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G-protein based ELISA as a potency test for rabies vaccines

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ABSTRACT

The NIH test is currently used to assess the potency of rabies vaccine, a key criterion for vaccine release. This test is based on mice immunization followed by intracerebral viral challenge. As part of global efforts to reduce animal experimentation and in the framework of the development of Sanofi Pasteur next generation, highly-purified vaccine, produced without any material of human or animal origin, we developed an ELISA as an alternative to the NIH test. This ELISA is based on monoclonal antibodies recognizing specifically the native form of the viral G-protein, the major antigen that induces neutralizing antibody response to rabies virus. We show here that our ELISA is able to distinguish between potent and different types of sub-potent vaccine lots. Satisfactory agreement was observed between the ELISA and the NIH test in the determination of the vaccine titer and their capacity to discern conform from non-conform batches. Our ELISA meets the criteria for a stability-indicating assay and has been successfully used to develop the new generation of rabies vaccine candidates. After an EPAA international pre-collaborative study, this ELISA was selected as the assay of choice for the EDQM collaborative study aimed at replacing the rabies vaccine NIH *in vivo* potency test.

1. Introduction

Rabies is a viral disease of the central nervous system caused by rabies viruses that is invariably fatal after clinical signs appear [1]. Rabies disease can be prevented using human vaccines in a pre- and post-exposure context. The rabies vaccine production process includes a potency testing step to ensure that these vaccines have the capacity to induce a protective immune response in vaccinated individuals [2]. Potency testing of inactivated rabies vaccines is traditionally performed using intra-cerebral challenge in mice. The method was developed by the National Institute of Health (NIH) and is currently required for vaccine release [2,3].

The NIH test has a number of limitations such as the use of mice and virulent virus, its long duration and cumbersome nature [4]. During the International Workshop on Alternative Methods for Human and Veterinary Rabies Vaccine Testing in 2011 (Ames, USA), worldwide rabies experts agreed on the need to replace the NIH test by an enzyme-linked immunosorbent assay (ELISA) [5]. The European monograph (Pharmacopoeia, 07/2014) additionally recommends an immunochemical method, such as a Single Radial Immunodiffusion (SRID) assay, or tests based on antibody binding to assess vaccine potency as well as antigen content.

Several ELISAs have been proposed as alternatives to the NIH test, such as a method for rabies G-protein estimation using monoclonal antibodies (mAbs) directed against site III [6,7] or site II [8,9] of this protein. Both sites were identified as important to achieve protection against the virus [10,11]. Assuming that the mAb recognizes a correctly folded G-protein, the G-protein content is indicative of the vaccine potency.

Two rabies vaccines manufactured by Sanofi Pasteur are licensed, the Human Diploid Cell Rabies Vaccine (HDCV or, Imovax rabies™) and the Purified Vero Cell Rabies Vaccine (PVRV or Verorab®). Sanofi Pasteur has improved the current Verorab® vaccine to develop a next generation, highly-purified vaccine, produced without any material of human or animal origin (next-generation purified Vero cell rabies vaccine [PVRV-NG]). During this vaccine optimization production process, we developed and validated an ELISA which can be used as release test and which is described here.

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This ELISA is based on two neutralizing mAbs that target G-proteins sites II and III and has been used to monitor vaccine blending. The ELISA detects specifically the native form of the G-protein and does not respond to unfolded or chemically modified forms, thus discriminating between potent and sub-potent vaccine lots.

2. Materials and methods

2.1. Materials

2.1.1. Monoclonal antibodies

MAb D1-25 was obtained from P. Perrin (Pasteur Institute, France) [12,13] and mAb WI 1112 from the Wistar Institute (also referred to in the literature as 1112 or TJU 1112 from B. Dietzschold, Jefferson University, USA) [14,15]. Biotinylated mAb D1-25 was obtained by coupling biotin *N*-hydroxysuccinimide ester to primary amine groups [16]. Biotinylated mAb was diluted in 50% (v/v) glycerol and stored at −20 °C. Surface Plasmon Resonance (SPR) experiment was performed to verify that biotinylation did not affect virus recognition (Supp. Fig. 1.).

2.1.2. Reference antigen

In-house produced bulk vaccine lots were used as an internal reference, which was titrated against the WHO International Rabies standard (NIBSC ref. 07/162).

2.1.3. Rabies vaccine samples

All vaccine batches used in the study correspond to PVRV-NG batches produced in-house.

2.1.4. Virus strains

Flury LEP, Challenge Virus Standard (CVS11) and Pitman-Moore (PM) lyssavirus strains are viral suspensions from Sanofi Pasteur produced from different cell lines (VERO for Flury LEP, BHK-21 for CVS and MRC-5 for PM).

