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A versatile *in vitro* ELISA test for quantification and quality testing of infectious, inactivated and formulated rabies virus used in veterinary monovalent or combination vaccine

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ABSTRACT

Regulatory potency test for rabies vaccines requires mice vaccination followed by challenge with a live virus *via* intracerebral route. An alternative *in vitro* test, consistent with the "3R's" (Reduce, Replace, Refine) was designed to quantify active glycoprotein G using seroneutralizing monoclonal antibodies. This versatile ELISA targets well conformed neutralizing epitopes. Therefore, it quantifies only the trimeric prefusion form of glycoprotein G known to elicits the production of viral neutralizing antibodies. The ELISA makes it possible to quantify the rabies antigen during all steps of the product cycle (*i.e.* viral cultivation, downstream process, formulation and product stability in the presence of aluminum gel or other vaccine valence). Moreover, the batch-to-batch consistency of our active ingredients and formulated products could be demonstrated.

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1. Introduction

Regulatory requirements for rabies vaccines potency testing imply mice vaccination followed by challenge with a live virus *via* intracerebral route [1]. An alternative *in vitro* test, faster, less variable, more consistent and allowing the reduction of lab animal use in compliance with the "3R's" (Reduce, Replace, Refine) should be implemented to replace the *in vivo* potency test [2,3].

Glycoprotein G is the only surface-exposed viral coat protein, responsible for host cell receptor recognition and membrane fusion even if this mechanism is still unclear [4–7]. In its native trimeric form, anchored to the membrane, it elicits the production of viral neutralizing antibodies (VNAb) and induces protection against challenge [8,9]. Post-translational modification, such as acylation and glycosylation occurs in the ectodomain during the maturation phase. Four or five glycosylation sites are present, depending on the rabies strain, and create antigenicity determinants. [10,11]. Glycosylation occurs on asparagin residues at 2 major sites: Asn319 and Asn37. Different glycoforms were determined on the same strain by their ability to migrate in electrophoresis [12]. Deletion of all sequons completely blocks surface expression of G and glycosylation of one sequon affects glycosylation of others [13].

Vaccine formulation is based on the quantification of this antigen using immunochemical tests such as Single Radial Diffusion (SRD) and ELISA for human [14–16] or veterinary products [17,18]. Current tests are not polyvalent as they are not suitable for the studies of different products (human *vs.* veterinary, unformulated *vs.* adjuvanted). Since the trimeric native form of glycoprotein G is the main rabies protein and the target of neutralizing antibodies, we have deeply characterized several monoclonal antibodies and performed a selection regarding their biochemical profiles and seroneutralizing index in order to develop an ELISA able to quantify only this form of glycoprotein G. This ELISA was shown to work at different steps of the manufacturing process, including the live or inactivated virus and the formulated antigen in the final product. Due to the versatility and reproducibility of this ELISA, it could be used to follow the batch-to-batch consistency between the various steps of the manufacturing process.

2. Materials and methods

2.1. Rabies active ingredients and vaccines

The Pitman-Moore (PM) rabies virus strain is a vaccine strain obtained from the Wistar Institute-of Philadelphia. The virus was

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grown on NIL2 hamster cells, inactivated with betapropiolactone, treated (active ingredient) and formulated with aluminum hydroxide (vaccine) according to standard industrial processes.

Batch 4 of the Biological Reference Preparation (BRP4) was purchased from EDQM (Strasbourg, France) [19].

2.2. Preparation and selection of monoclonal antibodies

The monoclonal antibodies were obtained as described by Lafon [20] after immunization of Balb/c mice (IFFA-CREDO) with solubilized inactivated rabies viral proteins. Specific antibody secreting hybridomas were first selected by indirect ELISA using inactivated rabies as antigen and specificity was confirmed by indirect immunofluorescence (IFI) on BHK21 cells infected with PM rabies strain or recombinant vaccinia (Rhône–Mérieux–Wistar Institute–Transgène SA) expressing Rabies glycoprotein G. Specific monoclonal antibodies were further characterized by studying their reactivity in western-blot against aliquots of rabies inactivated virus subjected to SDS-PAGE in absence or presence of β – mercaptoethanol as previously described [20]. The monoclonal antibodies (MAbs) were purified on a protein A chromatography column and were conjugated to horseradish peroxidase using sodium periodate according to methods previously described [21].

