Effect of formaldehyde inactivation on poliovirus

Thomas Wilton1#, Glynis Dunn1, David Eastwood2, Philip D. Minor1 and Javier Martin1

Division of Virology1 and Biotherapeutics2, National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, United Kingdom.

Running title: Inactivated Polio Vaccine

Abstract and Importance: 328 words

Text: 4910 words

# Corresponding author’s mailing address: Thomas Wilton. Division of Virology, National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, United Kingdom

Phone (44) 1707 641178, Fax: (44) 1707 641 050. Email: Thomas.Wilton@nibsc.org
ABSTRACT

Inactivated polio vaccines, which have been used in many countries for more than 50 years, are produced by treatment of live poliovirus (PV) with formaldehyde. However, the molecular mechanisms underlying virus inactivation are not well understood. Infection by PV is initiated by virus binding to specific cell receptors which results in viral particles undergoing sequential conformational changes that generate altered structural forms (135S and 80S particles) and leads to virus cell entry. We have analysed the ability of inactivated PV to bind to the human poliovirus receptor (hPVR) using various techniques such as ultracentrifugation, fluorescent-activated cell sorting (FACS) flow cytometry and real-time RT-PCR. The results showed that although retaining the ability to bind to hPVR, inactivated PV bound less efficiently in comparison to live PV. We also found that inactivated PV showed resistance to structural conversion in vitro as judged by measuring changes in antigenicity, ability to bind to hPVR and viral RNA release at high temperature. Furthermore, viral RNA from inactivated PV was shown to be modified, as cDNA yields obtained by RT-PCR amplification were severely reduced and no infectious virus was recovered after RNA transfection into susceptible cells.

IMPORTANCE

This study represents a novel insight into the molecular mechanisms responsible for poliovirus inactivation. We have shown that inactivation with formaldehyde has an effect on early steps of viral replication as it reduces the ability of PV to bind to hPVR, decreases the sensitivity of PV to convert to 135S particles and abolishes the infectivity of its viral RNA. These changes are likely responsible for the loss of infectivity shown by PV following inactivation. Techniques used in this study represent new approaches for the characterisation of inactivated PV products and could be useful to help developing improved methods for the production and quality control testing of inactivated polio vaccines. Measuring the
antigenicity, capsid stability and RNA integrity of inactivated PV samples could help establishing the optimal balance between loss of infectivity and preservation of virus antigenicity during inactivation.

INTRODUCTION

The incidence of paralytic poliomyelitis in the last 30 years has been significantly reduced by the use of oral live-attenuated (OPV) and inactivated poliovirus vaccines (IPVs) [1]. Although both provide protection against poliomyelitis, OPV became the favoured vaccine in many countries due to the ease of oral administration, the lower costs of production and its ability to replicate in the gastrointestinal tract, stimulating both local secretory IgA in the pharynx and gastrointestinal tract and circulating IgG [2-5]. The drawback of OPV is that Sabin strains are genetically unstable. In rare cases, the attenuated strains can revert to neurovirulence whilst replicating in the vaccinee, causing vaccine-associated paralytic poliomyelitis (VAPP). Additionally, in populations with low immunity against PV, the attenuated vaccine viruses can also give rise to neurovirulent and transmissible vaccine-derived poliovirus (VDPV) strains which have caused outbreaks in 17 countries during the last 10 years [6]; [2]; [7]. As a “killed” vaccine, IPV is not associated with the risks of VAPP or VDPV outbreaks. Consequently, as the Global Polio Eradication Initiative (GPEI) approaches the end-game, there is a growing consensus that OPV vaccination must cease alongside a transition to worldwide IPV vaccination [3, 8-11].

Commercial IPVs are produced by incubation of live wild-type PV with formaldehyde for 12 days at 37°C [12]. Inactivation results in some loss of immunogenicity which may be due to the partial destruction of specific antigenic epitopes by formaldehyde [13, 14]. However, the molecular mechanisms responsible for the destruction of virus infectivity during inactivation are poorly understood.
Binding of PV to hPVR is the initial step in the viral replication cycle. The hPVR gene was characterised as CD155, a glycosylated single-span cell surface molecule belonging to the Ig super family [15, 16]. Cloning of the cDNA of CD155 has enabled the expression of soluble hPVR in relevant cell systems. Soluble hPVR preparations have been used in a range of biological and structural studies, including cryo-electron microscopy and three dimensional image-reconstruction techniques, to analyse the interaction between PV and hPVR [17-22]. Furthermore, L20B cells, transformed mouse Ltk cells that express the hPVR, were also developed [23]. Expression of the receptor at the cell surface, otherwise only present in primate cells, renders L20B cells susceptible to infection with PV.

After binding to hPVR, PV virions undergo a series of conformational changes to enter a host cell. Binding of PV to hPVR at physiological temperature catalyses conformational changes that result in the formation of altered particles. These particles sediment in sucrose gradients more slowly (135S) than native PV virions (160S) and thus are sometimes called 135S particles [24]. Following the formation of 135S particle PV undergoes a second conformational change where the viral RNA is ejected resulting in the production of an empty particle which sediments at 80S [25, 26]. The viral RNA is then released into the cytoplasm to initiate transcription and replication.

