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Innovative *in cellulo* method as an alternative to *in vivo* neurovirulence test for the characterization and quality control of human live Yellow Fever virus vaccines: A pilot study

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ABSTRACT

Live attenuated vaccines have proved to be mostly valuable in the prevention of infectious diseases in humans, especially in developing countries. The safety and potency of vaccine, and the consistency of vaccine batch-to-batch manufacturing, must be proven before being administrated to humans. For now, the tests used to control vaccine safety largely involve animal testing. For live viral vaccines, regulations require suppliers to demonstrate the absence of neurovirulence in animals, principally in non-human primates and mice. In a search to reduce the use of animals and embracing the 3Rs principles (Replacement, Reduction, Refinement in the use of laboratory animals), we developed a new Blood-Brain Barrier Minibrain (BBB-Minibrain) *in cellulo* device to evaluate the neuroinvasiveness/neurovirulence of live Yellow Fever virus (YFV) vaccines. A pilot study was performed using the features of two distinct YFV strains, with the ultimate goal of proposing a companion test to characterize YFV neurovirulence. Here, we demonstrate that the BBB-Minibrain model is a promising alternative to consider for future replacement of YFV vaccine *in vivo* neurovirulence testing (see graphical abstract).

1. Introduction

Vaccination is one of the most cost-effective health investments, with proven strategies to protect from infectious diseases and increase the live expectancy of humans. Vaccines are sometimes prepared using live non-virulent viruses [1]. Their efficacy and the need for limited injections compared to inactivated vaccines make them useful tools. Before becoming licensed for human immunization, the seeds of live viral vaccines, such as those of Yellow Fever virus (YFV), Measles or Poliovirus need to be tested for efficacy and safety, including monitoring for the absence of neurovirulence [2,3]. Currently, safety testing is mainly performed in animal models [4,5]. There are a number of reasons to seek alternatives to this, namely; the cost of animal studies, the difficulties associated with measuring pathology in animal brains, and the ethical questions regarding the use of animals in experimentation, particularly non-human primates. All these reasons are strong drivers for the development of alternative methods. The 3Rs approach (Replacement, Reduction, Refinement of the use of animals in scientific research) is clearly a priority for health authorities globally, and poses a challenge for scientists to develop alternative *in cellulo* tests (i.e. a device used to conduct experiments with cells outside the organism (animal) and not in glassware) [6,7] that obviate the need for animal experimentation [8–12]. Cellular tests, which could amplify and detect the neurovirulence phenotypic traits of live vaccines, would be highly desirable. Virus-mediated neurological disease entails quite complex polygenic traits, requiring the acquisition of neuroinvasiveness (entry into the central nervous system [CNS]), by passage for instance through the blood-brain barrier (BBB), neurotropism (the capacity to infect neural cells and propagate in the nervous system), and virus-induced neuronal homeostasis perturbation (neurovirulence) [13,14]. The growth, within the CNS, of a pathogenic viral population could be the result of it acquiring neuropathogenic properties that originate from fixation of a few genomic mutations [15]. Moreover, neurological disorders (i.e. neuroinflammation, neurotoxicity, perturbation of neuronal homeostasis) result in the modulation of distinct clusters of cellular genes, activated by neuropathogenic viruses and not by their non-

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neurovirulent counterparts [16,17].

Cerebral endothelial cells, forming tight and adherent junctions, constitute the main anatomical basis of the BBB. There is a dynamic interaction between the brain endothelium, other neighboring cells (such as astrocytes, pericytes, perivascular microglia) and neurons which are pivotal to maintaining the integrity of the BBB. The three major functions of the BBB are the creation and maintenance of ionic homeostasis for neural functions, the supply of the CNS with nutrients, and protection from exogenous toxic injuries including those inflicted by pathogens [18,19]. Some neurotropic viruses (e.g. HTLV, AAV9, HIV, WNV) have acquired neuroinvasive capacity, that is to directly or indirectly cross the BBB [20,21].

Our objective was to design an *in cellulo* test capable of monitoring the absence of neurovirulence of a live YFV vaccine batch. Such a test must be highly sensitive and capable of firstly, revealing rare viral neuroinvasive/neurovirulent emerging events that enable the virus to enter the brain through the BBB; secondly, amplify the mutant population in a model, mimicking brain parenchyma, after its passage through the BBB; and finally, be able to detect specific features of the neuroinvasive/neurovirulent mutants.

We recently designed and developed a BBB-Minibrain culture device (da Costa et al., in preparation), which appears potentially suitable for such a test. The device consists of a *in cellulo* model of BBB formed by endothelial cell (hCMEC/D3)-coated Transwells® porous filters and cell culture wells, containing a mixed culture of human brain cells (neurons, astrocytes and microglia) called Minibrain (Fig. 1A). The Minibrain triculture system thus mimics a simplified cerebral parenchymal environment, with the exception of oligodendrocytes. We anticipate this device responding to all three requirements; selecting and amplifying rare variants exhibiting neuroinvasive/neuropathogenic properties, and detecting homeostasis perturbations induced by the neuroinvasive/neurovirulent mutants in the sub-compartment containing the Minibrain.