3. Deep characterization of the monoclonal antibodies

3.1. SPR (Surface plasma resonance) assays

Trials were performed on a BIAcore T200 (GE Healthcare Bio-Sciences AB, Sweden) using kits and buffers provided by the supplier according to the manufacturer's instructions. CM3 chip was immobilized with a rabbit anti-mouse monoclonal antibody. MAbs on test were diluted in running buffer and injected on the chip. Equivalent loading was checked for each mouse anti-rabies monoclonal antibody. Thus, the antigens were sequentially injected on the chip and the resonance unit (RU) obtained with each pair of anti-rabies antibody and antigen was monitored.

3.2. Seroneutralizing assays

Mabs were tested using an adapted Fluorescent Antibody Virus Neutralization (FAVN) method [22]. The second International Standard for Rabies Immunoglobulin, with a potency of 30 IU per ampoule is used as standard [23] and SN index are expressed in IU/ml.

3.3. ELISA titration method

120 μl of a solution of monoclonal antibodies at 2 μg/ml in carbonate buffer pH 9.6 was coated overnight at 4 °C and washed. After saturation with skim milk 5% in PBS pH 7.2, serial dilutions of antigen in TEB pH 7.2 were applied over 3 h at 37 °C. After washing, 100 μl of HRP labelled antibody were applied over 1 h at 37 °C and plates were revealed using TMB (Synbiotics, France) substrate for 30 min at 20 °C. The reaction was stopped by the addition of concentrated sulphuric acid and plates were read at 630/450 nm. Titres were expressed as log 10 per milliliter of the dilution giving 50% of the Maximum Optical Density ($\log_{10}OD_{50}$/ml). Each plate ELISA is validated by the measure of $\log_{10}$ OD_{50} titer and slope calculation of a control antigen followed by a control chart.

3.4. Buffer and pH adjustments

Inactivated antigens were diluted in TEB buffer and adjusted to target pH using NaOH 1 M or HCl 1 M. After incubation overnight at 4 °C, pH was checked and antigens were analyzed by ELISA or SPR. Remaining antigens were readjusted to pH 7.5 in order to study the reversibility of structural change.

3.5. Thermal degradation of antigens

Inactivated antigens were incubated at 60 °C or at 37 °C and samples were taken at 15 min; 30 min; 1H and 3H for analysis.

3.6. Deglycosylation assays

An EDEGLY kit (Sigma-Aldrich, Missouri, ASU) was used according to the manufacturer's instructions. Enzymes were used alone or mixed in order to obtain partial or complete deglycosylation. Enzymatic treatment efficiency was checked by SDS-page.

3.7. Glycoprotein G solubilization

Sucrose cushion purified inactivated antigen was diluted in PBS containing the non-ionic detergent mulgofen BC270 (GAF Corp., USA) at a final concentration of 2%. The solution was incubated for 2 h at 20 °C under agitation. Solubilized proteins were purified from nucleocapsids by ultracentrifugation for 2 h at 175,000 g.

3.8. Soluble glycoprotein G production

pFR266 was designed in order to remove the trans-membrane domain of glycoprotein G. Synthetic plasmid was ordered from Geneart (Life Technology). Briefly, 2×10^5 CHO cells cultivated in MEM glutamax + 2 mM proline were transfected with 12 μg/ml of plasmid pFR266 and 12 μg/ml of lipofectamine (Sigma Aldrich). DNA complexing was carried out over 20 min at room temperature. After 3 days of cultivation at 37 °C, cells and supernatant were harvested. A negative control was carried out using a plasmid without the transgene. Samples were stored at 4 °C before analysis.

4. Statistical analysis

Linearity study: a Cochran's test and a Bartlett's test were applied to check the homogeneity of the variances of ELISA titers at each tested dilution. A linear regression between the observed titers and the logarithmic values of the dilution factors was carried out and the lack of fit test was used to check the linearity of the technique.

Precision study: Cochran's test and Bartlett's test were applied to check the homogeneity of the variances of ELISA titers at each group of titers (one group= one session, one operator). Session and operator effects were estimated using analysis of variance. Standard deviations of repeatability and intermediate precision were calculated.

5. Results

5.1. Antibody selection and ELISA feasibility

The first selection of hybridomas of interest among 66 different clones was performed to determine their ability to specifically recognize rabies antigen by IFI using recombinant vaccinia expressing glycoprotein G as the sole rabies component and confirmed on cell cultures infected with PM rabies virus strain. Two monoclonal antibodies (RAG216A5F and RAG218D11H), recognizing infectious and inactivated virus, were selected for their neutralizing capacity and their ability to bind conformational epitopes for ELISA feasibility. When the antigen was completely linearized (western-blot in reducing conditions), monoclonal antibodies were unable to bind glycoprotein G, but in non-reducing condition, glycoprotein G doublet (62 kDa and 67 kDa) was detected, suggesting recognition of

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