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Citrate-mediated disaggregation of rotavirus particles in RotaTeq® vaccine

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Abstract

For the routine manufacture of live virus vaccines, virus is diluted into a formulation buffer to stabilize it for long-term storage, and to facilitate vaccine administration. The characteristics of this buffer are dependent on the storage temperature of the vaccine, as well as the desired characteristics of the product. The formulation buffer for RotaTeq®, Merck's live, pentavalent, oral rotavirus vaccine to prevent rotavirus gastroenteritis was developed as a fully liquid solution that requires no pre-feeding prior to administration, and is stable for 24 months at refrigerated temperatures. In studying the effects of the formulation buffer on the live virus contained within RotaTeq®, we observed that the formulation buffer also directly impacts the state of rotavirus aggregation. This observation, termed "the matrix effect," was first noted as an ~50% increase in measured in vitro infectivity, following dilution of the virus into the buffer. Subsequent experiments confirmed that citrate in the formulation buffer facilitates the disaggregation of viral particles, likely through a carboxylic-acid mediated interaction. For vaccine manufacture, bulk virus is titered and subsequently diluted to a target concentration for dosing. Aggregation of the virus and subsequent inaccurate measurement of the amount of virus contained in either the bulk sample or in the final dosing container could lead to an inability to accurately predict final vaccine concentrations. Thus, discerning the nature and extent of the matrix effect was key principally for providing an accurate prediction of final virus concentration upon dilution, to ensure a robust manufacturing process. In addition, understanding potential contributions of the formulation buffer to clinical efficacy of the vaccine was critical. Clinical data have confirmed that the citrate-mediated disaggregation had no measurable impact on vaccine safety, immunogenicity, or efficacy.

Keywords: Rotavirus vaccine; RotaTeq®; Formulation; Citrate; Disaggregation

1. Introduction

Rotavirus is a major causative agent of infantile gastroenteritis, and infects most children worldwide before the age of five. The disease causes considerable mortality, mostly in the developing world (~500,000 deaths annually), and significant morbidity in the United States and Europe (Parashar et al., 2003). Rotavirus is a triple-layered, non-enveloped, double-stranded (ds) RNA virus in the family Reoviridae (Estes, 2001). The rotavirus genome consists of 11 segments of dsRNA, most coding for a single gene product, and reassortment of these segments on co-infection in vivo is a significant source of evolution of the virus (Estes, 2001; Yolken et al., 1988). The two outer-most structural proteins of the virus, VP7 and VP4, elicit independent neutralizing antibodies and are used to classify the serotypes of the virus (Ciarlet and Estes, 2002). The VP7 classification is designated as the "G" type (for glycoprotein), and the VP4 classification is designated as the "P" type (for protease-sensitive). To date, 10 G serotypes and 11 P serotypes have been identified for human rotaviruses (Ciarlet and Estes, 2002; Hoshino et al., 2002).

RotaTeq® is Merck's live, pentavalent, oral vaccine against rotavirus gastroenteritis, and has been shown to be generally well-tolerated and effective in large-scale clinical trials (Clark et al., 2003, 2004a; Vesikari et al., 2005a,b; Itzler et al., 2005). The vaccine consists of five human-bovine reassortants, generated by co-infection of cell culture with human and bovine (WC3) strains, and subsequent reassortment of the segmented genome (Clark et al., 1996). Each of the vaccine's five reassortants shares most of its genes with the parental bovine (WC3) strain. The exceptions are VP4 and VP7, which are not in com-

Abbreviations: MOI, multiplicity of infection; M-QPA, multivalent quantitative PCR-based cell infectivity assay; TNC, Tris, sodium chloride, calcium buffer; PFU, plaque forming unit; IU, infectious unit; R.S.D., relative standard deviation; EM, electron microscopy; DLS, dynamic light scattering; S.D., standard deviation; TLP, triple-layered particle; DLP, double-layered particle

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mon among all five reassortants. Four of the five reassortants contain a gene for VP7 that comes from different human viruses, whereas the fifth reassortant contains a gene for VP4 that comes from a human virus. The serotypes of the viruses contained within the vaccine are [P7, G1], [P7, G2], [P7, G3], [P7, G4], and [P1A, G6]. For convenience, the five human-bovine reassortants are referred to here as G1, G2, G3, G4, and P1, respectively.

For vaccine manufacture, these five reassortant viruses are grown in culture, harvested and processed, and diluted into a formulation buffer for shelf-life storage and administration. The formulation buffer contains sucrose, sodium citrate, sodium phosphate, polysorbate-80, and tissue culture media (Burke and Volkin, 1999). The precise composition of this buffer was devised to neutralize the gastric contents of the vaccinee, and subsequently eliminate the need for pre-feeding of infants prior to vaccination (Clark et al., 2003). Both the pH of the vaccine formulation and the presence of the sodium citrate contribute to the neutralizing capacity of the formulation. The formulation buffer was designed to stabilize the virus for long-term storage at 2–8 °C.

