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Evaluation of the bioactivity of influenza vaccine strains *in vitro* suggests that the introduction of new strains in the 2010 Southern Hemisphere Trivalent Influenza Vaccine is associated with adverse events

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ABSTRACT

In Australia, during the 2010 Southern Hemisphere (SH) influenza season, there was an unexpected increase in post-marketing adverse event reports of febrile seizures (FS) in children under 5 years of age shortly after vaccination with the CSL trivalent influenza vaccine (CSL 2010 SH TIV) compared to previous CSL TIVs and other licensed 2010 SH TIVs. The present study describes the outcomes of a series of *in vitro* experiments directed at elucidating the root cause. The scientific investigations found that a subset of paediatric donors displayed elevated cytokine/chemokine responses to the CSL 2010 SH TIV but not to previous CSL TIVs nor other 2010 SH TIVs. The induction of elevated cytokines/chemokines in paediatric whole blood correlated with elevated NF-κB activation in a HEK293 cell reporter assay. The data indicate that the introduction of the B/Brisbane/60/2008 strain within the CSL manufacturing process (such as occurred in the preceding 2009/10 NH season) appears to have raised the pyrogenic potential of the CSL 2009/10 NH TIV but that this was insufficient to elicit FS in children <5 years. The 2010 SH season coincided with the first introduction of the H1N1 A/California/07/2009 in combination with the B/Brisbane/60/2008 strain. Our data demonstrates that the introduction of the H1N1 A/California/07/2009 (and to a much lesser degree, H3N2 A/Wisconsin/15/2009) in combination with B/Brisbane/60/2008 (as expressed through the CSL method of manufacture) combined and likely compounded the bioactivity of the CSL 2010 SH TIV. This was associated with stronger immune responses, which in a proportion of children <5 years were associated with FS. The assays and systems developed during these investigations should greatly assist in determining the bioactivity of new influenza strains, and thus aid with the manufacture of CSL TIVs indicated for use in the paediatric population.

Abbreviations: APC, antigen presenting cell; BPL, beta-propiolactone; CI, confidence interval; DS, double stranded; GMC, geometric mean concentration; HA, haemagglutinin; HEK, human embryonic kidney; HI, haemagglutination inhibition assay; MPH, monovalent pooled harvest; NH, Northern Hemisphere; NP, nucleoprotein; PAMP, pathogen associated molecular patterns; PKR, protein kinase R; PRR, pattern recognition receptor; RNA, ribonucleic acid; RT, room temperature; SEAP, secretory alkaline phosphatase; SH, Southern Hemisphere; SS, single stranded; TDOC, sodium taurodeoxycholate; TGA, Therapeutic Goods Administration; TIV, trivalent influenza vaccine; TLR, toll-like receptor; WA, Western Australia; WHO, World Health Organization.

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1. Introduction

In 2010, the World Health Organisation (WHO) recommended three new strains for inclusion in the 2010 influenza vaccine intended for use in the Southern Hemisphere (SH) season: A/California/07/2009 (H1N1), A/Wisconsin/15/2009 (H3N2) and B/Brisbane/60/2008 (B-strain) [1]. All TIVs, irrespective of manufacturer, contain similar amounts of antigen based on haemaggutinin (HA) content (7.5 μg per strain per dose for children 6 months–3 years and 15 μg per strain per dose for children above three years and adults). Methods of manufacture for all registered inactivated influenza vaccines are unique to each manufacturer [2].

In the 2010 SH season, CSL's 2010 SH TIV (CSL 2010 SH TIV), also known as Fluvax® 2010 SH, was associated with an unexpected increase in post-marketing reports of fever and febrile convulsions, compared to previous seasons, predominantly in children <5 years of age. The adverse events were first identified in April 2010 in the state of Western Australia during the third year of a government sponsored paediatric influenza vaccination programme [3,4]. The rate of febrile seizures (FS) was estimated to be as low as 3.3/1000 [3] and as high as 5–7/1000 [4], with the majority occurring in healthy children (median age: 1.5 years) within 12 h of vaccination [5]. As would be expected with the age-related nature of FS, FS were not evident in children over 5 years of age or in adults following vaccination with the CSL 2010 SH TIV. In contrast, only one FS which was temporally associated with a TIV was reported in Western Australia between 2008 and 2009, where cumulatively 37,946 children (26,037 in 2008 and 11,909 in 2009) were vaccinated [3]. Based on all the available post-marketing surveillance and clinical study data prior to the 2010 SH season, one could not have predicted the increase in reports of FS in children <5 years that were associated with the CSL 2010 SH TIV. CSL commenced a thorough and systematic investigation to determine the root cause [6,7]. The investigation had three discrete, but interlinking components: (1) Clinical Safety Investigation to characterise the adverse event, identify risk factors and at-risk populations; (2) Manufacturing and Quality impact assessment of Safety, Quality, Identity, Purity and Potency to review all process parameters and process controls which could have contributed to the adverse event; (3) A Scientific Program to identify the molecular mechanism and assess the feasibility for *in vitro* or *in vivo* predictive tests to assist in future vaccine production. A comprehensive review of all aspects of the CSL manufacturing process and quality attributes did not identify any changes or deviations from the previous seasons formulations that could explain the increase in reports of FS associated with the CSL 2010 SH TIV [6]. Furthermore, there was no change in the level of the splitting agent (TDOC) used to prepare the CSL TIVs between the 2009 and 2010 SH seasons [6].

