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A comparative study of the effect of UV and formalin inactivation on the stability and immunogenicity of a Coxsackievirus B1 vaccine

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ABSTRACT

Type B Coxsackieviruses (CVBs) belong to the enterovirus genus, and they cause both acute and chronic diseases in humans. CVB infections usually lead to flu-like symptoms but can also result in more serious diseases such as myocarditis, aseptic meningitis and life-threatening multi-organ infections in young infants. Thus, CVBs have long been considered as important targets of future vaccines.

We have previously observed CVB1 capsid disintegration and virus concentration decrease with 12-day long formalin inactivation protocol. Here a scalable ion exchange chromatography purification method was developed, and purified CVB1 was inactivated with UV-C or formalin. Virus morphology and concentration remained unchanged, when the UV (2 min) or formalin (5 days) inactivation were performed in the presence of tween80 detergent. The concentration of the native and UV inactivated CVB1 remained constant at 4 °C during a six months stability study, whereas the concentration of the formalin inactivated vaccine decreased 29% during this time. UV treatment decreased, whereas formalin treatment increased the thermal stability of the capsid.

The formalin inactivated CVB1 vaccine was more immunogenic than the UV inactivated vaccine; the protective neutralizing antibody levels were higher in mice immunized with formalin inactivated vaccine. High levels of CVB1 neutralizing antibodies as well as IgG1 antibodies were detected in mice that were protected against viremia induced by experimental CVB1 infection.

In conclusion, this study describes a scalable ion exchange chromatography purification method and optimized 5-day long formalin inactivation method that preserves CVB1 capsid structure and immunogenicity. Formalin treatment stabilizes the virus particle at elevated temperatures, and the formalin inactivated vaccine induces high levels of serum IgG1 antibodies (Th2 type response) and protective levels of neutralizing antibodies. Formalin inactivated CVB vaccines are promising candidates for human clinical trials.

1. Introduction

Coxsackievirus B1 (CVB1) is a RNA virus belonging to the picornaviridae family and the enterovirus genus. Infections caused by the six Coxsackievirus B types are usually asymptomatic or lead to flu-like symptoms. However, they can also result in serious diseases such as myocarditis [1], aseptic meningitis [2], pancreatitis [3] and life-threatening multi-organ infections in young infants [4]. In fact, CVBs have constantly been among those 15 enteroviruses most commonly reported to CDC by diagnostic laboratories in US causing significant morbidity especially among young children [5]. In addition, CVBs have been linked to chronic diseases such as cardiomyopathies and type 1 diabetes [6–8]. Thus, CVBs have long been considered as potential targets of future enterovirus vaccines [9–11].

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Besides polio [12] and Enterovirus-71 [13] (EV71) vaccines, there are no clinically approved treatments or vaccines available against enteroviruses. To meet the need for preventing CVB associated diseases the clinical development of a vaccine against T1D associated CVB serotypes has recently started [7]. With this comes an increasing demand for improved CVB vaccine production methods allowing for large scale vaccine production.

We have previously developed optimized production and purification methods for CVB1, CVB6 [14,15] and CVB3 [16] viruses and formulated the purified viruses as formalin inactivated whole virus vaccines. These vaccines induced robust neutralizing antibody responses in mice and the CVB1 vaccines were shown to protect against both CVB1 infection and CVB1 induced diabetes in mouse models [14,15]. We have shown previously that a 12-day formalin inactivation period negatively affects the virus structure and concentration [14]. Therefore, we have used 254 nm ultraviolet (UV) light irradiation as an alternative virus inactivation method and studied the effects of the inactivation methods on the stability, integrity and immunogenicity of the viruses.

In the present study, a scalable ion exchange chromatography purification method and optimized 5-day long formalin inactivation method for CVB1 was developed. Virus morphology and concentration remained unchanged when UV or formalin inactivation was performed in the presence of tween80 detergent and as such, the length of formalin inactivation was decreased to five days from twelve days. The formalin treated particles were more resistant to elevated temperatures than the native or UV treated particles, providing stability at elevated temperatures by inhibiting the initial heat-induced capsid expansion. Here we also demonstrate, that the formalin inactivated CVB1 vaccine is more immunogenic than the UV inactivated CVB1 vaccine. The protective neutralizing antibody levels were higher and persisted for longer in mice immunized with formalin inactivated CVB1 vaccine.

2. Materials and methods

2.1. Virus production and purification

A wild CVB1 field isolate from Finland (kindly provided by Vactech Ltd.) was used and produced in Vero cells as described previously [14]. Virus particles were concentrated by pelleting through a 30% sucrose cushion using ultracentrifugation (175 000 g, 16 h at 4 °C). Pelleted virus was further purified with ion exchange chromatography using a strong AEX monolithic column (6.7 mM ID × 4.2 mM, V: 1 ml) from BIA Separations (Ljubljana, Slovenia) with quaternary amine (QA) chemistry as described previously [16]. The only exception was that the purification was done in the presence of 0.1% tween80 detergent. Purified viruses were characterized as described previously [14].

2.2. Vaccine production and characterization

Purified viruses were inactivated in 0.01% (vol/vol) formalin for 5 days at 37 °C or by UV-C irradiation at 254 nm (2.0 mWatts/cm^2) for 2 min in M199-0.1% tween80. The inactivation was confirmed by demonstrating the lack of infectious virus in green monkey kidney cells in TCID$_{50}$ end-point dilution assay as described earlier [14]. The vaccine was formulated in M199 medium containing 0.1% tween80 to contain 1 μg inactivated CVB1 per vaccine dose.

Transmission electron microscopy (TEM) analysis of the vaccines was performed as described previously [14]. Dynamic light scattering (DLS) analysis was performed with a Zetasizer Nano ZS instrument (Malvern Instruments Ltd.). The hydrodynamic diameter of viruses was determined as the average of three measure-

ments (each measurement containing 10–20 × 10 s datasets at 25 °C). Total protein concentration measurement (Pierce BCA assay), SDS-PAGE and Western blot analyses were performed as described previously [14].