135S and 80S particles differ from mature PV virions in a number of characteristics. Both are antigenically distinct from native virus particles and are unable to bind to hPVR [25-29]. However, PV 135S and 80S particles differ in their sensitivity to RNase [27, 30]. It is known that viral RNA is still present and protected within 135S particles, while 80S particles either lack RNA or it is exiting from them, which exposes it to RNase [25, 31, 32]. The conformational changes that lead to 135S and 80S particles can be triggered in vitro in the absence of hPVR by incubating PV at super-physiological temperatures in a hypotonic medium [30, 31].
While a considerable amount of research has been carried out to explore the interaction between PV and hPVR and the entry of PV into cells, as of yet it has not been determined whether PV is still able to bind to the hPVR and can undergo the necessary conformational changes following treatment with formaldehyde. Previous observations in our laboratory and elsewhere indicated that antigenic site 1 of serotype 2 PV (PV2) is partially modified during inactivation with formaldehyde [13, 33, 34]. It is not clear whether such modifications may alter or disrupt the interaction between PV and the hPVR and subsequent steps in infection.

The aim of our work was to assess the effect of inactivation on PV biological properties that are essential for the early stages of PV replication. These include the interaction between PV and its cellular receptor, the ability of PV to undergo conformational changes and the integrity and infectivity of viral RNA. Our studies were conducted using purified live and inactivated PV samples, soluble hPVR protein preparations, L20B cells and a number of molecular, immunochemical and cellular techniques.

**MATERIALS AND METHODS**

**Cells**

HEp-2c, L20B, Ltk-, 293-T and 293-CD155-AP cells were used. 293-CD155-AP cells are transformed 293-T cells that express the human poliovirus receptor (CD155 or hPVR) protein in soluble form as a fusion protein also containing the sequence and activity of alkaline phosphatase (AP) enzyme. This fusion protein is hereby referred in this paper as hPVR-AP.

**Viruses and virus inactivation with formaldehyde**

The MEF-1 wild-type PV2 strain was used in all experiments except for the analyses by FACS flow cytometry because biocontainment requirements meant the Sabin 2 vaccine strain had to be used in these experiments. Stocks of PVs were grown in HEp-2C cells, purified by
ultracentrifugation on a 30% sucrose cushion and virus titres determined as previously described [35]. Virus titres were expressed as the log$_{10}$ of the 50% Tissue Culture Infectious Doses (TCID$_{50}$) per ml. Inactivated PV samples were prepared by incubation with formaldehyde (1:4000) at 37°C for 12 days following standard procedures described previously [14]. Briefly, virus preparations were resuspended in inactivation medium (Pasteur Merieux) to a final concentration of 5 × 10$^9$ TCID$_{50}$/ml and filtered through a 0.2 µm filter (PALL) prior to inactivation. Formaldehyde was added to the purified virus solutions to give a final dilution of 1:4000 of the concentrated stock. Inactivation was carried out for 12 days at 37°C in a constant-temperature water bath. At day 6 virus samples were again filtered through a 0.2 µm filter. At day 12 a 1:8 dilution of sodium bisulphite (35% w/v) (Sigma-Aldrich) was added to the inactivated PV preparations at a 1:100 ratio to neutralise any remaining HCHO.

**Determination of D-Antigen content of poliovirus preparations.**

The term D-Antigen refers to the antigen found in a sucrose gradient fraction that contains native virus particles, which are the target of neutralizing antibodies [36]. D-Antigen units were originally defined on the basis of an agar precipitin test performed with D-antigen-specific polyclonal sera. A vaccine preparation that produced a precipitin line at the distance of 25 millimetres from the centre was arbitrarily assigned a value of 600 D-Antigen units using a particular antibody at a particular concentration. This test was used in the initial calibration of reference materials. The D-Antigen content of PV samples is currently determined by an ELISA test [37]. Anti-PV2 MAbs used in ELISA tests were sourced from the collection at NIBSC. The Combistats statistical package was used to carry out the sigmoid curve ANOVA analysis on the assay data [38]. The European Pharmacopeia BRP batch 2 for IPV was used as the reference standard in these assays [39].

**Preparation and characterisation of soluble human poliovirus receptor**
293-CD155-AP cells were propagated and the supernatant containing hPVR-AP fusion protein in soluble form collected after 5 days. The hPVR-PA samples were assayed for specific activity by measuring the levels of alkaline phosphatase enzymatic activity and the neutralization titre against PV as described elsewhere [40]. The concentration of hPVR-AP protein was determined by using the Agilent High Sensitivity Protein 250 kit with the Agilent 2100 Bioanalyser (Agilent Technologies). Samples with the highest concentration/activity of hPVR-AP protein were chosen for further experiments.

**Analysis of the interaction between poliovirus and hPVR-AP by ultracentrifugation**

Live and inactivated PV preparations (50 D-Ag) were incubated with varying amounts of hPVR-AP protein samples (0.5-20 μg) in a total volume of 4.0 ml for 120 min at +4°C. The reaction samples were centrifuged through a 1.0 ml 30 % sucrose cushion (SW 50 Beckman rotor, 40,000 rpm, 240 min, +4°C). The resulting pellets were resuspended in Tris-HCl (0.01 M) and the amount of bound hPVR-AP was quantified by a colorimetric AP determination assay as described previously [40].

**Analysis of binding of poliovirus to L20B cells by FACS flow cytometry**

L20B or Ltk- cells (1x10^6 per reaction) were mixed with varying amounts of live or inactivated PV (1-100 D-Ag) in 1.0 ml MEM with 2 % FCS and incubated in suspension for 120 min at room temperature (RT, 18-20°C). Cells were pelleted (1105 g, 5 min, RT) and washed twice with PBS flow buffer (PBS with 5 % FCS and 1 % sodium azide, PFB). Cells were then incubated with anti-PV2 MAb 267, known to react equally with live and inactivated PV, in PBS for 30 min at RT. After this, cells were washed twice with PFB before being incubated with anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate (FITC) (Sigma-Aldrich) in PBS. After 20 min incubation in the dark at RT cells were washed twice with PFB before 200 μl of FACS FIX (150 ml PBS, 325 ml water and 25 ml formaldehyde) was added to each well. Cells were analysed using a BD FACS Canto II flow
cytometer (BD Biosciences) following manufacturer’s instructions. The mean fluorescence intensity and percentage of cells showing fluorescence levels above background were recorded.

