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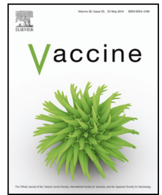

An inactivated yellow fever 17DD vaccine cultivated in Vero cell cultures

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ABSTRACT

Yellow fever is an acute infectious disease caused by prototype virus of the genus *Flavivirus*. It is endemic in Africa and South America where it represents a serious public health problem causing epidemics of hemorrhagic fever with mortality rates ranging from 20% to 50%. There is no available antiviral therapy and vaccination is the primary method of disease control. Although the attenuated vaccines for yellow fever show safety and efficacy it became necessary to develop a new yellow fever vaccine due to the occurrence of rare serious adverse events, which include visceral and neurotropic diseases. The new inactivated vaccine should be safer and effective as the existing attenuated one. In the present study, the immunogenicity of an inactivated 17DD vaccine in C57BL/6 mice was evaluated. The yellow fever virus was produced by cultivation of Vero cells in bioreactors, inactivated with β-propiolactone, and adsorbed to aluminum hydroxide (alum). Mice were inoculated with inactivated 17DD vaccine containing alum adjuvant and followed by intracerebral challenge with 17DD virus. The results showed that animals receiving 3 doses of the inactivated vaccine (2 μg/dose) with alum adjuvant had neutralizing antibody titers above the cut-off of $PRNT_{50}$ (Plaque Reduction Neutralization Test). In addition, animals immunized with inactivated vaccine showed survival rate of 100% after the challenge as well as animals immunized with commercial attenuated 17DD vaccine.

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1. Introduction

Yellow fever is a non-contagious acute infectious disease caused by the yellow fever virus, genus *Flavivirus*, family *Flaviviridae* [1,2]. It is a serious public health problem in the Americas [3] with clinical symptoms that may vary from mild to severe disease characterized by sudden onset of fever and jaundice. The most severe forms also include hepatorenal syndrome, bleeding, shock and eventually evolve to death [2,4,5]. Approximately 15% of infected people develop moderate or severe clinical disease [6]. There is no specific treatment and vaccination is the most effective method of preventing yellow fever [7,8].

The attenuated vaccine was developed in 1936 by Theiler & Smith by attenuating yellow fever virus Asibi strain through serial passages in tissue culture and animals, originating the 17D attenuated vaccine. Nowadays, there are two licensed vaccines available in the international market, the first vaccine uses the 17D-214 strain and the second uses the 17DD strain [9]. Bio-Manguinhos has been producing yellow fever attenuated 17DD vaccine for more than 6 decades being the world largest producer. The attenuated vaccine is administered in a single subcutaneous dose and confers immunity for at least 10 years [10].

Despite the good records of efficacy and safety, rare cases of serious adverse events (SAEs) have been reported in every yellow fever attenuated strains employed in vaccine production worldwide. The SAEs include the yellow fever vaccine-associated viscerotropic disease (YEL-AVD) and the yellow fever vaccine-associated neurotropic disease (YEL-AND). The YEL-AVD is a fulminant infection in the liver and other vital organs with 60% of case fatality rate. The YEL-AND is caused by the vaccine virus neuroinvasion and has 1–2% of case fatality rate [8,9,11].

Aiming to produce a vaccine with an improved safety profile, Bio-Manguinhos is developing an inactivated vaccine for yellow fever from the 17DD virus strain. The aim of this work was to evaluate the humoral immune response of an inactivated yellow fever 17DD vaccine in C57BL/6 mice.

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2. Materials and methods

2.1. 17DD virus production in bioreactor

Yellow fever 17DD virus derived from the vaccine batch 035VFA035P, Fiocruz, Brazil, was produced through cultivation in Vero (African green monkey kidney) cells in a 3 L bioreactor Bioflo model 110 (New Brunswick Scientific, USA) operated in batch mode using a commercial serum-free medium VP-SFM (Gibco, USA) and 3 g/L Cytodex microcarriers (GE Healthcare, USA).

2.2. Virus clarification and purification

Clarification was performed by filtration through 8 μm, 3–0.8 μm and 0.45–0.22 μm (Sartorius, USA) series of cellulose acetate membranes. Virus purification was performed by ion exchange chromatography (Äkta Purifier, Amersham Bioscience) yielding a purified batch named VINFLAP001/2010. Under operating conditions, the purifications steps are quite efficient, showing high product recovery and efficient DNA clearance as described before [12]. The level of host cell protein (HCP) was considered high indicating the need for targeting HCP in a subsequent step. Quantification of total proteins was carried out using the BCA protein assay kit (Pierce®) according to manufacturer's instructions.