The thermal stability of the virus particles was characterized by a thermofluorometric dye-binding assay using the protein binding dye SYPRO orange (Invitrogen) and the nucleic acid binding dye Midori Green (Nippon Genetics). Reaction mixtures of 25 μl containing 4.0 μg CVB1, 6 × SYPRO orange or 10 × Midori Green with PBS were mixed and heated from 25 to 110 °C, with fluorescence reads taken at 1 °C intervals every 30 s within the Biorad quantitative PCR system. SYPRO Orange, a fluorescence dye that binds to the hydrophobic amino acid residues, was used to analyse the unfolding or denaturation of the capsid proteins to study the conformational stability of the viruses. The fluorescence intensity of the dye in the presence of viruses was plotted as a function of the temperature, and melting temperatures (Tm) of the viruses were derived from the inflection points of the transition curve using the Boltzmann equation [17]. The melting temperature at which the genome of virus becomes accessible (T_{RNA}), was determined from the fluorogram of Midori Green.

2.3. Mouse immunizations and CVB1-challenge

C57BL/6J mice were housed in specific pathogen-free conditions at Karolinska Institutet, Stockholm, Sweden. All experiments were conducted in accordance with the NIH Principles of Laboratory Animal Care and national laws in Sweden and were approved by the local ethics committee. Two vaccination experiments were performed. C57BL/6J mice were vaccinated interscapularly (i.s.) on days 0, 21 and 35 with 1 μg of UV or formalin inactivated CVB1 (n = 5 / experiment). In the second vaccination experiment mice were challenged with 1×10^6 plaque forming units (PFU) CVB1 (intraperitoneal injection; i.p.) on day 60 as described earlier [15].

2.4. Neutralization assays and CVB1 specific ELISA

Neutralizing antibodies against CVB1 were measured by standard virus plaque reduction assay in green monkey kidney (GMK) cells as previously described [6]. Sera from C57BL/6J CVB1 vaccinated mice was tested for CVB1 specific IgG and IgG subtype antibodies by ELISA according to the previously described procedures [18]. Briefly, 96-well half-area polystyrene plates (Corning Inc., Corning, NY) were coated with 50 ng of CVB1 virus-like particles (CVB1-VLPs) per well. CVB1-VLPs were produced with insect-cell baculovirus expression system and were concentrated from the culture supernatant by tangential flow filtration and were purified with the combination of anion and cation exchange chromatography steps (Hankaniemi et al, manuscript submitted). Antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich, St. Louis, MO), IgG1 (Invitrogen) or IgG2a (Invitrogen) and SIGMA FAST OPD substrate (Sigma-Aldrich). Optical densities (OD) at 490 nm were measured by Victor^2 microplate reader (PerkinElmer).

2.5. Statistical analyses

All statistical analyses were performed using GraphPad Prism version 5.02. Neutralizing antibody titers and CVB1-specific antibody responses were analysed with Mann-Whitney U test. Plaque assay virus titrations were analysed by Kruskal-Wallis test.

3. Results

3.1. Ion-exchange purification yields a highly pure CVB1

CVB1 was produced in Vero-cells and the virus was concentrated and partially purified from the clarified cell culture supernatants by 30% sucrose cushion pelleting. In our previous study we discovered that tween80-detergent increases the virus yield and stability [14]. Therefore, the virus was further purified with anion exchange chromatography (AEX) in the presence of 0.1% tween80, which was also compatible with the chromatography. CVB1 virus was eluted from the AEX-column at 60 mM NaCl (Fig. 1a). SDS-PAGE analysis and subsequent detection of proteins with stainfree staining method revealed protein bands of approximately 31 kDa and 26 kDa that corresponded with the CVB1 capsid proteins VP1 and VP3 [14]. The purity of the virus was >95% as measured by densitometric analysis of SDS-PAGE gel (Fig. 1b, middle panel). Viral VP1 capsid protein was also detected by Western blotting using a mouse anti-enterovirus mAb (clone 5-D8/1, DAKO) (Fig. 1b, right panel). According to the TEM images, the particles were intact and had the expected morphology with an average diameter of 30 nm (Fig. 1c).

3.2. Optimization of the CVB1 inactivation methods and evaluation of the conformational stability of the native, UV and formalin treated viruses

We have previously shown that formalin inactivation of CVB1 negatively affects the virus preparations [14]. Here, we evaluated whether a shorter duration of the formalin inactivation period and a different virus buffer composition could lead to better preservation of the virus integrity. We also explored the inactivation of the virus using potentially less harsh inactivation method, UV-C irradiation, and examined the effect of the different protocols on CVB1 morphology, stability, particle size, particle size distribution and concentration. To this end, CVB1 virus was formalin inactivated (5 days in 0.01% formalin at 37 °C) or UV irradiated (2 min with intensity of 2.0 mWatts/cm^2) followed by analyses by Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS) and SDS-PAGE. Inactivation was done in the presence of 0.1% tween80 detergent for all protocols. The previously observed virus capsid disintegration and decrease of the virus concentration [14] was avoided when the UV or formalin inactivation was performed in the presence of tween80 detergent. According to TEM, we found three virus forms with differential staining patterns (Fig. 2a–c, Supplementary Fig. 1). All samples represented ~30 nm capsids with the correct icosahedral morphology. We suggest that the viruses which were impermeable to uranyl acetate stain represent solid capsids, partly permeable viruses represent slightly porous capsids, whereas fully permeable viruses represent very porous capsids. The native (Fig. 2a) and UV inactivated (Fig. 2b) viruses contained similar proportions of impermeable (75%) as well as partly permeable (25%) particles (Supplementary Fig. 1a and b). Formalin inactivated (Fig. 2c) virus preparations were composed of 60% of particles that were fully permeable to the stain and 40% of particles that were partly permeable to the stain (Supplementary Fig. 1c). These results differed considerably from those with the native and UV inactivated viruses and indicate that formalin treatment appears to induce capsid porosity.