**Analysis of binding of poliovirus to L20B cells by real-time RT-PCR**

This assay was based on that described by Jonsson *et al* [41]. L20B or Ltk- cells (2.5 x 10^5 per reaction) were mixed with varying amounts of live and inactivated PV (0.02-2 D-Ag) in 0.550 ml MEM with 2 % FCS and incubated in suspension for 120 min at RT. Cells were pelleted (425 g, 10 min) and washed twice with MEM with 2 % FCS before resuspension in MEM. Supernatants from the washing steps were harvested and pooled. Viral RNA was extracted from both the sedimented cells and the pooled supernatants and quantified by real-time RT-PCR as described below. Primers VP1-2521 and VP1-2737 (Table 1) giving a 200 nt DNA product were used in this case. In some experiments, PV samples were pre-incubated with soluble hPVR-AP or anti-PV MAbs before binding to cells.

**Poliovirus RNA extraction**

Poliovirus RNA was extracted from PV samples or PVs associated with cells using the Kingfisher automated particle processor (Thermo Electron Corporation) and a MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche) following manufacturer’s instructions. The concentration of extracted viral RNA samples was determined using a nanodrop spectrophotometer at 230 nm (NanoDrop® ND-1000 spectrophotometer, NanoDrop Technologies).

**Quantification of viral RNA by real-time RT-PCR**

Real-time RT-PCR assays were performed to quantify RT-PCR products obtained from viral RNA extracted from experimental PV or PV-cell samples. For this purpose, the Rotorgene 3000 platform and a QuantiTect® SYBR® Green RT-PCR kit (Qiagen) were used. Pairs of oligo-nucleotide primers, of equal length and temperature melting point, which yielded RT-
PCR products from MEF-1 PV genome between 200-800 nucleotides (nt) in size were designed using the online program “Primer3 Input version 0.4.0” [42] (Table 1). The genome copy number of RT-PCR products from the viral RNA samples was estimated using a calibration curve generated with MEF-1 cDNA of known concentration. The limit of detection of PV RNA in our real-time RT-PCR assay was between 2-3 logs of genome copy number, equivalent to the viral RNA content in 10^1-10^2 TCID₅₀ of live PV.

**Transfection of viral RNA from live and inactivated poliovirus**

The recovery of infectious PV from viral RNA was assessed by transfection [43] or electroporation [44] of HEp-2C monolayers followed by incubation at 35°C and 5% CO₂. The presence of virus cytopathic effect (CPE) was monitored for 7 days. In both experiments, serial dilutions of RNA from live PV were used as positive controls. The limit of detection in transfection/electroporation assays was that corresponding to the amount of RNA in 10^4 TCID₅₀ of live PV.

**In vitro conversion of poliovirus to 135S and 80S particles**

Conversion of PV to 135S and 80S particles was performed as described by Curry *et al.* [30]. Live and inactivated PV preparations (0.2 D-Ag) were diluted to a final volume of 50 µl with prewarmed 20 mM Tris–2 mM CaCl₂–0.1% Tween 20 (pH 7.5) (TCT) buffer and incubated at 50°C or 60°C for 3 min or 20 min, respectively.

**Sensitivity of poliovirus particles to RNase treatment**

The sensitivity of PV to RNase A was used as a measure of the presence of viral RNA in PV particles. Treated and untreated live and inactivated PV samples diluted to 0.2 D-Ag / 50 µl in TCT buffer were incubated with RNase A (0.001 µg / µl, Sigma-Aldrich) at 50°C or 60°C for 3 min or 20 min, respectively. PV preparations were then placed on ice and RNase inhibitor (4 U/µl, Roche) was added. Viral RNA was extracted from the treated samples and
PCR products quantified by real-time RT-PCR as described above. Primers VP1-2521 and VP1-2737 (VP1 gene, Table 1) giving a 200 nt DNA product were used.

RESULTS

Inactivation of poliovirus with formaldehyde

Live serotype 2 PV samples (5x10⁹ TCID₅₀/ml) were inactivated by incubation with formaldehyde (1:4000) at 37°C for 12 days in 25 ml reactions. The absence of infectious virus in sequential aliquots and inactivated samples was confirmed by virus titration assays in HEp-2C cells and by the addition and passage of treated virus samples on HEp-2C cell monolayers for periods of up to three weeks. The results showed that infectivity was not detected at or after 120 hours of incubation with formaldehyde (data not shown). The D-Ag content of the laboratory inactivated PV samples was determined by ELISA using a standard assay. Inactivated PV preparations contained approximately 250 D-Ag/ml. There was no significant change in D-Ag content before and after inactivation (data not shown).

Analysis of PV interaction with soluble hPVR-AP by ultracentrifugation

Laboratory preparations of expressed soluble hPVR-AP protein were obtained as described in Materials and Methods. Samples containing approximately 5 µg/ml of hPVR-AP were capable of neutralising between 4.0 and 5.0 log₁₀ TCID₅₀ of PV in cell culture assays. No anti-PV activity was detected in cell supernatants from 293-T cells which did not express hPVR-AP.

To analyse the interaction between PV and soluble hPVR-AP, samples containing soluble hPVR-AP were incubated at +4°C for 120 min in the presence or absence of PV and then the reaction samples were subjected to ultracentrifugation through a 30% sucrose cushion in conditions that PV is sedimented in the pellet and soluble hPVR-AP is not. The presence of hPVR-AP bound to PV in the pellet was measured by an AP enzymatic assay.
Preliminary experiments were performed by mixing live PV2 (50 D-Ag) with varying amounts of soluble hPVR-AP (0.5-20 µg) (Fig. 1A). Non-saturating conditions were then used to compare binding of live and inactivated PV with hPVR-AP (50 D-Ag PV + 10 µg hPVR-AP). As shown in Fig. 1B, both live and inactivated PV bound to soluble hPVR-AP whereas only background AP activity was detected in the pellet in the absence of PV. The results suggested that, in these conditions, inactivated PV bound approximately 31% less amount of hPVR-AP than live PV.