We assessed the BBB-Minibrain culture device as an *in cellulo* test, to isolate and amplify rare neuroinvasive/neurovirulent variants potentially present in YFV strains. Two YF live vaccine strains were included in this study: 17D strain and the French Neurotropic Virus (FNV) strain. They were obtained by passaging with Asibi and French Viscerotropic Virus (FVV) strains, respectively, in the mid-1930s [22–24]. The 17D vaccine is a safe, and efficacious, live-attenuated vaccine still in use today [24]. FNV, although slightly more efficacious than 17D, was found to cause post-vaccination neuropathogenesis in children (0.3–0.4%), and thus was discontinued in 1980 [22,23]. We chose this pair of YFV vaccine strains for our test: the FNV strain as a prototype of neuroinvasive/neurovirulent YF viruses, and 17D strain as a prototype of non-neuroinvasive/neurovirulent virus [25]. We compared the capacity of FNV and 17D viruses to cross the BBB, in the BBB-Minibrain model, we assessed their ability to infect the Minibrain, multiply in it, and perturbate its homeostasis. Our results demonstrate that it is feasible to detect neurovirulent variants, reliably and timely *in cellulo* with the BBB-minibrain. This system represents a promising development, and could ultimately enable the replacement of animals for neurovirulence testing of vaccine from which live virions can cross the BBB.

2. Material and methods

2.1. Cells

hCMEC/D3 cells are human cerebral microvessel endothelial cells [26]. The cells were purchased from Tebu-Bio (France). They were maintained at 37 °C on rat collagen (0.1 mg/ml in water, Cultrex, 3443-100-01, R&D Systems, U.K.) in EndoGro medium (Merck Millipore, SCME004, France) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin-streptavidin (Thermofisher, 15140, France).

For experiments, 5.10^4 or 2.10^5 hCMEC/D3 cells were seeded onto collagen-coated cell culture Transwell® inserts, 12- or 6-well plate inserts, respectively (Corning, 3460; Corning, 3450, USA) and grown for 6 days. Under these conditions, hCMEC/D3 cells formed a confluent monolayer after 3 days and formed a barrier, with tight junctions, after 6 days [26].

NT2-N/A, a mixed culture of neurons and astrocytes [17,27], was differentiated from Ntera-2clD/1 cells (ATCC CRL1973), as described elsewhere [28,29]. A tri-culture was obtained by adding human CHME microglial cells [30] at a 1:10 ratio. Phase-contrast microscopy pictures of Fig. S1A show Ntera-2clD/1 (upper panel), NT2-N/A, mixed culture of neurons and astrocytes (middle panel) and CHME, microglial cells (bottom panel). Of note, the CHME we used were phenotyped to prove that they were of human origin (*Homo sapiens* CCNT1 phenotyping) and not of rat (*Rattus norvegicus*) origin as claimed by Garcia-Mesa Y. et al. [31]. This tri-culture, called Minibrain, was maintained at 37 °C in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM–F12) (Thermofisher, 31330, France) supplemented with 5% FBS (EuroBio, CVFSVF01-01, France), 1% L-glutamine (Thermofisher, 25030, France) and 1% penicillin-streptavidin (Thermofisher, 15140, France).

African green monkey kidney cells Vero (ATCC CRL-1586, positive control cell line for Yellow Fever viruses) were maintained at 37 °C in DMEM supplemented with 5% FBS and 1% penicillin-streptavidin.

2.2. hCMEC/D3-Minibrain co-culture

For the co-culture experiments between hCMEC/D3 on inserts, and Minibrain in the cell culture well, the hCMEC/D3 and Minibrain cells were seeded the same day, on inserts or wells respectively, in EndoGro medium supplemented with 5% FBS and 1% penicillin-streptavidin. Each day, during the 6 days of culture needed by the hCMEC/D3, inserts containing hCMEC/D3 cells were placed in wells containing Minibrain cells. On the sixth day, the paracellular permeability of hCMEC/D3 was tested for each condition by monitoring the permeability to lucifer yellow (LY).

2.3. Assessment of the restrictive paracellular permeability with LY

The restrictive paracellular permeability of hCMEC/D3 was assessed by their low permeability to the non-permanent fluorescent LY (Sigma Aldrich, L0259, USA).

Briefly, for 12-well inserts, hCMEC/D3 monolayers cultivated for 6–7 days on inserts, were transferred to 12-well plates containing 1.5 mL of transport medium [(HBSS, Thermofisher, 14025-100, France) supplemented by 10 mM of hepes (Thermofisher, 15630-080, France) and 1 mM of sodium pyruvate (Thermofisher, 11360)] per well (abluminal compartment). Transport medium (0.5 mL) containing 50 µM of LY was then added to the luminal compartment. Incubations were performed at 37 °C, 5% CO_2, 95% humidity. After 10, 25 and 45 min, the inserts were transferred into new wells, previously filled with 1.5 mL of transport medium. After 45 min, aliquots were taken for each time point, from both compartments and the concentration of LY determined using a fluorescence spectrophotometer (Tecan Infinite F500, USA). For 6-well inserts, the same method was used with 1.5 mL in the luminal compartment and 2.5 mL in the abluminal compartment.

The endothelial permeability coefficient (P_e) of LY was calculated in centimeters/min (cm/min), as described by Siflinger-Birnboim et al. [32]. To obtain a concentration-independent transport parameter, the clearance principle was used. Briefly, the average volume cleared was plotted versus time, and the slope was estimated by linear regression. Both insert permeability (PS_f, for insert only coated with collagen) and insert plus endothelial cell permeability (PS_t, for insert with collagen and cells) were taken into consideration, according to the following formula: $1/PS_e = 1/PS_t - 1/PS_f$. The permeability value for the endothelial monolayer was then divided by the surface area of the porous membrane of the insert to obtain the endothelial permeability coefficient (P_e) of the molecule (in cm/min^{-1}). As a quality control of our