The stability of the native and differentially treated CVB1 viruses was analysed by monitoring changes in particle size distribution by DLS. All CVB1 vaccines (that originated from the same CVB1 stock) were stored at 4 °C in M199-tween80 for six months and were analysed by DLS on day 0 and at the end of the six-month storage period. The samples were gently mixed before
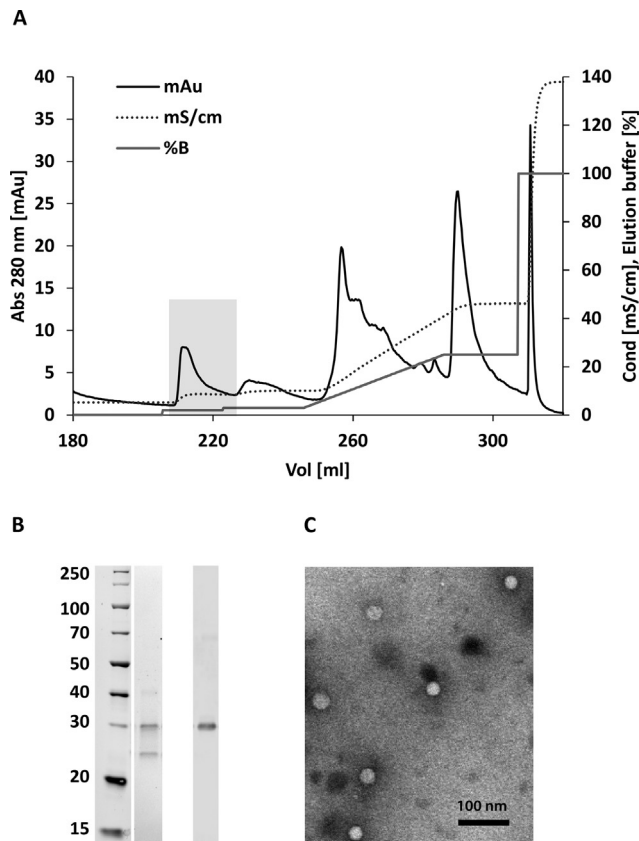
Fig. 1. Characterization of chromatography purified CVB1 virus. (A) CVB1 virus concentrated by 30% sucrose pelleting, was loaded onto an anion exchange column. The bound virus and impurities were eluted from the column with the combination of stepwise and linear gradients. Grey area marks the elution peak of CVB1. (B) SDS-PAGE and Western blot analyses of the purified viruses. Middle panel shows the total protein stained CVB1 preparation and the right panel the VP1 protein stained with 5-D8/1 mAb. (C) TEM image of the purified CVB1 virus. Bar: 100 nm. Image captured from 25000 × magnification.

DLS analyses to obtain a representative sample and avoid exclusion of particles that may have sedimented during storage. Native CVB1 virus contained 79% 37 nm particles (Scattering Intensity (SI) 63,689 kcps) on day 0 and 67% 43 nm particles (SI 101,335 kcps) after six months storage at 4 °C (Fig. 2d). UV inactivated CVB1 contained 76% 41 nm particles (SI 64,499 kcps) on day 0 and 66% 44 nm particles (SI 98,024 kcps) after six months storage at 4 °C (Fig. 2e). Formalin inactivated CVB1 contained 58% 43 nm particles (SI 81,161 kcps) on day 0 and 28% 49 nm particles (SI 107,935 kcps) after six months storage at 4 °C (Fig. 2f). During the six months storage period, the volume of ~40 nm particle population decreased (Fig. 2g), whereas the mean sizes of the different particle populations were found to increase slightly (Fig. 2h). Inspection of the intensity-based particle size distribution revealed increases in the scattering originating from particles with diameter >1 μm. These results indicate that during storage, a portion of the virus samples became insoluble. This effect was most notable in the case of the formalin inactivated particles (Fig. 2f). In comparison, the native and UV inactivated viruses were relatively stable when stored at 4 °C for six months (Fig. 2d,e).

Concentration of the differentially formulated CVB1 vaccines was also assessed after six months storage at 4 °C by densitometric analysis. Equal volumes of the vaccines (stored at 4 °C in M199-tween80 for six months) from both day 0 and the month 6 time point were run on a SDS-PAGE gel and proteins were detected with a stainfree staining method. Based on densitometric analysis of the
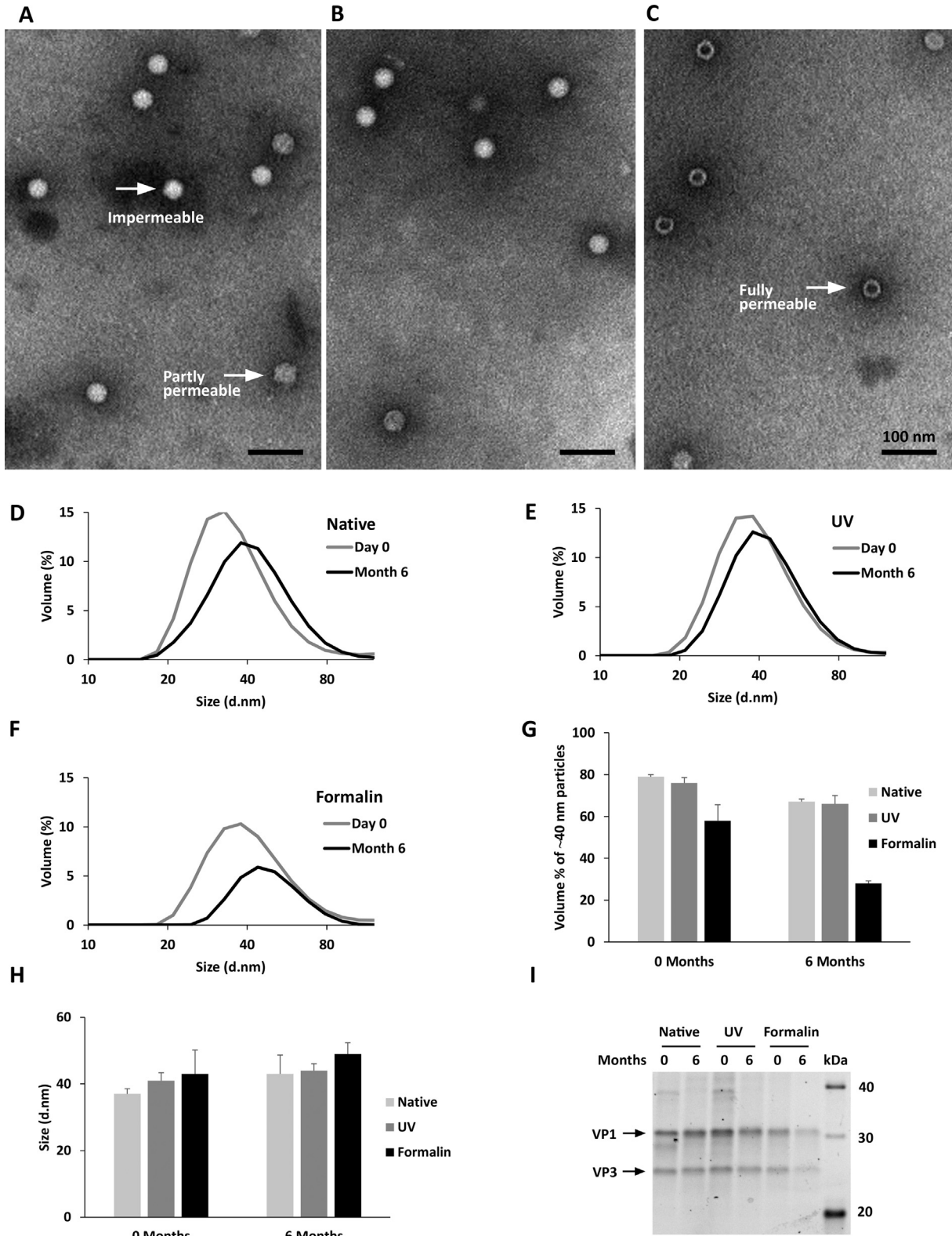
Fig. 2. Stability profiles of native CVB1 virus, UV and formalin inactivated CVB1 vaccines. TEM analysis of chromatography purified (A) native, (B) UV inactivated and (C) formalin inactivated CVB1 virus. Scale bars 100 nm. (D-H) CVB1 viruses formulated in the vaccine buffer (M199-0.1% tween80) stored at 4 °C for 0 and six months were analysed by dynamic light scattering (DLS) for their size and volume distributions: (D) Native CVB1, (E) UV inactivated CVB1 and (F) Formalin inactivated CVB1. (G) The volume of ~40 nm particle population at day 0 and after 6 months storage period for the different CVB1s. (H) The size of the particles at day 0 and after 6 months storage period for the different CVB1s. (I) SDS-PAGE analysis of the native CVB1 virus and CVB1 vaccines from day 0 and after storing at 4 °C for 6 months. Two μg of virus or vaccine were loaded per well.