Analysis of poliovirus interaction with L20B cells by FACS flow cytometry

To further assess the effect of inactivation on the ability of PV to bind hPVR, a FACS flow cytometry assay which specifically detects PV bound to cells was designed. As shown in Fig. 2A, cells incubated with either live or inactivated PV showed increased fluorescence in FACS assays in a virus dose-dependent manner demonstrating that both live and inactivated PV were able to bind to L20B cells. The results were quantified and expressed both as the mean fluorescence intensity shown by cells after incubation with virus and the percentage of cells showing fluorescence above background levels (Fig. 2). The results suggested that inactivated PV bound less efficiently to L20B cells than live PV. For example, when cells were incubated with 10 D-Ag of either virus preparation, the cells treated with inactivated PV showed reduced mean fluorescence intensity (31%) and percentage of fluorescent cells (29%) than those shown by cells incubated with live PV.

Our results indicated that PV binding to L20B cells was likely due to interaction of PV with hPVR expressed on the cell surface as no PV binding to Ltk- cells, which lack expression of hPVR, was observed at any virus concentration. Ltk- cells in any combination with live or inactivated PV showed fluorescence levels similar to those shown by Ltk- or L20B cells alone (≤ 70 fluorescence intensity units). Furthermore, as shown in Fig. 2C, addition of soluble hPVR-AP prevented virus binding to L20B cells, as a reduction of 84%
and 93% in fluorescence levels was observed for cells mixed with inactivated PV and live PV
that had been pre-incubated with soluble hPVR-AP, respectively.

**Analysis of poliovirus interaction with L20B cells by real-time RT-PCR**

The interaction between live and inactivated PV and L20B cells was also analysed using a
real-time RT-PCR assay that measured the amount of viral RNA associated to cells. As
shown in Fig. 3A, both live and inactivated PV were able to bind to L20B cells as judged by
this assay. As with the results in the FACS flow cytometry assays shown above, inactivated
PV appeared to bind less efficiently to L20B cells showing a 51-78% reduction in binding
with respect to live PV. Again, the results indicated that PV binding to L20B cells was likely
due to interaction of PV with hPVR expressed on the cell surface as pre-incubation of PV
with soluble hPVR-AP or anti-PV2 MAbs prevented any significant interaction of PV with
L20B cells (Fig. 3B). Interestingly, MAb 436 failed to inhibit binding of inactivated PV to
L20B cells but did block the interaction between live PV and L20B cells. This was not
unexpected as MAb 436 (site 1) did not react with PV in ELISA following inactivation (data
not shown) which indicates that antigenic site 1 was partially modified by formaldehyde as
previously shown [13]. This result was further confirmation that our assay was measuring the
physiological interaction between PV and hPVR. Furthermore, MAb 520, specific for
serotype 3 PV, was not able to prevent binding of either live or inactivated PV to L20B cells.

**In vitro conversion of poliovirus to 135S and 80S particles**

To assess the effect of inactivation on the ability of PV to undergo essential
conformational changes, purified live and inactivated PV samples were incubated at super-
physiological temperatures in a hypotonic medium to trigger such changes. The degree of
conversion of the treated PV samples to 135S and 80S virus particles was measured by
characterising their antigenic properties in ELISA, assessing their ability to bind to L20B
cells in real-time RT-PCR assays and determining the presence of viral RNA in the treated
viral particles by measuring their sensitivity to RNase A using a real-time RT-PCR assay. Following incubation at 50°C, live PV exhibited properties characteristic of 135S particles as they lost the ability to interact with anti-PV2 MAbs (Fig. 4). Additionally, live PV treated at 50°C lost the ability to bind to L20B cells but retained viral RNA in the virus particle, also properties associated with 135S particles (Table 2). Conversely, inactivated PV was still able to interact with anti-PV2 MAbs (Fig. 4) and bind to L20B cells (Table 2) following incubation at 50°C suggesting that inactivated PV had failed to convert to 135S particles in these conditions and maintained the native 160S state. As expected, inactivated PV samples treated at 50°C also contained viral RNA. Following incubation at 60°C, as shown in Fig 4 and Table 2, neither live nor inactivated PV retained any antigenicity or cell-binding activity. No viral RNA could be detected in any of the two preparations in these conditions. These properties are similar to those of 80S PV particles which suggest that both live and inactivated PV treated at 60°C had undergone the conformational changes to reach this state.

Effect of inactivation on viral RNA

The effect of inactivation with formaldehyde on PV RNA was assessed in a series of real-time RT-PCR assays which were used to estimate the PV genome copy number present in sequential virus samples taken during the inactivation process. Primers which amplified RT-PCR products of different sizes were used (Table 1). As shown in Table 3, the yield of RT-PCR DNA product decreased as incubation with formaldehyde progressed. Results were similar when target sequences in three different genomic regions were analysed: 5’NCR, VP1 and 3’NCR. The reduction in DNA yields was more obvious when larger DNA fragments were amplified. However, it was possible to obtain RT-PCR products from all laboratory and commercial inactivated IPV samples tested. Nucleotide sequence analysis of the DNA
products confirmed the successful extraction of viral RNA from these samples (data not shown).