2.3. Virus inactivation

For viral inactivation the batch was previously filtered through a 0.22 μm membrane and the pH was adjusted to 8.5 using 1 N NaOH. The β-propiolactone inactivating agent, 1:3000 dilution (Natalex, 97% purity), was added to the suspension, followed by incubation at 4 °C under constant agitation of 100 rpm for 24 h. For hydrolysis of the β-propiolactone after the inactivation procedure, the suspension was maintained at 37 °C for 2 h. The inactivation process resulted in the batch, purified and inactivated VINFLAPI001/2010 virus sample which was used in the immunogenicity assay. Kinetics of yellow fever 17DD virus inactivation process in the range of 10 to 200 mL show that after 4 h of virus incubation with β-propiolactone no infectious particle is detected (data not shown).

2.4. Testing of residual live virus

Virus inactivation was confirmed through different methodologies: virus titration (plaque assay), immunofocus and passages in cell culture (cytopathic effect analysis). The sample was considered inactivated only when approved in the three trials. The same reference virus titer 8.46 Log_{10} PFU/mL was used as positive control in all inactivation tests.

2.4.1. Virus titration

To perform a plaque assay, ten-fold dilutions of the inactivated viral suspension were prepared, and 200 μL aliquots were inoculated onto Vero susceptible cell monolayers (6 well plates) with a density of 1.0×10^5 cells/cm^2, in a total of 18 replicates. After 7 days of incubation (37 °C/5% CO_2), the monolayer was observed for the presence or absence of lysis plaques.

2.4.2. Immunofocus

In order to perform the immunofocus assay, Vero cell monolayers with 1.0×10^5 cells/cm^2 density were prepared 24 h in advance in 6 well plates. Each well plate was inoculated with 200 μL of viral suspension, accounting a total of 12 replicates. After 7 days of incubation (37 °C/5% CO_2) revelation was performed using a specific monoclonal antibody for yellow fever (2D12).

2.4.3. Serial passage in cell culture

In the cell-based assay, 5 mL of the inactivated viral suspension were initially inoculated into five 175 cm^2 cell culture flasks containing 1.0×10^5 cells/cm^2 each. The flasks were incubated at 37 °C/5% CO_2 and the cell monolayer was assessed daily using an inverted microscope to verify the occurrence of cytopathic effect (CPE). Every 7 days, 5 mL of the viral supernatant was removed from each bottle and inoculated in new flasks. Three propagations into cell cultures were performed. Thereby, the suspension was evaluated for a total period of 21 days, the absence of CPEs during this period confirmed that the viral inactivation had been successful.

2.5. Formulation of inactivated vaccine with alum adjuvant

For the immunogenicity assay, the viral batch was formulated with 0.2% aluminum hydroxide (Alhydrogel; Brenntag, EUA) on the day prior to immunization. Samples containing 17DD virus in the absence of adjuvant and a vial containing only adjuvant in HEPES buffer were used as controls. Samples were left for 2 h in constant agitation at 4 °C, after this period 500 μL were collected from each vial for process control by enzyme immunoassay (ELISA). After agitation vials were kept at 4 °C until use in immunogenicity assay. Sample was immediately storage under −70 °C after harvest, clarification, purification by ion exchange chromatography and inactivation by β-propiolactone. Under these operating conditions, no modification on virus stability was observed.

2.6. Animals

C57BL/6 mice (female, 4 weeks old) were provided by the Laboratory Animals Breeding Center (CECAL-FIOCRUZ), the supply was carried out using a protocol approved by the Institutional Committee of Animal Care and Experimentation (CEUA-FIOCRUZ: LW-28/11).

2.7. Mouse immunization studies

For humoral immune response and challenge study, a total of one hundred forty four mice (9 groups, 16 animals each) were inoculated as follows; groups 1 and 2 received three doses of 100 μL of HEPES buffer and alum respectively. Groups 3–8 were immunized using 100 μL of purified inactivated vaccine (batch VINFLAPI001/2010) in the presence or absence of alum adjuvant at a concentration of 2 μg/dose. A positive control group (group 9) was immunized with a single dose of 2.33 Log_{10} PFU/100 μL of the commercial live attenuated yellow fever 17DD vaccine (Bio-Manguinhos-FIOCRUZ, Brazil) at day 0. Immunizations were performed at days 0, 14 and 28 by the subcutaneous route.

2.7.1. Neutralization assay

Serum samples were collected before the first immunization (pre-immune sera) and on 12, 26 and 40 days post infection (DPI) to analyze the humoral response. Initially, samples were treated for 30 min at 56 °C, subsequently antibody titers were determined by 50% PRNT50 on Vero cells, considering the cut-off of 794 mIU/mL or 2.9 Log_{10} mIU/mL as described by Simões. $PRNT_{50}$ was conducted in serial four-fold dilutions starting at 1:16 in 6 well tissue culture plates, as described elsewhere [13]. Seroconversion was considered by a four-fold increase in serum neutralizing antibody titers.

2.8. Protection against lethal challenge

Lethal challenge by intracerebral injection occurred at 42 DPI. Fourteen days after the last immunization with purified inactivated vaccine, one hundred eight animals (4 animals in each group

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