Characterization work has now been completed to further understand how the formulation buffer impacts the viral reassortants in RotaTeq®. The work here describes a phenomenon in which the formulation buffer causes an increase in measured infectivity in vitro for each of the five reassortant viruses contained within the vaccine. The mechanisms for this observation as well as implications are discussed.

2. Materials and methods

2.1. Viruses, cells, and the formulation buffer

The five viral reassortants, WI79-9, SC2-9, WI78-8, BrB-9, and WI79-4 (designated G1, G2, G3, G4, and P1, respectively for simplicity) were generated as described using bovine and human parental strains (Clark et al., 1996; Offit et al., 2003). All reassortants are composed of the bovine rotavirus strain WC3 (P7[5],G6) genome background expressing human outer capsid glycoproteins of G1 (derived from the G1 human rotavirus [HRV] strain WI79), G2 (derived from the G2 HRV strain SC2), G3 (derived from the G3 HRV strain WI78), or G4 (derived from the G4 HRV strain BrB) serotype specificity, or expressing the human spike protein of P1A[8] (derived from the P1A[8] HRV strain WI79) serotype specificity (Clark et al., 1996; Offit et al., 2003). Viral samples for analysis were produced by infecting African green monkey kidney Vero cells in the presence of varying concentrations of trypsin. Three days post-infection, the virus was harvested and further processed. Processing included freeze/thaw of the cell lysates, filtration, and concentration. The final concentrated virus was in a mixture of cell lysate and tissue culture media, and was stored at −70 °C for subsequent use.

The standard formulation buffer into which the virus is diluted for final vaccine administration has varied throughout clinical development of the vaccine, and includes sucrose, sodium citrate, sodium phosphate, polysorbate-80, and tissue culture media. The buffer and its development and preparation is described in US Patent 5,932,223 (Burke and Volkin, 1999).

2.2. Purification of virus by CsCl gradient

Viral purification from the concentrated cell lysates was carried out as described in Patton et al. (2000) with minor modifications. The viral particles were pelleted from the supernatant using an SW28 rotor at 26,000 rpm for 2 h, with a cushion of 25% sucrose in TNC (10 mM Tris, pH 7.4; 140 mM NaCl, 10 mM $CaCl_2$). Following removal of the supernatant, the viral pellet was suspended in TNC. Cesium chloride was added and dissolved by gentle shaking, and the solutions were subsequently centrifuged at 34,000 rpm for 20 h in an SW55 rotor to form CsCl gradients. Visible double-layer particle (DLP) and triple-layer particle (TLP) bands were harvested using needle aspiration. Two methods for removing the CsCl from the viral bands were used: high-speed centrifugation (SW28, 26,000 rpm, 2 h, using a 25% sucrose cushion) or dialysis against TNC at 4 °C.

2.3. Multivalent quantitative PCR-based cell infectivity assay (M-QPA) and plaque assay

The multivalent quantitative PCR-based cell infectivity assay (M-QPA) was carried out using Vero cells as described with minor modifications (Ranheim et al., in press). An additional reference for the basic principles of this method can be found in Wang et al. (2005). For this method, quantitative PCR technology is used to measure viral replication following infection of cells, as compared with a homologous reference standard. Infectivity of the sample is calculated by interpolation of the sample results to the reference standard curve. The infectivity of the reference standard used for the standard curve was independently assigned using the plaque assay (Ranheim et al., in press).

In more detail, for this assay individual 96-well cell preparations are infected with either a sample or four dilutions of a reference standard. Following 24 h of infection, the cells are lysed and primer/probe sets specific for each reassortant (G1, G2, G3, G4, or P1) are used to quantitate viral mRNA in each well using reverse-transcriptase quantitative PCR technology (RT-QPCR). As with all Q-PCR, a C_t or Cycle-threshold value, is measured to indicate the relative amount of nucleic acid in the starting sample. To report a value for the sample, the C_t value obtained from the Q-PCR of each dilution point of the standard is plotted versus the assigned infectivity of each point (assigned previously via plaque assay). In this way, a straight line is obtained for the standard curve, in which C_t is plotted against infectivity. The potency of the sample relative to the standard can then be reported using straight-forward interpolation of the C_t value obtained for the sample to the standard curve. Rather than the standard measure of plaque forming unit (pfu), this assay reports values in “Infectious Units” or “IU.” Since the standard is originally assigned its infectivity using the plaque assay, for the purposes of this discussion 1 IU is essentially equal to 1 pfu.