The chemical alteration of viral RNA induced by formaldehyde appeared to be partially reversible as RT-PCR yields increased up to 15 times after incubation of RNA at 70°C prior to the RT-PCR assays (Fig. 5). This was determined by analysis of RT-PCR products by agarose gel electrophoresis and real-time RT-PCR using primers VP1-2521 and VP1-3125 (Table 1) that gave a 600 nt DNA product.

Additionally, viral RNA from inactivated PV appeared not to be biologically active as no infectious PV was recovered from cells transfected/electroporated with viral RNA from inactivated PV even after pre-treatment of viral RNA at 70°C for 60 min. Only viral RNA from samples taken before or at 12 hours of incubation with formaldehyde led to CPE and virus recovery when transfected into susceptible cells (data not shown).

DISCUSSION

Inactivation with formaldehyde is commonly used for the production of commercial human and animal viral vaccines such as those against polio, hepatitis A, Enterovirus 71 and influenza viruses [45-47]. However, little is known about the molecular mechanisms underlying virus inactivation by formaldehyde. As shown in this paper, inactivation of PV with formaldehyde did not prevent PV interacting with hPVR, the first step required for PV infection. Inactivated PV was able to interact with soluble hPVR-AP and bind to L20B cells, although at an apparent reduced efficiency with respect to live PV. The degree of the reduction in binding observed between inactivated and live PV varied depending on the experiment used. This might be due to the different nature of the experiments which
measured either the quantity of virus bound to soluble hPVR-AP or L20B cells using an enzymatic or immunofluorescence assay, respectively, or the amount of viral RNA associated to cells using a real-time RT-PCR method. As expected, binding of PV to L20B cells was specifically inhibited by pre-incubation of PV with anti-PV antibodies or soluble hPVR-AP. However, MAb 436 which did not interact with inactivated PV in ELISA could not inhibit binding of inactivated PV to cells.

The reasons and significance of the observed differences in binding to hPVR between live and inactivated PV are not clear. Treatment with formaldehyde is known to result in the alteration of antigenic epitopes of PV, particularly in antigenic site 1 [13, 34]. Antigenic site 1, modified in inactivated PV, is located in the BC loop at the north rim of the canyon, a narrow surface depression that surrounds the 5-fold axis of symmetry and interacts with hPVR [48-52]. Viral variants that are adapted to grow in cells expressing mutant forms of hPVR contain mutations at the BC loop [19]. Although amino acids in the BC loop are located outside the footprint of the receptor, it has been proposed that this sequence might modulate the flexibility of the capsid to facilitate the recognition and binding of PV to hPVR. Thus, chemical crosslinking between capsid proteins and/or viral RNA induced by formaldehyde could prevent effective binding of inactivated PV to hPVR by restricting capsid structural movements [53].

The possibility that treatment with formaldehyde affects the flexibility of the PV capsid is consistent with our observation that inactivated PV was more stable at high temperatures than live PV. Incubation of live PV in hypotonic solution at 50°C led to conformational changes typical of PV 135S particles: loss of antigenic properties and loss of ability to bind to hPVR but retention of viral RNA. However, when treated in the same conditions, inactivated PV maintained the ability to bind to MAbs and L20B cells, similarly to untreated 160S native PV particles. Conversion to 135S is an essential step for PV
infection [24] and its alteration or inhibition by inactivation could be a reason for the loss of infectivity shown by inactivated PV. Recent results by Lin et al. [54] using immune-cryogenic electron microscopy, suggest that a neutralizing MAb (C3) inhibits infection by poliovirus by binding two adjacent BC loops which restricts capsid movements inhibiting conformational changes in the viral particle [54]. However, it remains to be established whether inactivated PV can enter susceptible cells and release viral RNA into the cytoplasm. Recent results by Martin-Acebes et al. [55] have shown that Foot-and-Mouth-Disease-Virus (FMDV) inactivated with binary ethylenimine can bind to BHK-21 cells. Similar to the results shown here, binding of inactivated particles to BHK-21 cells was blocked by pre-incubation with either a FMDV-specific MAb or a synthetic peptide spanning the integrin-binding viral motif Arg-Gly-Asp (RGD). Furthermore, FMDV virus particles were internalized through endocytosis but the fate of viral RNA was not analysed.

As discussed above, it is well established that treatment with formaldehyde results in alteration of the antigenic structure of PV as it has been shown for several other viruses such as herpes virus-1, Newcastle disease virus, influenza virus and Rift Valley fever virus [56-59]. The effect of formaldehyde on viral RNA is not so clear. We and others have shown that the release of viral RNA from inactivated PV particles using phenol-chloroform standard methods was severely impaired [14, 53]. This is thought to be due to crosslinking between viral proteins and RNA and can be compared with the poor extraction of RNA from formalin-fixed tissue samples [60]. Formalin-fixed tissues are generally resistant to solubilisation by chaotrophic agents such as phenol or guanidinium thiocyanate. However, Masuda et al. [61] found that pre-treatment with proteinase K completely solubilised fixed tissues and enabled the extraction of almost the same amount of RNA as that from fresh samples [61]. In their example, the extracted RNA did not show apparent degradation but successful RT-PCR amplification was still limited to short targets. Resistance to RT-PCR amplification was
found to be due to chemical modification of RNA templates as all four nucleoside bases showed addition of mono-methylol groups that probably interfered with RT polymerase activity [61]. It was also shown that these chemical modifications induced by formaldehyde could be partially removed at high temperature (70°C). Our results showed that a similar effect on PV RNA had likely occurred following incubation of PV with formaldehyde. After extraction of viral RNA using proteinase K-based assays we were able to obtain RT-PCR DNA products from both laboratory and commercially inactivated PV samples. Similar to RNA extracted from formalin-fixed tissue samples, RNA from inactivated PV showed partial resistance to RT-PCR amplification. The chemical modifications in RNA molecules from inactivated PV appeared to be randomly distributed throughout the PV genome as no differences were found in the quantity of amplified DNA products from different genomic regions. As in the example above, the RNA modifications induced by formaldehyde could be partially removed as increased RT-PCR yields were obtained by incubating RNA from inactivated PV at 70°C prior to RT-PCR amplification. It is likely that changes induced by formaldehyde were also responsible for rendering PV RNA non-infectious as it was not possible to recover infectious PV by transfection/electroporation of RNA from inactivated PV samples into susceptible cells, even after pre-treatment of viral RNA at 70°C. Thus, the destruction of the biological activity of viral RNA by chemical alteration induced by treatment with formaldehyde could be another reason why inactivated PV is non-infectious.