SDS-PAGE gel, the concentration of the native CVB1 virus and the UV inactivated vaccine did not change during the six months storage period (Fig. 2i). Two prominent proteins of approximately 31 kDa and 26 kDa corresponding to the CVB1 capsid proteins VP1 and VP3 were detected in the vaccines by total protein staining (Fig. 2i). However, the concentration of the soluble formalin inactivated CVB1 vaccine decreased by 29% during the 6 months storage period. These results show that virus morphology and protein concentrations remained relatively stable when the native virus or UV inactivated virus was stored in M199 medium in the presence of tween80 detergent. However, storage at $-20\,^{\circ}$C or lower temperatures might be advisable for the long-term storage of formalin inactivated CVB1 which decreased in concentration at $4\,^{\circ}$C. To find out if storage at $4\,^{\circ}$C affects the immunogenicity of the vaccine, separate studies should be performed.

3.3. Thermal stability of native CVB1, UV and formalin inactivated CVB1 vaccines

Desired vaccine candidates are stable during storage, especially at elevated temperatures. Polioviruses have been shown to undergo antigenic switch from the native (N- or D-antigenic) form to a non-native or heated (H- or C-antigenic) form when heated above $50\,^{\circ}$C [19]. The latter form (H- or C-antigenic) is not able to elicit a robust neutralizing antibody response and as such, there must be a constant amount of poliovirus D-antigen in formalin inactivated poliovirus vaccines to maintain immunogenicity [20]. Due to the influence of temperature on vaccine stability, the ability of the native, UV or formalin inactivated CVB1 preparations were evaluated by Differential Scanning Fluorimetry (DSF). Two fluorescent dyes were employed in the assay: SYPRO Orange, a fluorescence dye that binds to hydrophobic amino acid residues, indicating unfolding or denaturation of the capsid proteins and Midori Green, a fluorescence dye that binds to the nucleic acid, indicating the access to the viral RNA. The fluorescence intensity of the respective dyes in the presence of virus was plotted as a function of the temperature, and melting temperatures (Tm) of the viruses were derived from the inflection points of the transition curve using the Boltzmann equation [17]. SYPRO orange had two peaks in the fluorogram (Fig. 3a), where Tm_1 indicated capsid expansion and the exposure of hydrophobic residues, whereas Tm_2 indicated denaturation of the viruses [21] (Fig. 3b). The virus melting temperatures differed between the differentially treated CVB1 viruses. According to Tm_1 values, formalin treated particles were more resistant to elevations in the temperature during the initial temperature induced unfolding event than the native virus. Contrastingly, the UV treated particles were less resistant than the native virus in the same conditions. The Tm_2 data indicated that UV treated particles were also less resistant to elevations in temperature up to the final denaturation temperature (Fig. 3b).