Due to the flexibility of the 96-well platform, various assay formats are easily achievable in the M-QPA. For all data points, at least two duplicate wells are plated. For the experiments

described here, a format of "$Y \times Z$" indicates the number of plates/day and the number of days for any given assay, respectively. For example, a "2×1" assay format is a single sample tested in duplicate wells on each of two 96-well plates on a single day. A "1×12" format would describe a single sample tested in duplicate wells on independent plates on each of twelve different days. A "6×1" format would describe a sample that had been plated in two replicate wells across each of six plates, all plated on a single day.

For experiments described in which monovalent or pentavalent samples were formulated prior to assay, virus was diluted into the indicated solutions, samples were then inverted gently to mix completely, and in most cases subsequently frozen to await assay.

Plaque assays were performed in African green monkey kidney MA-104 cells using standard methods as described (Clark et al., 2003). Virus samples in the various solutions to be tested were diluted to appropriate concentrations using cell culture media prior to plating and assay. Assays for samples of each viral preparation were performed at the same time by the same operator, using cells planted from the same cell suspension. For each sample, the assays were completed in triplicate on each of 2 days (3×2).

2.4. Impact of the formulation buffer and its individual components

G1, G2, G3, G4, and P1 viral preparations generated as described in Section 2.1 were diluted into tissue culture media and measured using the M-QPA in a 1×12 format. These same viral preparations were then mixed to generate a pentavalent mixture, and diluted into the sucrose/citrate/phosphate/PS-80 formulation buffer. The development of this formulation buffer is described by Burke and Volkin (1999). The buffer contains tissue culture media, sucrose, sodium citrate, sodium phosphate, and polysorbate-80, with a final pH of ~6.4. The formulated, pentavalent samples were then measured using the M-QPA in a 1×12 format. The average matrix effect was calculated as a percentage increase in in vitro infectivity for each formulated preparation over the infectivity measured for each viral preparation in tissue culture media. This entire procedure was repeated for two independent viral preparations of each of the reassortants.

For the data described in Fig. 1, each viral preparation was diluted into the sucrose/citrate/phosphate/PS-80 formulation buffer at least nine independent times for each reported experimental result. Each of the nine replicates was tested using the M-QPA in a 1×12 format. For Fig. 1, therefore, a total of at least 108 values were averaged for each lot of each reassortant shown.

Further experiments were carried out in which P1 virus prepared in two ways, as a concentrated lysate as described in Section 2.1 and using CsCl purification as described in Section 2.2, was diluted into formulations of either tissue culture media, sucrose/citrate/phosphate/PS-80 formulation buffer, or formulation buffer missing one of each of the formulation components. The average matrix effect was calculated as a percentage

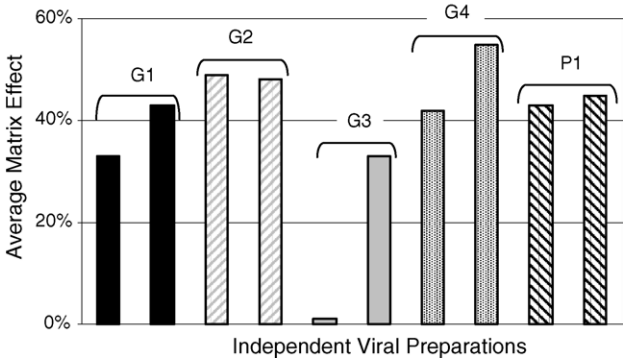


Fig. 1. Average matrix effect for different preparations of the five reassortants. Two independent preparations of viral samples from each of the five reassortants were measured before and after dilution into the formulation buffer, using a 1×12 M-QPA. The averages of these results are reported as a percentage increase in in vitro infectivity for each preparation over the infectivity measured for each viral preparation in tissue culture media.

increase in in vitro infectivity for each formulated preparation as compared with the infectivity of the sample diluted into tissue culture meda.

To titrate specifically the effects of citrate in the formulation buffer, a P1 virus preparation generated as described in Section 2.1 was formulated either into media, "full" formulation buffer (containing all sucrose/citrate [1×]/phosphate/PS-80 components), or formulation buffer containing 0×, 0.25×, 0.5×, or 1× citrate. To eliminate pH effects, each formulation was standardized to pH 6.2 before addition of virus. All samples were assayed in a 6×1 M-QPA format to assign potency as described in Section 2.3.

To titrate the effects of succinate, a pentavalent virus preparation was diluted into citrate-free formulation buffer with the following levels of succinate: 0, 0.005, 0.05, 0.1, 0.2, 0.4, 1.4 M succinate. All formulations were standardized to pH 6.2 prior to adding virus, and all where stored at 4 °C overnight prior to being frozen and tested by M-QPA in a 2×2 format as described in Section 2.3.