We conclude that inactivation with formaldehyde reduces the ability of PV to bind to hPVR, decreases the sensitivity of PV to convert to 135S particles and abolishes the infectivity of its viral RNA. The results and techniques shown here could be useful to help developing improved methods for IPV production and quality control. Methods that result in the modification of viral nucleic acids with minimal alteration of viral proteins are the ideal mechanisms of virus inactivation for vaccine production as they ensure the permanent
destruction of virus infectivity but the preservation of vaccine immunogenicity. This is particularly relevant at present as inactivated polio vaccines are going to be the vaccines of choice for the endgame of polio eradication and changes in the manufacturing process such as the use of different poliovirus strains as vaccine seeds are expected. Manufacturers are also considering the use of alternative chemicals such as beta-propiolactone and binary ethyleneimine for inactivation as they are used for the production of other viral vaccines such as influenza, rabies and foot-and-mouth disease virus [62-64]. Analysis of the protein and RNA viral properties as shown here, could help assessing the impact of changes in antigenicity and stability of candidate vaccines as well as detecting residual infectivity during the inactivation process. This would help finding the optimal balance between loss of infectivity and preservation of virus antigenicity during inactivation and comparing the quality of different vaccine preparations.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Steffen Mueller and Dr Eckard Wimmer (State University of New York, Stony Brook, NY) for providing us with 293-CD155-AP cells.

REFERENCES


**FIGURE LEGENDS**

**Figure 1.** Analysis of the interaction between PV and soluble hPVR-AP. (A) Live PV2 (50 D-Ag) was incubated with soluble hPVR-AP (0.5-20 μg) at +4°C for 120 min. Samples were then ultracentrifuged through a 30% sucrose cushion. The resulting pellets were
resuspended in Tris-HCl and the amount of bound CD155-AP was quantified by a colorimetric AP reaction. The average OD values at 405 nm of two determinations are shown with the standard error. (B) Live and inactivated MEF-1 preparations (50 D-Ag) were incubated with soluble hPVR-AP (10 μg) and processed as described above. The results were compared to those with samples centrifuged with soluble hPVR-AP alone (lane -). The average OD values at 405 nm of three determinations are shown as bars with standard errors.

Figure 2. Quantification of binding of poliovirus to L20B cells by FACS flow cytometry. Samples containing 1-100 D-Ag of live (grey columns) or inactivated (white columns) PV were mixed with 10^6 L20B or Ltk- cells followed by incubation with anti-PV2 MAb 267 and FITC-labelled anti-mouse IgG. For each sample, the fluorescence of 10^4 cells was measured by FACS flow cytometry analysis. The mean fluorescence intensity (A) and the proportion of cells showing fluorescence levels above background (B) are shown. The data represent results from three independent experiments. The average values are shown as columns. Standard deviations are indicated as error bars. In (C), 10 D-Ag of live or inactivated PV were incubated with 10^5 L20B cells in the presence or absence of soluble hPVR-AP (5 μg/ml) before addition of MAb 267 and FITC-labelled anti-mouse IgG. Histograms show the fluorescence intensity on the surface of 10^4 L20B cells measured by FACS flow cytometry analysis. The results represent values from single data points of duplicate assays with comparable results.

Figure 3. Quantification of binding of poliovirus to L20B cells by real-time RT-PCR. (A) Samples containing 0.02-2 D-Ag of live (grey columns) or inactivated (white columns) PV were mixed with 2.5x10^5 L20B or Ltk- cells. Following incubation at RT, cells were sedimented by centrifugation and the amount of associated PV quantified by a real-time RT-
PCR. The estimated amount of PV bound to cells is shown. (B) Samples containing 0.2 D-Ag of live (grey columns) or inactivated (white columns) PV were mixed with 2.5x10^5 L20B cells in the presence or absence of soluble hPVR-AP (5 μg/ml) or anti-PV MAb. The antigenic site specificity of anti-PV2 MAb is indicated in brackets. The data represent results from two independent experiments. The average values of the percentage of binding inhibition are shown as columns. Standard errors are indicated as error bars.

**Figure 4. Antigenic properties of native and heat-treated poliovirus.** The reactivity of live (light lines) and inactivated (dark lines) PV samples with anti-PV2 MAb was measured by ELISA. Serial dilutions of PV samples were added to 96-well plates covered with capture anti-PV2 polyclonal antibody. Anti-PV2 MAb 267 (A) or 1050 (B) was then added and the reactivity assessed by measuring the chemical reaction following incubation with peroxidase-conjugated anti-mouse IgG. The graph shows OD average values of duplicate wells at 492 nm of PV samples incubated at 20°C (solid lines), 50°C (dashed lines), or 60°C (dotted lines).