The melting temperature (T_{RNA}) at which the genome of UV inactivated, native and formalin inactivated CVB1 becomes accessible to fluorescent dye, was determined with DSF (Fig. 3c). T_{RNA} (Fig. 3d) was higher than Tm_1 and lower than Tm_2 (Fig. 3b) in all of the vaccine preparations. The increase in Tm_1 seen in the formalin inactivated CVB1 virus was matched by an increase in T_{RNA}, whereas the decrease in the Tm_1 value for the UV inactivated CVB1 did not alter the T_{RNA}. This observation could be explained by the fact that as the thermal stability of CVB1 increases, alterations in the capsid are delayed and as such the accessibility of RNA which is synchronized with the initial unfolding of the capsid, is altered. In conclusion, the results from the thermal stability assay employed here (Tm_1 and T_{RNA} results), formalin treatment protected the virus against temperature induced capsid expansion.
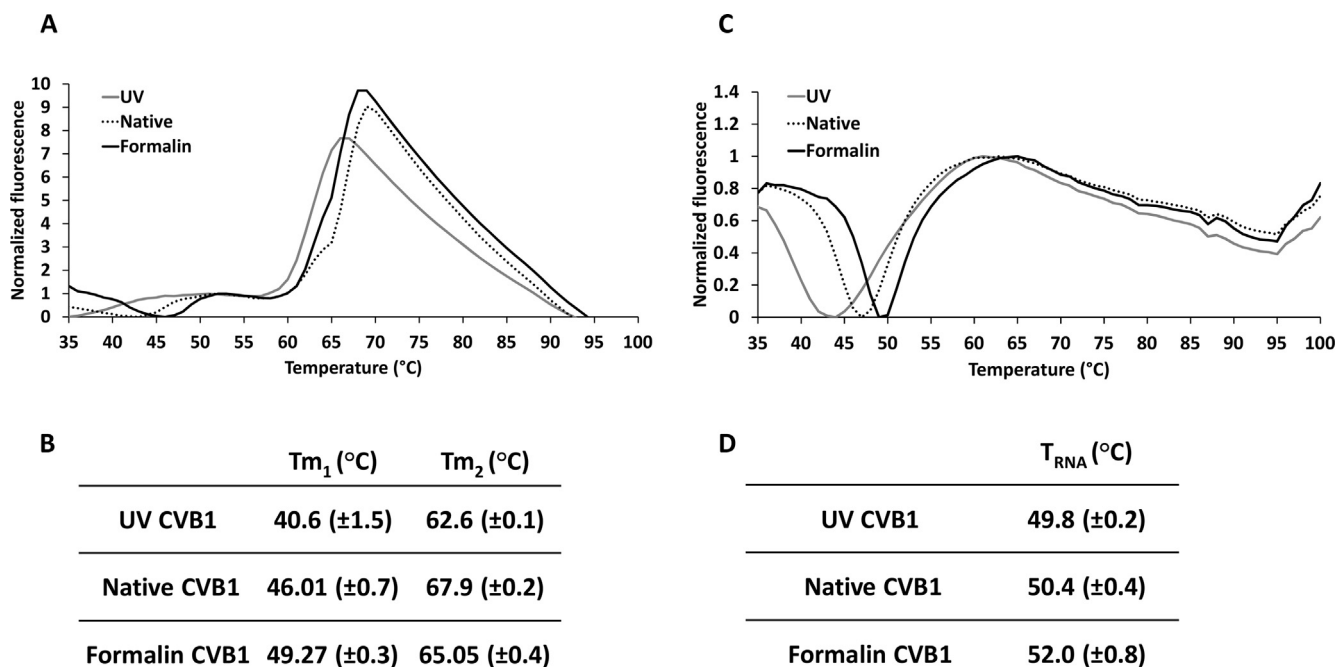
3.4. UV and formalin inactivated CVB1 vaccines are immunogenic and protect against viremia following infection with CVB1

No information is available on whether the different CVB inactivation methods during the vaccine production process affect the vaccine immunogenicity. Therefore, the immunogenicity of the UV and formalin inactivated CVB1 vaccines was tested in C57BL/6J mice following the experimental timeline shown in Fig. 4a. Sera from CVB1 vaccinated mice were evaluated for CVB1 neutralizing ability *in vitro* (n = 10). All vaccinated mice had already generated neutralizing antibodies by day 21 (after one vaccination) (Fig. 4b). Neutralizing geometric mean titers (GMTs) were 338 and 446 at day 21 for mice immunized with the UV and formalin inactivated CVB1 vaccines respectively. After three immunisations (on day 49) the neutralizing GMT was 891 for the UV inactivated CVB1 vaccine and 3104 for the formalin inactivated CVB1 vaccine. The neutralizing antibody response was stronger in the mice that were immunized with formalin inactivated CVB1 vaccine on days 42, 49 and 60 (after three immunisations) compared to the animals that were immunized with UV inactivated CVB1 vaccine (Mann-Whitney *U* test, p = 0.026, 0.031 and 0.019 respectively) (Fig. 4b).

In the second vaccination experiment the neutralizing antibody levels were followed until day 60. Neutralizing antibody GMTs in sera from UV inactivated CVB1 vaccinated mice decreased from 776 to 256 between day 49 and day 60 and the same values changed from 7132 to 5405 in mice immunized with formalin inactivated CVB1 vaccine (Fig. 4b). Mice were infected with CVB1 on day 60 to establish whether the vaccines would prevent viremia in the blood on day 3p.i. or virus replication in the heart and pancreas on day 5p.i., viral titers are expressed as PFU/ml of blood or PFU/mg of tissue. All mice immunized with formalin inactivated CVB1 vaccine (5/5) were protected against viremia (Fig. 4c) and no replicating virus was detected in their pancreas (p = 0.0093) (Fig. 4d) or heart (Fig. 4e). In contrast, only 2/5 mice immunized with UV inactivated CVB1 vaccine were protected against viremia as determined by standard plaque assay analysis of the blood sampled on day 3p.i. (Fig. 4c). Moreover, replicating virus was not detected in the pancreas of the two mice without viremia on day 3p.i. whereas the remaining 3/5 UV inactivated CVB1 vaccinated mice had replicating virus in their pancreas (Fig. 4d). All buffer treated mice had replicating virus in the pancreas on day 5p.i. (Fig. 4d), 4/5 mice were viremic on day 3p.i. (Fig. 4c) and 3/5 had replicating virus in the heart (Fig. 4e). Overall, vaccination with formalin treated virus provided more efficient protection against infection as compared to that obtained with UV treated virus.

3.5. CVB1 vaccination results in an IgG1 oriented immune response

Sera of C57BL/6J mice immunized with UV or formalin inactivated CVB1 vaccines or control vaccine buffer treated mice were analysed for CVB1-specific serum IgG, IgG1 and IgG2a 49 days after the prime vaccination. All immunized mice were positive for CVB1-specific IgG by ELISA which was not found in the control animals. The magnitude of the IgG response was high in both groups. GMTs were 11,143 and 6400 in the groups receiving UV and formalin inactivated vaccines respectively (Fig. 5a). However, no statistically significant difference (Mann-Whitney *U* test, p = 0.58) was observed in the magnitude of IgG responses induced by the UV or formalin inactivated vaccines. CVB1-specific IgG1 and IgG2a immunoglobulin subtypes which are indicators of Th2 and Th1 type immune responses were also investigated. Both vaccines induced a strong Th2-type (IgG1) response (GMTs were 1600 and 3676 respectively in the groups receiving UV and formalin inactivated vaccines), whereas the Th1-type (IgG2a) responses were very low in both groups (Fig. 5b). Similar to IgG responses, no significant differences (Mann-Whitney U test) were observed in the magni-