Scientific investigations included exploration of several *in vivo* animal models, including non-human primates, ferrets, rabbits and newborn rats as potential models of fever and/or FS [6,7]. None of the TIVs tested, including the CSL 2010 SH TIV, induced symptoms consistent with FS in any of the *in vivo* models examined. An alternative approach was to utilise cytokine/chemokine release from paediatric whole blood as well as a NF-κB HEK293 reporter assay as *in vitro* surrogate assays to investigate and identify the root cause of the increased fever and FS observed in a proportion of children <5 years of age that were associated with the CSL 2010 SH TIV. Although not definitive, the observation that individuals who have undergone FS frequently demonstrate elevations in systemic cytokine levels, provides a logical basis for the use of these *in vitro* models to evaluate the reactogenic potential of TIVs and MPHs. In this regard, cytokines and other immune mediators have been implicated in either inducing fever or lowering the threshold for FS in both animal models [8–10] and humans [11–20]. Pro-inflammatory cytokines are significantly elevated in the serum or central nervous system (CNS) of children following FS, including those induced in response to influenza [11–17,19,20], but rarely in fever only control groups suggesting that fever in the absence of systemic cytokines is insufficient to trigger FS. Furthermore, elevated levels of cytokines in the serum or CNS of children with FS as compared to healthy controls, is also observed *in vitro* using either unfractionated Whole Blood (WB) or Peripheral Blood Mononuclear Cells (PBMC) [18]. It has been proposed that systemic cytokine release in response to pyrogens may be viewed as pro-convulsant factors in children and FS as a state of active neuroinflammation [12,21–25]. The development and use of *in vitro* assay systems to model and predict *in vivo* pyrogenicity induced by therapeutic products is an evolving field that is gaining acceptance [26–29]. The present study has used several *in vitro* assays and identified that a small subset of paediatric whole blood donors displayed elevated cytokine/chemokine responses to the CSL 2010 SH TIV (as compared to previous CSL TIVs or other 2010 SH TIVs), with the remainder displaying low cytokine/chemokine responses to all TIVs tested. The scientific investigations have also provided insight into the component(s) of the CSL 2010 TIV that potentially contributed to the increase in reports of FS observed in 2010 as described in the accompanying manuscript [30].

2. Materials and methods

2.1. TIV and MPH preparations tested

TIVs: CSL 2005/2006–2011/12 TIV (see Supplemental Table 1), CSL Panvax® monovalent H1N1, new CSL 2010 SH TIV (where A/California/07/2009 replaced by A/Brisbane/59/2007), licensed split virion 2010 SH TIV made by another manufacturer (Comparator A), licensed sub-unit 2010/11 NH TIV made by another manufacturer (Comparator B) and MPHs: H1N1 (A/Brisbane/59/2007 and A/California/07/2009), H3N2 (A/Uruguay/716/2007, A/Wisconsin/15/2009 and A/Victoria/210/2009) and B strains (B/Brisbane/60/2008 and B/Florida/04/2006) were tested in the *in vitro* assays with the appropriate controls as described below.

2.2. Paediatric subjects and in vitro whole peripheral blood assays (WBA)

Healthy children aged 1–10 years undergoing an elective surgical procedure at the Royal Children's Hospital, Melbourne, Australia were eligible for the study. EDTA whole blood samples were aliquoted into 48-well tissue culture plates, diluted ½ in Dulbecco's PBS and cultured in duplicate, in the presence of either a test vaccine (TIV, final dilution 1/10), its MPH components (10.6 μg HA/mL), TIV diluent (Nil control) or Endotoxin (USP, Merck, 11.1 EU/mL). See Supplemental Materials and Methods for further details.

2.3. In vitro NFκB HEK293 reporter assay

The NF-κB/SEAPorter HEK293 cell line (NF-κB HEK 293) was maintained in the presence of selection agents according to manufacturer's instructions (Imgenex Corp., USA). For *in vitro* stimulation, cells were seeded into 96 well plates at a cell density of 4×10^4 cells/well and 16 h later, TIV or MPH added to a final concentration of 10.6 μg/mL HA in a volume of 200 μL (or 2.12 μgHA/well). See Supplemental Materials and Methods for further details.

2.4. Statistical analysis

A data set comprising protein concentrations of 21 cytokines/chemokines from WBA of 9 donors stimulated with 14 stimuli and Nil control, and 1 donor stimulated with 13 stimuli