2.2. Methods

2.2.1. Determination of monoclonal antibody neutralizing activity

Neutralizing rabies-specific mAb titers were determined by the Rapid Focus Fluorescent Inhibition Test (RFFIT) using the Nunc™ Lab-Tek™ II chamber slide™ system (ThermoFischer scientific, ref 154534). Approximately 100 $CCID_{50}$ of challenge rabies virus (Flury LEP, CVS11 and PM) in 100 µl were added to 100 µl of successive dilutions of mAbs and incubated for 90 min in a humidified cell culture incubator (36 °C; 5% CO_2). BHK-21 cells (100 µl; 10^5 cells/ml [9]), were then added to the virus/mAb mixture and incubated for 24 h. Non-neutralized virus was detected by staining BHK-21 cells with FITC anti-rabies monoclonal globulin (Fujirebio Diagnostics Inc., ref. 800-092). Titers were determined by comparing the results obtained for the mAbs with those obtained using the WHO international standard for anti-Rabies Immunoglobulin (NIBSC code: RAI). Neutralizing potency was determined by comparing the 50% neutralizing titer for the mAb to the 50% neutralizing titer of the standard (2 IU/ml).

2.2.2. Generation of modified vaccine batches

2.2.2.1. Reduction/alkylation of inactivated virus. Inactivated virus (vaccine bulk; 300 µg/ml) was dialyzed against buffer A (20 mM Tris, 150 mM NaCl, pH 8.0) using 10 kDa cut-off membranes (Slide-A-Lyzer™ Dialysis Cassettes, ThermoFischer Scientific); dithiothreitol was added to a final concentration of 30 mM. The resulting samples were incubated at 37 °C under stirring for 60 min, then at 4 °C for 5 min. Iodoacetamide was then added to a final concentration of 120 mM. The mixture was kept at room temperature, under stirring and in darkness, for 30 min, then dialyzed exhaustively against buffer A and stored at 4 °C.

2.2.2.2. Excessive inactivation using beta-propiolactone. Bulk samples of non-inactivated virus were inactivated using beta-propiolactone (BPL) at various final concentrations superior to those used in vaccine production, i.e. 1/2000 to 1/500 (v/v) vs 1/4000 (v/v). Samples were incubated at 12 °C for 32 h with the mixtures containing different BPL concentrations. BPL was neutralized by the addition of sodium thiosulfate (80 g/L) and the mixture was incubated at 37 °C for 2.5 h. Samples were then concentrated to 150 µg protein/ml using Amicon® Ultra Centrifugal Filters with a 10 kDa cut-off (Millipore).

2.2.2.3. Heat treatment of inactivated virus. Vaccine doses (lyophilized product) were incubated at 65 °C for 3 days. Product was reconstituted in 0.4% (w/v) sodium chloride solution. A mixture of equal volume of heated and non-heated product was made to generate sub-potent batches.

2.2.2.4. ELISA. Maxisorp ELISA plates (Nunc) were coated with mAb WI 1112 in carbonate–bicarbonate buffer (pH 9.6, 100 µl/well) and incubated at 4 °C for 16 h. Plates were then washed three times with PBS containing 0.05% (v/v) Tween-20 and blocked with 100 µl/well of 1% (w/v) bovine serum albumin (BSA) in PBS at 37 °C for 1 h. Standard antigen or rabies vaccine dilutions (100 µl) were added and plates were incubated at 37 °C for 1 h. Plates were then washed and 100 µl of dilution buffer (PBS, 0.05% (v/v) Tween-20, 0.1% (w/v) BSA) containing biotin-labelled mAb D1-25 were added to each well, followed by incubation at 37 °C for 1 h. Dilution buffer (100 µl) containing streptavidin-peroxidase polymer (Southern Biotechnology Associates) was added to each well and incubated at 37 °C for 1 h. After washing, 100 µl of chromogen substrate solution (4 mg/mL O-phenylenediamine, Sigma–Aldrich) in 0.05 M citrate buffer, pH 5.0, 0.009% (v/v) H_2O_2 was added; plates were incubated in the dark at room temperature for 30 min. The reaction was stopped by adding 50 µl of 2N sulphuric acid. The OD_{492nm} was determined using an ELISA reader (Molecular Devices). The G-protein content, estimated as international units (IU/ml), was calculated by the parallel line method, according to the European Pharmacopoeia [17].

2.2.2.5. ELISA validation. Specificity was assessed using the vaccine final bulk matrix (i.e. not containing antigen) and spiking the antigen of interest. Standard rabies vaccine (WHO International standard, NIBSC code: 07/162) was spiked in matrix. Linearity and accuracy assessment was carried out by triplicate analyses on 5 test formulations containing different quantities of standard rabies vaccine. Repeatability was assessed by conducting sextuple replicates by the same operator on the same day. These 6 independent titrations were repeated 3 times by different operators, on different days to assess intermediate precision.

2.2.2.6. Mouse potency assay. Mouse potency tests were performed according to the European Pharmacopoeia guidelines [3]. The assay was conducted in accordance with the EU Directive 2010/63/EU for animal experiments. The NIH test involved intraperitoneal injection (500 µl) of mice (16 OF-1 mice weighting from 11 to 15 g per dilution) with vaccine dilutions 1/25, 1/125, 1/625 and 1/3125. After immunizations at days 0 and 7, mice were challenged at day 14 with an intracerebral injection (30 µl) of 30-fold the lethal dose of rabies strain CVS11. Mice were observed until day 28 and the number of animals surviving were used to calculate the ED_{50} of the vaccine, which is normalized with the ED_{50} of the reference (internal reference calibrated against WHO International standard NIBSC ref