2.5. Electron microscopy and dynamic light scattering

Electron microscopy was carried out using standard methods following PTA staining. For the samples that were examined, virus was prepared as described in Sections 2.1 and 2.2. Dynamic light scattering (DLS) experiments were performed using a particle analyzer (Model 4700, Malvern Instruments Ltd., Malvern, Worcester, UK). TLPs and DLPs in varying formulations were diluted 1:5 into tissue culture media to reduce the effect of viscosity contributed by the sucrose and sodium citrate in the formulation. Particle size and size distribution were assessed using DLS, in which a laser power of 0.25–0.5 W at 488 nm and a scattering angle of 90° were employed in all experiments. The temperature of the refractive index matching fluid was maintained at 25 °C. For each sample analyzed, ten consecutive measurements were taken for 10 s each. Measured viscosity for each individual diluted formulation condition was used correspondently during the measurements. Data shown are from

the mean of 10 measurements for each individual sample. Latex beads (96 nm) were 1:1000 diluted into the corresponding formulations and used as a reference to determine the accuracy of the measurements.

2.6. Centrifugation experiments

For experiments to separate aggregates via centrifugation, CsCl-purified virus preparations prepared as described in Section 2.2 were diluted in media and centrifuged for 10 min at 10,000 rpm at 4 °C in a microcentrifuge. The supernatant was removed by pipetting and transferred to a clean centrifuge tube. The pellet was suspended with the remaining media. No visible pellet was observed in these experiments. OD_{260} readings of the samples at each step were recorded using spectrophotometry to confirm the removal of the viral aggregates. Light scattering was also conducted at each step to monitor the changes within the virus particle suspensions. The fractionated purified preparations were further formulated into media, sucrose/citrate/phosphate/PS-80 formulation buffer, or citrate-free formulation buffer to examine changes in infectivity. M-QPA (2×4 format) was used to monitor the viral infectivity under each condition as described in Section 2.3.

3. Results

3.1. The formulation for RotaTeq® enhances in vitro infectivity, as measured by the M-QPA

A study was initiated to determine whether or not the sucrose/citrate/phosphate/PS-80 formulation buffer for RotaTeq® had any impact on the in vitro infectivity of the various reassortants contained within the vaccine. Multiple pentavalent preparations were diluted either into tissue culture media or into formulation buffer, and infectivity of the samples was measured using the multivalent quantitative PCR-based cell infectivity assay (M-QPA). This assay was chosen for its ability to distinguish individual reassortants within a pentavalent mixture, and its superior operating characteristics (good reproducibility and corresponding low variability). Fig. 1 shows the average results of this set of experiments. Results are reported as the percent increase in infectivity of virus diluted in formulation buffer as compared to the infectivity of the same volume of virus diluted in tissue culture media.

The results show that for all five reassortants, dilution into the sucrose/citrate/phosphate/PS-80 formulation buffer resulted in increases in measured in vitro infectivity. We have termed the observed enhancement in infectivity following dilution into the formulation buffer the "matrix effect." G1, G2, G4, and P1 reassortants behaved similarly, although not identically, with respect to the magnitude of the effect. The effect appeared to be less pronounced for G3 (ranging from ∼0 to 30%), whereas for the other reassortants it ranged from ∼30 to 60% in various experiments. The average matrix effect from all testing, including data from samples not included in Fig. 1, was as follows: G1, 38%; G2, 60%; G3, 14%; G4, 53%; P1, 47%. For individual experiments, the magnitude of the effect varied, consistent with assay variability. RSD for this assay format is ∼8%.

3.2. The plaque assay confirms the presence of the matrix effect

Because the M-QPA is a relatively new method for determination of viral infectivity, the traditional plaque assay was also carried out on monovalent samples to confirm the matrix effect of the formulation buffer. In this experiment, two independent preparations of each of the monovalent viruses were diluted identically into either tissue culture media or into the formulation buffer, and assayed for infectivity in the plaque assay using MA-104 cells. Monovalent rather than pentavalent samples were used since the plaque assay cannot distinguish between reassortants.

The results indicated that for each of the reassortants, the infectivity observed following dilution in formulation buffer was higher than when samples were diluted in cell culture media. The average relative differences in potency between sample in formulation buffer versus media for each reassortant was as follows: G1, 48%, G2, 49%, G3, 46%, G4, 43%, P1, 23%. The relative standard deviation for these assays ranged from ∼20 to 60%, averaging around 35%. These data are qualitatively consistent with the observations from the M-QPA, and confirm that the matrix effect is not due to assay artifact or cell type.