A

C

B

	Tm$_1$ (°C)	Tm$_2$ (°C)
UV CVB1	40.6 (±1.5)	62.6 (±0.1)
Native CVB1	46.01 (±0.7)	67.9 (±0.2)
Formalin CVB1	49.27 (±0.3)	65.05 (±0.4)

D

	T$_{RNA}$ (°C)
UV CVB1	49.8 (±0.2)
Native CVB1	50.4 (±0.4)
Formalin CVB1	52.0 (±0.8)

Fig. 3. Thermal stability profile of UV inactivated, native and formalin inactivated CVB1-vaccines. UV inactivated, native and formalin inactivated CVB1 virus were analysed with Differential Scanning Fluorometric assays using SYPRO orange protein binding dye and Midori green nucleic acid-binding dye. (A) Relative fluorescence of SYPRO orange and (B) melting temperatures (Tm$_1$ and Tm$_2$) of different CVB1 preparations determined from fluorogram. (C) Relative fluorescence of Midori green and (D) the dye accessibility temperature for RNA (T$_{RNA}$) of different CVB1 preparations determined from fluorogram.

tude of the IgG1 (p = 0.67) or IgG2a (p = 0.37) responses induced by UV or formalin inactivated vaccines.

4. Discussion

In this study, our objective was to optimize the purification, formulation and inactivation of CVB1 to produce stable and immunogenic inactivated virus vaccines. We also wanted to investigate the extent to which UV and formalin inactivation treatments affected the virus stability and immunogenicity.

In our previous study we developed a scalable three-step purification method for the production of a CVB1 vaccine, which relied on 30% sucrose cushion pelleting, gelatin affinity chromatography and 30/50% sucrose cushion pelleting [14]. Although the method can be scaled up to purify several litres of virus containing supernatant, the method developed in the current study is more suitable for industrial scale purification, because it consists of only two steps (30% sucrose cushion pelleting and AEX). We showed previously that the addition of tween80 detergent in the purification process increases virus yield and stability [14] and as such this detergent was included in all the steps of the purification process.

Inactivation with formalin is commonly used to produce commercial human virus vaccines such as those against polio [22]. Formalin has an effect on both genome and proteins. It acts as an alkylating agent by crosslinking RNA to capsid proteins, causing a block to genome reading, and also as crosslinker by formation of inter- and intra-molecular methylene bridges between primary amino groups. [23] We have shown previously that the 12 day formalin inactivation protocol used for the inactivation of poliovirus vaccine [22] causes a dramatic change in CVB1 integrity and that ~95% of the CVB1 virus particles dispersed into smaller units following inactivation [14]. Therefore, in the current study, we have examined whether UV irradiation could serve as an alternative inactivation method and addressed whether decreasing the formalin inactivation length from 12 to five days would prevent the particle disintegration. Based on wavelength, UV light can be

subdivided into three classifications: UV-A (320–400 nm), UV-B (280–320 nm) and UV-C (200–280 nm). UV-C can lead to formation of dimers between two adjacent pyrimidines (uracil and thymine). Formation of pyrimidine dimers can put a strain on the sugar backbone of the genome, which possibly leads to breaks in the genome [23]. UV-C may also crosslink proteins [24] and induce crosslinking between nucleic acids and proteins [25]. Here, a comparative study on the effects of 254-nm UV-C inactivation and 5-day long formalin inactivation on CVB1 was performed. Shorter formalin inactivation (3–5 days) periods have been utilized with other enterovirus vaccines including EV71 [26] and CAV vaccines (serotypes 6, 10 and 16) [27]. Also, UV inactivation has been used in the inactivation of EV71 and polio vaccines [26,28,29]. In the present study, we show that when UV or formalin inactivation was performed in the presence of tween80-detergent and the length of formalin inactivation was decreased to five days (instead of the 12 day inactivation protocol employed previously [14]), virus morphology was preserved, and the virus concentration remained unchanged when measured immediately after the inactivation period, highlighting the improvement in the methodology. Although the TEM analysis showed that the diameters of the native, UV and formalin inactivated viruses were approximately ~30 nm with icosahedral symmetry (characteristic to enteroviruses [16]), the physical appearance of the formalin inactivated virus was different to native and UV inactivated viruses. Approximately 60% of the formalin-inactivated viruses exhibited a ring-like appearance due to stain incorporation into capsids, suggesting that formalin treatment caused physical changes to the virus resulting in capsid porosity.