**Figure 5. Demodification of viral RNA from inactivated poliovirus.** Aliquot samples containing viral RNA extracted from inactivated PV (0.2 D-Ag/sample) were incubated at RT or 70°C for periods ranging from 10 to 120 min. The DNA products obtained by RT-PCR were analysed by agarose-gel electrophoresis (A) and real-time RT-PCR (B). Primers VP1-2521 and VP1-3125, giving a DNA product of approximately 600 nt in size, were used. The data represent results from three independent experiments. The average genome copy number values are shown. Standard errors are indicated as error bars. M in A indicates DNA marker. Open circles in B show results at RT and closed circles indicate increase in DNA product (%) after incubation at 70°C.
TABLE 1. Nucleotide sequences of primers used for RT-PCR reactions.

<table>
<thead>
<tr>
<th>Region</th>
<th>Orientation</th>
<th>Code</th>
<th>Primer sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'NCR</td>
<td>Sense</td>
<td>5NCR-44</td>
<td>GCG GCC AGT ACA CTG GTA TT</td>
<td>44-63</td>
</tr>
<tr>
<td>5'NCR</td>
<td>Sense</td>
<td>5NCR-153</td>
<td>ACT GGT TTG TAC CCC CTC CT</td>
<td>134-153</td>
</tr>
<tr>
<td>5'NCR</td>
<td>Sense</td>
<td>5NCR-252</td>
<td>TCT CGA AGT ACA TGA GCG GAT A</td>
<td>231-252</td>
</tr>
<tr>
<td>Antisense</td>
<td>5NCR-455</td>
<td>GCC GGA GGA CTC TCA GGT A</td>
<td>437-455</td>
<td></td>
</tr>
<tr>
<td>5'NCR</td>
<td>Sense</td>
<td>5NCR-654</td>
<td>ATC AAA TTC TCA CCG GAT GG</td>
<td>635-654</td>
</tr>
<tr>
<td>5'NCR</td>
<td>Sense</td>
<td>5NCR-861</td>
<td>GCT CGC AGA ATC CCT GGA ATA A</td>
<td>840-861</td>
</tr>
<tr>
<td>VP1</td>
<td>Sense</td>
<td>VP1-2521</td>
<td>ACG AGA AAT GCC TTG ACA CC</td>
<td>2521-2540</td>
</tr>
<tr>
<td>VP1</td>
<td>Sense</td>
<td>VP1-2737</td>
<td>AAG CTC CTC TTG CGA AGA AA</td>
<td>2718-2737</td>
</tr>
<tr>
<td>VP1</td>
<td>Antisense</td>
<td>VP1-2937</td>
<td>ATT TAG TGC GTG CCC ATT GT</td>
<td>2918-2937</td>
</tr>
<tr>
<td>VP1</td>
<td>Antisense</td>
<td>VP1-3125</td>
<td>ACT TTG GCA AAC CCA TCG TA</td>
<td>3106-3125</td>
</tr>
<tr>
<td>VP1</td>
<td>Antisense</td>
<td>VP1-3338</td>
<td>TAG TCA ACC CCT GGT CCG TA</td>
<td>3319-3338</td>
</tr>
<tr>
<td>3'NCR</td>
<td>Antisense</td>
<td>3NCR-7412</td>
<td>ACA ACA GTA TGA CCC AAT CCA A</td>
<td>7391-7412</td>
</tr>
<tr>
<td>3'NCR</td>
<td>Antisense</td>
<td>3NCR-7288</td>
<td>AAG ATT AGA AGT GTG CCA ATC G</td>
<td>7288-7309</td>
</tr>
<tr>
<td>3'NCR</td>
<td>Antisense</td>
<td>3NCR-7201</td>
<td>AGA TCC CAG AAA CAC TCA GGA T</td>
<td>7201-7221</td>
</tr>
<tr>
<td>Sense</td>
<td>3NCR-7011</td>
<td>CTA GCC CAA TCA GGA AAA GAC T</td>
<td>7011-7033</td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>3NCR-6782</td>
<td>ATT ATC TGA ATC ACT CGC ACC A</td>
<td>6782-6803</td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>3NCR-6583</td>
<td>AAG AAC CCA GGT GTA GTG ACA A</td>
<td>6583-6604</td>
<td></td>
</tr>
</tbody>
</table>

* Nucleotide position in the MEF-1 genome (Gene accession: AY238473)

TABLE 2. Characterisation of viral RNA from heat-treated PV samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Viral RNA from PV associated with L20B cells</th>
<th>Viral RNA following treatment of PV with RNase A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 °C</td>
<td>60 °C</td>
</tr>
<tr>
<td>Live PV</td>
<td>1.4 ± 1.0</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Inactivated PV</td>
<td>67.2 ± 17.1</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>
The amount of viral RNA was quantified by real-time RT-PCR as. Numbers in the table represent the genome copy number as a percentage relative to that obtained with an equivalent amount of untreated PV.

Data represents results from two independent experiments with the standard deviation.

**TABLE 3.** Quantification of viral RNA in PV samples by real-time RT-PCR

<table>
<thead>
<tr>
<th>Sample</th>
<th>Aliqt. (hour)</th>
<th>[RNA] (ng/μl)</th>
<th>5' NCR (nt)</th>
<th>VP1 (nt)</th>
<th>3' NCR (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>200 400 600 800</td>
<td>200 400 600 800</td>
<td>200 400 600 800</td>
</tr>
<tr>
<td>Live PV</td>
<td>0</td>
<td>31</td>
<td>9.0 9.2 8.9 8.5</td>
<td>8.6 8.8 8.2 8.1</td>
<td>9.1 8.9 8.8 8.2</td>
</tr>
<tr>
<td>IPV</td>
<td>12</td>
<td>21</td>
<td>8.8 8.6 8.2 7.6</td>
<td>9.0 9.0 8.4 8.2</td>
<td>9.1 8.6 8.4 8.0</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>25</td>
<td>8.8 8.4 7.8 7.3</td>
<td>8.6 8.8 7.9 7.5</td>
<td>8.9 8.5 8.0 7.0</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>21</td>
<td>8.6 8.0 7.4 6.6</td>
<td>8.5 8.5 7.4 7.4</td>
<td>8.8 8.1 7.6 6.6</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>25</td>
<td>8.8 8.1 6.8 5.5</td>
<td>8.8 8.4 7.1 6.3</td>
<td>8.5 7.9 6.8 5.8</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>28</td>
<td>8.4 7.5 6.1 4.7</td>
<td>8.2 7.9 6.3 5.3</td>
<td>8.6 7.4 6.1 4.8</td>
</tr>
<tr>
<td></td>
<td>288</td>
<td>26</td>
<td>8.2 7.1 5.7 4.0</td>
<td>7.9 7.4 5.4 4.4</td>
<td>8.2 6.9 5.5 4.0</td>
</tr>
</tbody>
</table>

