during subcloning, and the other 5 cell strains were cultured for mAb production. The mAbs had specific binding to the CTN, aG, Flury LEP, PM and PV strains [21,23]. In brief, each mAb was purified using ammonium sulfate fractionation, and mixed together at a 1:1 ratio for capturing and detecting the virus. The detecting antibodies, $4C_1$ and $5C_2$, were labeled with horseradish peroxidase (HRP) [21]. All the antibodies were obtained from Wuhan Institute of Biological Products Co. Ltd, Wuhan, China.

2.5. Immunocapture ELISA as a relative potency test

The five capture mAbs were diluted to 1–2 μg/ml in bicarbonate buffer (pH 9.6). Next, 96-well ELISA microplates were coated with 150 μl/well of the capture mAbs dilution (Xiamen YUNPENG Technology development Co. Ltd, China). After coating overnight at 4 °C, the plates were washed three times using PBS-T buffer. (8 mM Na_2HPO_4, 150 mM NaCl, 2 mM KH_2PO_4, 3 mM KCl, 0.05% Tween 20, pH 7.4). The plates were then blocked with 200 μl/well of 1% bovine albumin in PBS-T at 37 °C for 1 h. The plates were further blocked by adding 200 μl/well of 10% sucrose at 37 °C for 20 min. The vaccine samples were reconstituted using sterile water, then diluted at serial two-fold (0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 6.0). Each prepared sample was added at 100 μl per well, and incubated at 37 °C for 1 h. The plate was washed again as described above, and added 100 μl of HRP-conjugated $4C_1$ and $5C_2$ antibodies to each well and incubated for 30 min at 37 °C. The plate was visualized using the chromogen substrate tetramethylbenzidine solution at 50 μl per well (Sigma, St. Louis, MO). The reaction was stopped by adding 50 μl of 0.5 M sulphuric acid solution to each well. The optical densities (OD) were measured at 450 nm wavelength using a microtiter plate reader (BioTek, Winooski, VT).

We measured the glycoprotein content as ELISA units (EU/ml) using a parallel-line model by comparing test samples to the international standard or the in-house internal reference vaccine. Briefly, we identified the 'linear range' in the plotted ELISA and used linear regression to analyze the samples (log of vaccine dilution as predictive variable and log of OD as response/outcome variable).

2.6. Repeatability of using the ELISA for potency assay for human rabies vaccine lots

Our test was assessed by an interlaboratory trial. Six human rabies vaccine lots were elaborately calibrated: high ELISA potency (H1 and H2), medium ELISA potency (M1 and M2) and low ELISA potency (L1 and L2). Each sample was tested 10 times by the same assay in one testing site/laboratory, or in 12 independent assays in 2 sites/laboratories (Lab of Viral Vaccine by one technician and Dept. of Rabies Vaccine in WIBP by three technicians).

2.7. The NIH mouse protection tests

The potency tests were performed as described in China Pharmacopeia. In brief, the vaccines were prepared in triplicate at five-fold serial dilutions (25 ×, 125 × and 625 ×) using PBS buffer (0.01 M, pH 7.4). We used 96 outbred Kunming mice about 4 weeks old, each weighing 12 g–16 g. Mice were randomized into six groups, with 16 mice per group. Each mouse was injected intraperitoneally (ip) with 0.5 ml of the vaccine dilution, and the second inoculation was repeated a week later. Two weeks after the 2nd inoculation, the mice were challenged intracerebrally (ic) using 0.03 ml of CVS-infected mouse brain suspension ($\sim 10^{3-4}$ LD_{50} per ml). All animals were observed twice daily for 14 days. The median effective dose (ED50) for the reference and vaccine samples was calculated, and the retrospective potency of samples was assessed according to the formula: Potency of sample = ED50 of sample × potency reference/ED50 reference.

2.8. Correlation of relative potency by ELISA to the NIH test

The correlation between relative potency by ELISA and the potency with NIH test were analyzed using Pearson's correlation test. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Comparative quantification of rabies virus glycoproteins by ELISA

Using the reference vaccine 5th standard, we investigated an immunocapture ELISA for detecting rabies virus glycoproteins in various preparations, including uninfected Vero cell supernatants, rabies virus working seed stock, rabies virus harvested from cell culture, concentrated virus, inactivated virus in vaccine formulations, purified virus, and lyophilized rabies vaccine. The results in Fig. 1 presented comparable quantification curves for the test samples, which implied the OD value of ELISA reflecting the quantity of rabies virus