An important and desirable feature of a vaccine is stability during storage, especially at elevated temperatures. Some virus vaccines, such as inactivated poliovirus vaccines (IPVs) are temperature sensitive and require storage between 2 °C and 8 °C, whereas oral poliovirus vaccine (OPV) requires storage at −20 °C [30]. In the current study we show that the concentration of native CVB1 virus or UV inactivated CVB1 vaccine did not change during the six months
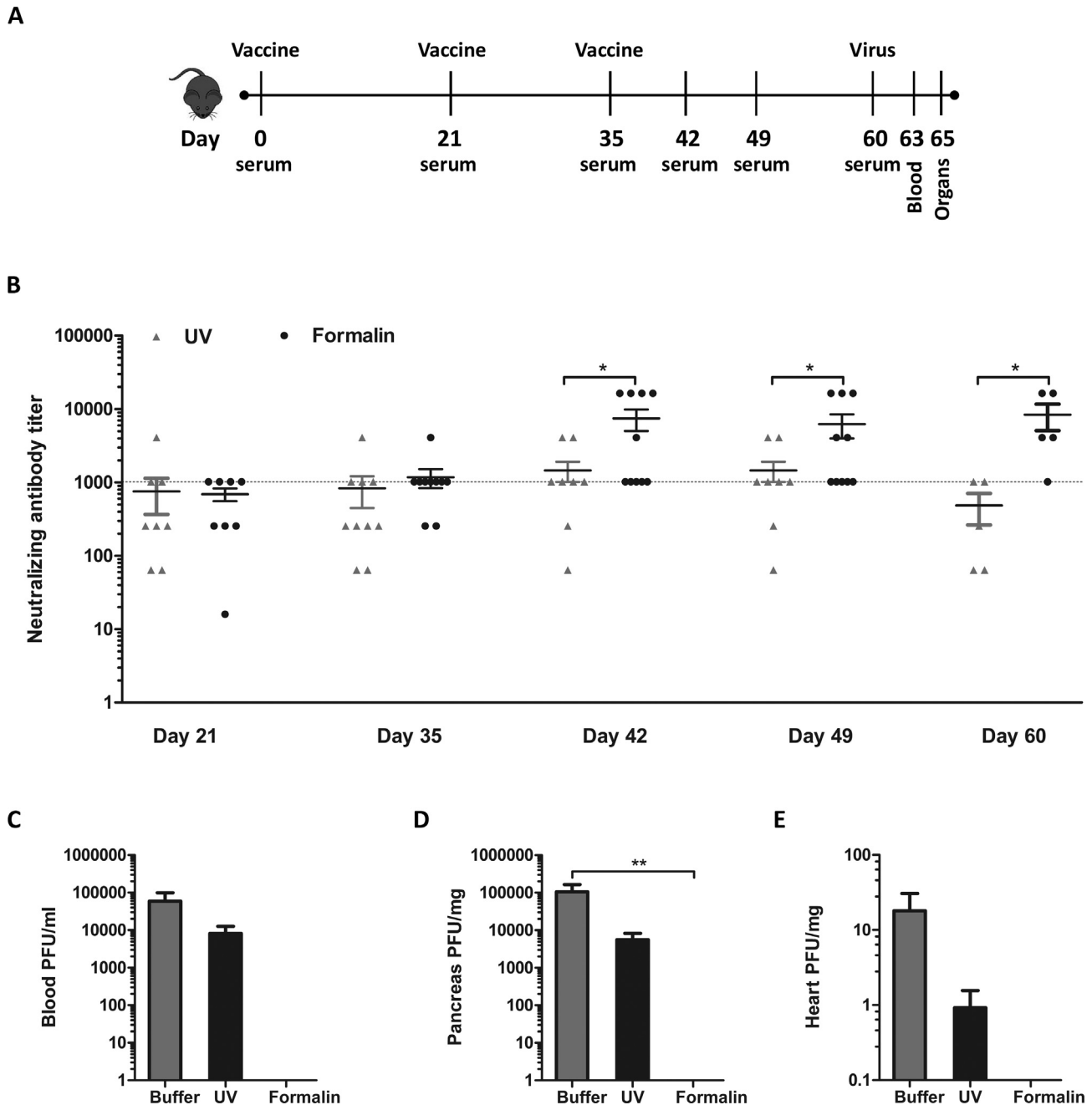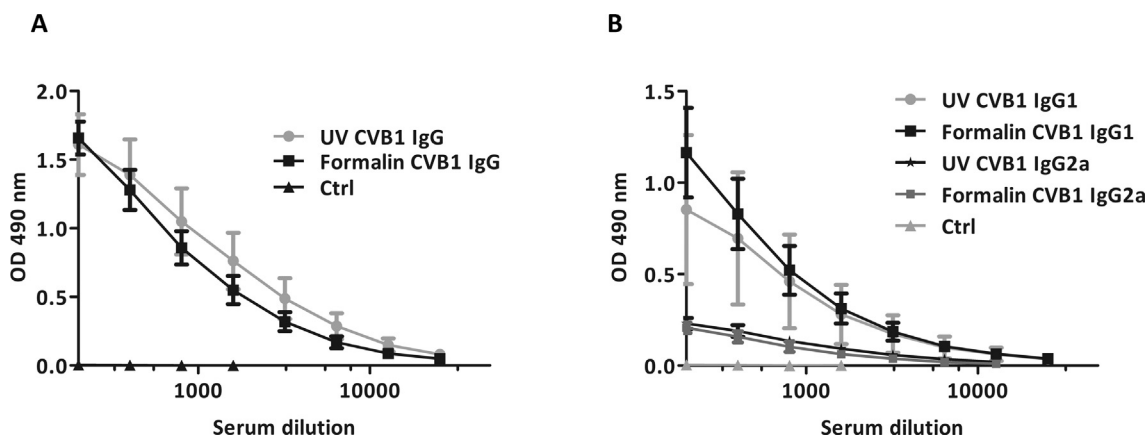
Fig. 4. UV and formalin inactivated CVB1 vaccines induce neutralizing antibody responses and protect against viremia following infection with CVB1 in C57BL/6J mice. (A) Schematic showing the experimental timeline of the immunisation strategy in C57BL/6J mice. The immunogenicity of the UV or formalin inactivated CVB1 was tested by injecting 3×1 μg non-adjuvanted vaccine i.s. to five mice in two separate experiments (n = 5 + 5). (B) CVB1 neutralizing antibody titers in the sera of mice immunized with CVB1 vaccines in samples taken days 21, 35, 42, 49 (n = 10) and 60 days (n = 5) after the prime vaccination. Mean neutralizing antibody titers are indicated by the line ± SEM; * = p < 0.05, compared to the other differentially formulated vaccines at each time point, as determined by Mann–Whitney U test. The dotted line represents the protective neutralizing antibody threshold (dilution 1024) and vaccinated mice at or above this level were protected against viremia (following infection with CVB1). (C) Cytopathic virus measured in the (C) blood, (D) pancreas and (E) heart of the buffer-treated (n = 5) or vaccinated (n = 5) mice on day 3 p.i. by standard plaque assay. Mean values ± SEM, **p < 0.01, Kruskal-Wallis test.

storage at 4 °C, whereas the concentration of the formalin inactivated CVB1 vaccine decreased by approximately 29% during the 6 months storage period at 4 °C. The latter vaccine was produced by inactivation with 0.01% formalin, formulated in M199-0.1% tween80 and because the initial formalin concentration in the inactivation process was relatively low, we decided not to neutralize the formalin after the 5-day inactivation period which may account in part for the decrease seen in the vaccine concentration. Furthermore, M199 contains components reacting with formalin, which should prevent further chemical crosslinking during extended storage. However, we observed decreased solubility for the formalin inactivated virus during the six months stability study that might have been caused by some reactive formalin present in the vaccine. In line with our results with UV inactivated and native CVB1, an EV71 VLP vaccine remained stable for at least five months storage in appropriate buffers [31]. A number of determinants that have capsid stabilising properties, such as stabilizing compounds [32] and amino acid substitutions [21] have been previously