3.3. Citrate in the formulation buffer is the major contributor to the matrix effect

The impact of multiple factors on the magnitude of the matrix effect was examined, including both physical and chemical factors. Various methods of mixing and freeze–thaw of both formulated and unformulated samples were examined, as were scale of formulation and time-in-solution. None of these variables were found to be significant contributing factors to the effect within detectable limits. As an alternative, a set of studies was completed that removed each component from the formulation buffer, one at a time, in an attempt to determine if a single excipient or combination of excipients was responsible for the increase in in vitro infectivity. For this experiment, both concentrated filtered virus preparations and CsCl-purified virus preparations were used. The data from this experiment suggested that individual removal of sucrose, phosphate, or polysorbate-80 had little or no effect on minimizing the enhancement caused by the formulation for either preparation. In contrast, the removal of citrate decreased the matrix effect by ∼60% for both the purified and unpurified viral samples. These data indicated that the citrate in the formulation might account for the observed enhancement in infectivity in vitro.

3.4. Titration of citrate or succinate in the formulation buffer leads to a titration in the matrix effect

To further explore the impact of citrate on the matrix effect, an experiment was performed in which a filtered P1 virus preparation was diluted into formulation buffer containing various

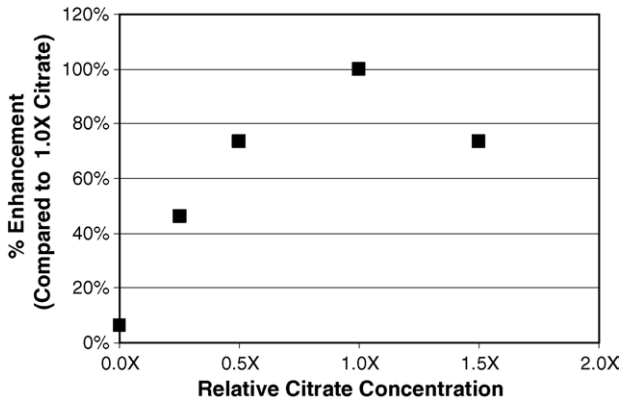


Fig. 2. Titration of matrix effect by varying citrate concentration. Monovalent P1 virus was diluted either into media or in sucrose/citrate/phosphate/PS-80 formulation buffer containing various levels of citrate as shown. Samples were stored overnight at 4 °C prior to transfer to −70 °C to await assay in the M-QPA in a 6 × 1 format. Results are reported as the % increase in potency observed following formulation as compared with dilution in media.

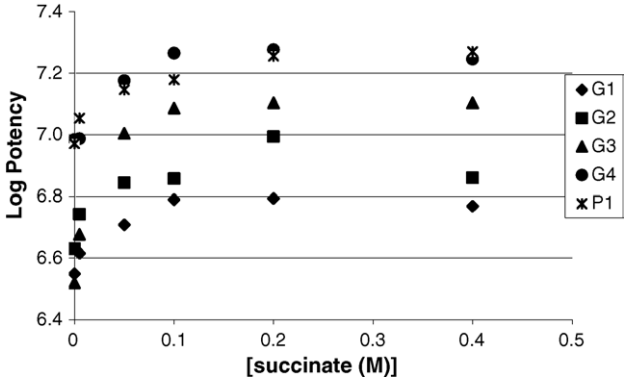


Fig. 3. Titration of matrix effect by varying succinate concentration. Pentavalent virus was diluted either into sucrose/phosphate/PS-80 formulation buffer (no citrate) containing various levels of succinate as shown. Samples were assayed in the M-QPA in a 2 × 2 format. Results are reported as the log of the infectivity in IU/mL.

levels of citrate. The results, shown in Fig. 2, demonstrate that the magnitude of the matrix effect titrated with citrate concentration, peaking at a citrate concentration of 1× (i.e., the amount found in the standard sucrose/citrate/phosphate/PS-80 formulation buffer). These data further demonstrate the role of citrate in the matrix effect phenomenon. These experimental results have also been confirmed for filtered virus preparations of G1, G2, G3, and G4 reassortants, as well as for CsCl-purified viral preparations.

The results in Fig. 3 demonstrate that substituting succinate for citrate in the formulation maintains the observation of the matrix effect. For this experiment, all five reassortants were diluted into citrate-free formulation buffer containing the noted amounts of succinate and assayed for infectivity. The data demonstrate a clear trend of increasing infectivity with increasing succinate concentrations for each of the reassortants within the pentavalent sample. The magnitude of the effect is consistent with that seen for citrate-containing formulation buffer.

3.5. Comparison of physical properties of purified rotavirus G1 viral particles in various formulations

To understand the physical mechanism of the matrix effect, a combination of light scattering, spectrophotometry, electron microscopy, and M-QPA was pursued. The goal of these studies was to correlate changes in infectivity caused by the formulation buffer to potential changes in particle size. We hypothesized simply that disaggregation of viral particles might be leading to decreased particle:infectivity in the sucrose/citrate/phosphate/PS-80 formulation buffer, and the subsequent increase in measured infectivity in vitro.