*The location in the genome and approximate size in nt of the amplified DNA is indicated. Values are coloured from high (green) to medium (yellow) and low (red).*

---

a The location in the genome and approximate size in nt of the amplified DNA is indicated. Values are coloured from high (green) to medium (yellow) and low (red).
Figure 1. Analysis of the interaction between PV and soluble hPVR-AP. (A) Live PV2 (50 D-Ag) was incubated with soluble hPVR-AP (0.5-20 μg) at +4°C for 120 min. Samples were then ultracentrifuged through a 30% sucrose cushion. The resulting pellets were resuspended in Tris-HCl and the amount of bound CD155-AP was quantified by a colorimetric AP reaction. The average OD values at 405 nm of two determinations are shown with the standard error. (B) Live and inactivated MEF-1 preparations (50 D-Ag) were incubated with soluble hPVR-AP (10 μg) and processed as described above. The results were compared to those with samples centrifuged with soluble hPVR-AP alone (lane -). The average OD values at 405 nm of three determinations are shown as bars with standard errors.
Figure 2. Quantification of binding of poliovirus to L20B cells by FACS flow cytometry. Samples containing 1-100 D-Ag of live (grey columns) or inactivated (white columns) PV were mixed with 10^6 L20B or Ltk- cells followed by incubation with anti-PV2 MAb 267 and FITC-labelled anti-mouse IgG. For each sample, the fluorescence of 10^4 cells was measured by FACS flow cytometry analysis. The mean fluorescence intensity (A) and the proportion of cells showing fluorescence levels above background (B) are shown. The data represent results from three independent experiments. The average values are shown as columns. Standard deviations are indicated as error bars. In (C), 10 D-Ag of live or inactivated PV were incubated with 10^6 L20B cells in the presence or absence of soluble hPVR-AP (5 μg/ml) before addition of MAb 267 and FITC-labelled anti-mouse IgG. Histograms show the fluorescence intensity on the surface of 10^4 L20B cells measured by FACS flow cytometry analysis. The results represent values from single data points of duplicate assays with comparable results.
Figure 3. Quantification of binding of poliovirus to L20B cells by real-time RT-PCR.

(A) Samples containing 0.02-2 D-Ag of live (grey columns) or inactivated (white columns) PV were mixed with 2.5x10^5 L20B or Ltk- cells. Following incubation at RT, cells were sedimented by centrifugation and the amount of associated PV quantified by a real-time RT-PCR.

(B) Samples containing 0.2 D-Ag of live (grey columns) or inactivated (white columns) PV were mixed with 2.5x10^5 L20B cells in the presence or absence of soluble hPVR-AP (5 μg/ml) or anti-PV MAbs. The antigenic site specificity of anti-PV2 MAbs is indicated in brackets. The data represent results from two independent experiments. The average values of the percentage of binding inhibition are shown as columns. Standard errors are indicated as error bars.
Figure 4. Antigenic properties of native and heat-treated poliovirus. The reactivity of live (light lines) and inactivated (dark lines) PV samples with anti-PV2 MAbs was measured by ELISA. Serial dilutions of PV samples were added to 96-well plates covered with capture anti-PV2 polyclonal antibody. Anti-PV2 MAb 267 (A) or 1050 (B) was then added and the reactivity assessed by measuring the chemical reaction following incubation with peroxidase-conjugated anti-mouse IgG. The graph shows OD average values of duplicate wells at 492 nm of PV samples incubated at 20°C (solid lines), 50°C (dashed lines), or 60°C (dotted lines).
Figure 5. Demodification of viral RNA from inactivated poliovirus. Aliquot samples containing viral RNA extracted from inactivated PV (0.2 D-Ag/sample) were incubated at RT or 70°C for periods ranging from 10 to 120 min. The DNA products obtained by RT-PCR were analysed by agarose-gel electrophoresis (A) and real-time RT-PCR (B). Primers VP1-2521 and VP1-3125, giving a DNA product of approximately 600 nt in size, were used. The data represent results from three independent experiments. The average genome copy number values are shown. Standard errors are indicated as error bars. M in A indicates DNA marker. Open circles in B show results at RT and closed circles indicate increase in DNA product (%) after incubation at 70°C.
AUTHOR CORRECTION

Correction for Wilton et al., Effect of Formaldehyde Inactivation on Poliovirus

Thomas Wilton,§ Glynis Dunn,ª David Eastwood,ª Philip D. Minor,ª Javier Martinª
Divisions of Virology,ª and Biotherapeutics,ª National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, United Kingdom

Volume 88, no. 20, p. 11955–11964. Page 11955: The byline should appear as shown above.

Page 11955, column 2, shaded box: The corresponding author e-mail and present address footnote should read “t.a.wilton@btinternet.com” and “*Present address: Thomas Wilton, Crucell Holland B.V., Leiden, Netherlands.”