Fig. 5. CVB1-specific serum IgG-, IgG1- and IgG2a-antibody responses. Mean end-point titration curves of anti-CVB1 (A) total IgG, (B) IgG1 and IgG2a antibodies in the sera of C57BL/6J mice vaccinated with UV or formalin inactivated CVB1 vaccines. Sera was collected on day 49 after the prime vaccination for each group. Mean titration curves with standard errors of the mean of the experimental groups are shown (n = 5). The positivity cut-off OD value was 0.1 (control mice mean OD + 3 × SD).

identified for poliovirus. For instance, two capsid mutations in VP1 (V87A and I194V) have been identified which enhance virus stability by preventing premature uncoating and the release of viral RNA at high temperatures [21]. In the current study we measured the capsid flexibility and denaturation temperature of the native and inactivated CVB1 preparations under continuously increasing thermal stress in a DSF-assay. We found that formalin treated particles were more resistant to temperature induced unfolding than the native particles, whereas the inverse was true for the UV treated particles. Although TEM analysis showed that all of the formalin inactivated particles were partially or completely permeable to the negative stain (which could be a sign of structural instability), we found that they were even more thermostable than the native or UV inactivated viruses. This demonstrates that formalin treatment can be used for the production of stabilized vaccine candidates.

Immunity to CVB is serotype-specific and the pathogenesis of the infection is not fully understood [33]. CVB infections induce rapid and strong neutralizing antibody responses. First IgM antibodies appear after 7–10 days in both humans [34] and mice [35], followed by the IgG antibodies. Neutralizing antibodies (antibodies mainly directed toward the CVB capsid proteins VP1, VP2 and VP3) approximately appear from the second week of infection and immunity may be life-long. [36] Thus, the most important feature of a CVB vaccine is the generation of long lasting protective levels of neutralizing antibodies. The loss of viral antigenicity has been observed in UV inactivated poliovirus showing both antigenic and morphologic change [28,29]. Formalin inactivation has been shown to alter the antigenicity of poliovirus through the modification of antigenic sites and the effects on different epitopes varied [28]. During the second immunization experiment in the current study, the neutralizing antibody levels were followed for 60 days and the mice were challenged thereafter. A significant difference in the level and longevity of the antibody response was found between the two vaccines. During day 49 and 60, the neutralizing GMTs decreased from 776 to 256 for the mice immunized with UV inactivated vaccine and from 7132 to 5405 for the mice immunized with formalin inactivated vaccine. The challenge virus dose (10^6 PFU) was optimized to ensure that a systemic infection was obtained, and the mice were immunized with relatively low dose of each vaccine (1 μg, respectively) to enable the detection of possible differences in the immunogenicity of the vaccines. We found that the protective neutralizing antibody level was 1024 for this dose of virus in C57BL/6J mice. All five mice immunized with formalin inactivated virus were protected against CVB1 challenge, whereas only 2/5 of the mice immunized with UV inactivated virus

were protected. 3/5 of the mice immunized with UV inactivated virus had neutralizing antibody titer lower than 1024 and these mice were not protected from viremia. This suggests that sufficient amounts of neutralizing antibodies are necessary to induce the main protective mechanism against enteroviruses. We hypothesise that the virus epitopes that induce the neutralizing antibodies and result in protective immunity against CVB1 might be better conserved when the virus is inactivated with formalin. Alternatively, we hypothesise that the capsid proteins and virus genome chemically modified by formalin act like adjuvants to trigger a stronger immune response. Moreover, a further explanation could be that the UV inactivated virus induces more antibodies that do not have neutralizing capacity and thereby lowers the capacity of neutralizing antibodies to prevent infection [37]. Both formalin and UV inactivation methods have been used in the production of EV71, polio and influenza vaccines [26,28,29,38], but the neutralizing antibody titers obtained with UV inactivated vaccines were reportedly lower than those obtained with the formalin inactivated vaccines [26,28,29,38].

Previous studies have investigated the IgG response to CVBs in humans and it has been reported that there is a very poor correlation between IgG and CVB neutralizing antibody titers. However, in assays specifically examining the IgG subclasses, CVB specific IgG1 and IgG3 antibodies were detected [39]. Furthermore, a study describing human IgG subclass responses to EV71 infections found that the neutralizing activity of human intravenous immunoglobulin is mainly mediated by the IgG1 subclass [40]. Therefore, we also studied the quality of the IgG response in the vaccinated mice. Although the formalin inactivated CVB1 vaccine elicited significantly higher neutralizing antibody titers against CVB1 than UV inactivated vaccine (after three immunizations), the IgG, IgG1 and IgG2a levels were comparable in both groups. Generally, antibody responses to soluble protein antigens primarily induce IgG1 which are accompanied by lower levels of the other IgG subclasses [41]. In the current study, both UV and formalin inactivated CVB1 vaccines induced high Th2-type (IgG1) responses in C57BL/6J mice characteristic for neutralizing antibody response. Similarly, monovalent and bivalent CAV16 and EV71 vaccines were shown to induce higher levels of IgG1 and IgG2b compared to other IgG subtypes [42]. Since the IgG subclass responses induced by both vaccines were similar, it remains unclear whether other subclasses were responsible for the higher level of neutralizing antibodies induced by the formalin inactivated vaccine.

In conclusion, this study demonstrates that formalin inactivated CVB1 vaccine induces more robust neutralizing antibody responses when compared to UV inactivated CVB1 vaccine and provides pro-

tection against viremia after CVB1 infection. We have shown that formalin treated particles are more resistant, whereas UV treated particles are less resistant to temperature induced unfolding event compared to the native virus, and formalin inactivated CVB1 vaccine induces predominantly Th2 type antibodies and a protective level of neutralizing antibodies. These results indicate that formalin inactivated CVBs are promising vaccine candidates for human clinical trials in the future.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: HH owns stocks and is a member of the board of Vactech Ltd, which develops vaccines against picornaviruses. HH and MFT serve on the scientific advisory board of Provention Bio Inc., which is developing an enterovirus vaccine. The other authors have no conflict of interest to declare.

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Contributors

MMH produced and characterized the vaccines and wrote the manuscript. VMS planned and performed the animal experiments. ASK coordinated neutralizing antibody analysis. SH coordinated IgG-antibody analysis. VM performed TEM-imaging. HH, VB, OHL, MFT and VPH contributed to the study design. All authors critically revised the manuscript and approved the final version.

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Appendix A. Supplementary material

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