Initially, viral preparations were examined using EM to determine if consistent aggregation and/or disaggregation under the various buffer conditions could be observed. While aggregation of the viral samples in media was apparent, technical difficulties hampered observation in the formulation buffer samples due to the high viscosity and osmolarity of the sucrose/citrate/phosphate/PS-80 solution. Instead, dynamic light scattering was used to examine gross physical characteristics of the viral samples. Briefly, the CsCl-purified G1 preparations originally formulated in media, formulation buffer, or citrate-free formulation were each further diluted 1:5 into tissue culture media. The diluted virus from the various formulations was then studied using M-QPA and light scattering.

The data from these initial experiments indicated that the matrix effect was evident as expected in the sucrose/citrate/phosphate/PS-80 solution. The measured in vitro infectivity in this experiment was increased by ∼80%. In addition, light scattering data indicated that the measured diameters of the particles (Z_{ave}) in media, citrate-free formulation, and sucrose/citrate/phosphate/PS-80 formulation were 142, 110, and 78 nm, respectively (Table 1). The results suggested that in media the rotavirus particles appear to be larger, with a wider range of size distribution (i.e., polydispersity), as compared to particles diluted into formulation buffer. Since the expected size of a single rotavirus particle should be ∼75 nm in diameter (Yeager et al., 1990), the results suggest that in media and in citrate-free formulation the virus particles are present as aggregates. In contrast, the measurements of samples in

Table 1
Dilution of pre-formulated viral preparations preserves the matrix effect

Formulation	Matrix effect (%)	Z_{ave} (nm)	S.D.	Polydispersity	S.D.
Media	–	142.0	6.0	0.53	0.02
Citrate-free formulation	10	109.9	6.4	0.47	0.05
Sucrose/citrate/phosphate/PS-80	79	78.0	5.4	0.06	0.02
DLPs in media	–	64.9	0.9	0.03	0.03

Table 2
Dynamic light scattering of aggregates separated by centrifugation

Sample ID	Formulation	Z_{ave} (nm)	S.D.	Polydispersity	S.D.
Pre-centrifugation	Media	159.9	5.9	0.49	0.03
Pre-centrifugation	Sucrose/citrate/phosphate/PS-80	76.5	1.8	0.14	0.06
Supernatant post-centrifugation	Media	71.2	2.6	0.20	0.04
Pellet post-centrifugation	Media	118	3	0.46	0.02

the sucrose/citrate/phosphate/PS-80 formulation buffer demonstrated more uniform size distribution and a measurement more closely matching the published particle size, suggesting that aggregation was not present appreciably, if at all, in this formulation.

As a control, double-layered rotavirus particles in media were measured. The measured size of this sample agreed with the published measurement of ~65 nm (Yeager et al., 1990). This is further indication of the validity of the measurements, and it suggests that the aggregation occurring in the samples described here is likely due to interactions with the VP4/VP7 protein layers that are not present in the double-layered particles. Overall, the outcome of combining light scattering with QPA strongly suggested that the matrix effect correlates well with disaggregation of viral particles.

3.6. Centrifugation can be used to further examine aggregates

In order to further explore this, an experiment was designed to separate viral aggregates from non-aggregated particles using centrifugation. CsCl-purified G1 virus preparations were fractionated by centrifugation and subsequently diluted into various solutions as described in Section 2.6. Light scattering (Table 2) and M-QPA were conducted after each step.

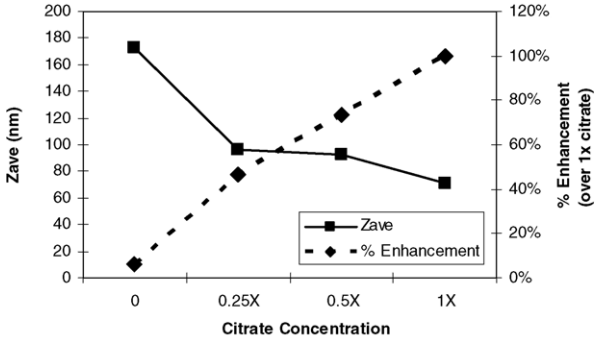
The viral particle size and size distribution data from the light scattering measurements confirmed that following centrifugation to pellet viral aggregates, the average size of particles in the media supernatant was ~71 nm, very close to the size of particles following dilution in the sucrose/citrate/ phosphate/PS-80 formulation buffer (~76 nm). This change in average size for the supernatant was accompanied by decreased polydispersity. The results suggest that large viral aggregates were formed in media and centrifuging the samples resulted in pelleting of these aggregates, leaving more uniform, unaggregated virus in solution. The DLS results from the pellets furthermore suggest the persistence of aggregation in the pelleted fraction. Z_{ave} for this sample was ~120 nm.

To further correlate disaggregation with the matrix effect, M-QPA was also performed using fractions of these samples that had been formulated in media, formulation buffer, or citrate-free formulation buffer, before and after centrifugation. The data confirmed that the magnitude of the matrix effect increased by ~250% in pelleted particles diluted into the sucrose/citrate/phosphate/PS-80 formulation buffer as compared with samples with no centrifugation. In addition, the magnitude of the matrix effect in the supernatant of centrifuged samples decreased. This suggests that centrifugation pelleted viral aggregates, and subsequent dilution of these aggregates into formulation buffer enhanced virus infectivity in vitro by disaggregating them. Supernatant samples contained fewer aggregates, hence the lower matrix effect on dilution into formulation buffer.

3.7. Impact of citrate concentration on particle size

To definitively prove the causal effect of increasing citrate in the formulation buffer on viral disaggregation, CsCl-purified G2 particles were diluted into media or formulation buffer containing various concentrations of citrate. The resulting samples were then measured for average size using light scattering. Fig. 4 shows the particle size and size distribution results from light scattering measurements in these various formulations.

The data demonstrate a reduction in particle size and size distribution concurrent with increasing citrate concentrations in the formulation buffer. The decrease in size correlates clearly with increasing measures of infectivity. The results are consistent with our earlier studies, and provide a clear link between the process of viral particle aggregation/disaggregation and chang-



Formulation	Z_{ave} (nm)	S.D	Polydispersity	S.D
Media	184.4	6.4	0.47	0.04
Citrate-free formulation	173.3	7.1	0.45	0.02
0.25 X Citrate formulation	95.9	1.7	0.25	0.03
0.5 X Citrate formulation	92.7	8.4	0.36	0.15
1 X Citrate formulation	71.1	5.3	0.20	0.14

Fig. 4. Correlation of citrate concentration with G2 particle size and matrix effect. Particle size and size distribution of CsCl-purified G2 virus formulated as shown were assessed using dynamic light scattering. Data shown are the mean of ten consecutive measurements of each individual sample condition. Overlayed are the P1 data from Fig. 3 describing the average matrix effect for the given concentrations of citrate.

ing in vitro infectivity. These data provide definitive evidence to support the hypothesis that the sucrose/citrate/phosphate/PS-80 formulation buffer enhances virus infectivity in vitro through citrate-mediated disaggregation.

4. Discussion and conclusions

4.1. Mechanism of enhanced infectivity

The data presented here confirm that the increase in measured infectivity in vitro brought about by the formulation for RotaTeq® is a result of citrate-mediated disaggregation of viral particles. This observation is apparent in two independent infectivity assays using two different cell lines, the plaque assay and the multivalent quantitative PCR-based potency assay (M-QPA). One question this observation raised was what part of the virus's life cycle was disrupted by aggregation. Unlike the plaque assay, where measured infectivity is based on infection of a single cell and subsequent viral spread to adjacent cells, in the M-QPA infectivity values are based mostly on detection of viral mRNA from a single round of dsRNA replication (Ranheim et al., in press). Thus if two (or more) virus particles enter the same cell, the M-QPA should register a correspondingly higher infectivity value since the assay should detect viral mRNA resulting from replication of both (or all) virus particles.

In the case described here, however, the higher infectivity value is not registered with the aggregated samples in the M-QPA. Instead, the aggregated particles are reported as less infectious as compared with particles in the disaggregated state. This observation suggests that the aggregated particles do not efficiently proceed to the steps of RNA replication and transcription, where they would be registered by the M-QPA. Instead, the aggregates are probably defective in the early steps in the virus's life cycle, likely binding/entry or uncoating. Given this, it seems probable that relief of this entry defect by disaggregation is what leads to the matrix effect, effectively resulting in decreased particle:infectivity in both the plaque assay and the M-QPA.

Inhibition of infectivity through aggregation has been reported for many other viruses, and has been found to take various pathways (Smith et al., 1993; Gómez-Puertas and Escribano, 1997; Barbas et al., 1992). Yuan and Parrish (2000) confirmed that aggregation of canine parvovirus by neutralizing antibody led to defects in uncoating. Others (Streckert et al., 1988) have shown that binding of neutralizing antibody can lead to aggregation and subsequent inability of the virus to bind its receptor. Aggregation, not mediated by neutralizing antibodies, has also been shown to prevent internalization of virus into cells in the Dengue virus system (Sithisarn et al., 2003). The precise step of entry that is blocked here is unclear from the present studies; however, the data suggest that the problem lies in the entry/uncoating process, rather than a later step in the virus's life cycle.

4.2. Mechanism of citrate-Induced disaggregation

Since the CsCl-purified samples exhibited the same effects as the filtered virus preparation, it seems likely that gross differences in viral preparations, specifically with respect to cellular components, have minimal effects in contributing to the magnitude of the matrix effect. In addition, double-layered particles that do not contain the VP7 and VP4 proteins did not appear to exhibit any aggregation under the conditions tested. Furthermore, the various reassortants apparently displayed different magnitudes in the effect. G1, G2, G4, and P1 appeared to exhibit average effects of ∼45%, whereas G3 exhibited a smaller average effect. Since the only differences between these reassortants are in the outer proteins of VP4 and VP7, this is an indication that the extent of aggregation/disaggregation observed is related directly to interactions between these proteins and the citrate in the formulation buffer.

The mechanism by which citrate induces disaggregation through the VP4 and/or VP7 proteins has been explored through experiments examining the impact of ionic strength, calcium concentration, and succinate on the matrix effect. Calcium was specifically chosen since it is known that citrate chelates metal ions such as calcium (Rex et al., 2002), and therefore it was hypothesized that calcium chelation by citrate might contribute to the matrix effect. It is also known that calcium plays a key role in the association of the outer proteins of the TLP to the inner protein layer of the DLP (Gajardo et al., 1997; Ruiz et al., 1996). Data from experiments examining the impact of added calcium and/or sodium chloride, however, indicate that ionic strength and free calcium levels have at most minimal impact on the effect (data not shown).

Succinate was also explored for its ability to induce the matrix effect, since like citrate, it is a carboxylic acid. Data from the experiments shown here suggest that succinate can cause the matrix effect to a similar extent as citrate. This result suggests that a commonality between citrate and succinate may lead to the disaggregation. The obvious similarity between the chemicals is their carboxy groups. As such, we propose that specific surface charge interactions with the viral particle and the carboxy groups on the succinate or citrate leads to the disaggregation observed.

4.3. Clinical implications of the matrix effect

All pivotal clinical studies to assess the final safety and immunogenicity of RotaTeq® were carried out using the sucrose/citrate/phosphate/PS-80 formulation buffer described here. As discussed, citrate is added to the formulation buffer to neutralize gastric acid during immunization to eliminate the need for pre-feeding. The formulation buffer also imparts 2–8 °C stability on the vaccine. A key question that arose from the research described here was whether or not the matrix effect caused by the formulation buffer in vitro had clinical impact in vivo.

In a controlled clinical study using citrate-containing formulation buffers, no significant impact of the formulation buffer on immunogenicity in infants was observed (Clark et al., 2003). Vaccine using the formulation buffer was well-tolerated, and immunogenicity was generally similar to the pre-fed control group who received vaccine that was not diluted into the buffer. In addition, a striking similarity in efficacy estimates existed across clinical studies that used a formulation buffer similar to the one discussed here and a formulation consisting largely of

tissue culture media (Clark et al., 2004b; Vesikari et al., 2002, 2005b; Itzler et al., 2005). For similar dose levels, point estimates of efficacy for two independent clinical studies of unbuffered formulations were 75% and 74%. In two independent Phase III clinical studies using vaccine in the sucrose/citrate/phosphate/PS-80 formulation buffer, the point estimates for efficacy were 73% and 74%. These data indicate that the matrix effect has minimal or no impact in vivo.

Presumably the disaggregation that occurs on dilution into the formulation buffer either: (1) also occurred on vaccination of subjects with virus diluted in other buffers or (2) is at a low enough level such that it has minimal clinical impact. Since RotaTeq® is an oral vaccine, the virus must travel through the gastrointestinal tract, and thus the former hypothesis is reasonable. The virus could simply disaggregate through effects of the gastrointestinal system, even if it is not initially diluted into the sucrose/citrate/phosphate/PS-80 formulation buffer.

4.4. Manufacturing implications of the matrix effect

The impact of the matrix effect on the manufacturing process of RotaTeq® is significant. For vaccine manufacture, bulk virus is diluted into formulation buffer and filled into final containers for storage, distribution, and administration. An ability to accurately target the potency of the vaccine in the final container is absolutely critical to consistent vaccine production. If one measurement is taken from the bulk virus in media, and a different "enhanced" measurement is taken from final vaccine in the sucrose/citrate/phosphate/PS-80 formulation buffer, the two measurements will not be comparable. To avoid this inconsistency, testing of the bulk virus in the M-QPA is conducted in the presence of formulation buffer. This measurement is then used to target the full-scale formulation and manufacture of the vaccine. Final manufactured product is necessarily also tested in the presence of formulation buffer. This method of vaccine targeting has been used successfully in clinical trials, and will continue to be used for subsequent large-scale manufacture.

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