Virology 485 (2015) 199–204

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Treatment of norovirus particles with citrate

Anna D. Koromyslova [a,b], Peter A. White [c], Grant S. Hansman [a,b,*]

[a] Schaller Research Group at the University of Heidelberg and the DKFZ, Heidelberg 69120, Germany
[b] Department of Infectious Diseases, Virology, University of Heidelberg, Heidelberg 69120, Germany
[c] Molecular Microbiology Laboratory, School of Biotechnology and Biomolecular Sciences (BABS), The University of New South Wales, Sydney 2052, Australia

ARTICLE INFO

Article history:
Received 30 April 2015
Returned to author for revisions
30 May 2015
Accepted 9 July 2015

Keywords:
Norovirus
Disinfectant
X-ray structure
Virus-like particles

ABSTRACT

Human norovirus is a dominant cause of acute gastroenteritis around the world. Several norovirus disinfectants label citric acid as an active ingredient. In this study, we showed that norovirus virus-like particles (VLPs) treated with citrate buffer caused the particles to alter their morphology, including increased diameters associated with a new ring-like structure. We also found that epitopes on the protruding (P) domain on these particles were more readily accessible to antibodies after the citrate treatment. These results suggested that citrate had a direct effect on the norovirus particles. Using X-ray crystallography, we showed that the P domain bound citrate from lemon juice and a disinfectant containing citric acid. Importantly, citrate binds at the histo-blood group antigen binding pocket, which are attachment factors for norovirus infections. Taken together, these new findings suggested that it might be possible to treat/reduce norovirus infections with citrate, although further studies are needed.

Introduction

Human noroviruses are the dominant cause of outbreaks of acute gastroenteritis in humans. Norovirus infection frequently occurs following consumption of foods susceptible to contamination with human faecal waste, including mussels, oysters, berries, and vegetables (De Keuckelaere et al., 2015; Made et al., 2013; Sarvikivi et al., 2012; Pan et al., 2012; Brassard et al., 2012; Lodo et al., 2014; Schaeffer et al., 2013; Pavoni et al., 2013; Benabbes et al., 2013; Le Guyader et al., 2012; Alfano-Sobsey et al., 2012; Kittigul et al., 2011; Ueki et al., 2010; Terio et al., 2010; Long et al., 2002). In developing countries, norovirus is a frequent infection in children, with most infected by one year of age. In this setting, norovirus causes 18% of childhood gastroenteritis in children less than five years of age (Ahmed et al., 2014). Screening for norovirus in foods and water only occurs once a link with a possible food- or water-borne contamination is suspected to be the cause of infections. At this stage, it is hard to further reduce transmission and outbreaks can ensue. At present, a tiny fraction of food is screened before consumption, usually by RT-PCR. However, another large source of norovirus infections occurs through food handlers, who can either be symptomatically or asymptomatically infected (Ozawa et al., 2007).

Several studies using surrogate noroviruses (feline calcivirus and murine norovirus) found that natural fruits or their components could inhibit or reduce infectivity (Horm and D'Souza, 2011; Su et al., 2010a, 2010b, 2010c; Whitehead and McCue, 2010). Moreover, an EPA approved disinfectant is reported to inhibit norovirus infections in 10 min and contains ~5% citric acid, where the active antiviral ingredient is described as silver dihydrogen citrate. However, the mode of inhibition in these reports has been lacking. Human noroviruses are enteric viruses and as such are stable between pH 3–7 (Ausar et al., 2006). This suggested that the citrate in these disinfectants might have a direct effect on the particles.

We recently showed that the norovirus capsid binds citrate (Hansman et al., 2012). The citrate binding site was located at the histo-blood group antigen (HBGAs) binding pocket, which are attachment factors that enhance norovirus infections (Jones et al., 2014). In the present study, we continued our investigations on citrate mode-of-action on human norovirus. We found that citrate caused the norovirus virus-like particles (VLPs) to enlarge and change morphology. We also showed that citrate from lemon juice and a disinfectant was able to bind to the capsid using X-ray crystallography. Interestingly, the citrate molecule bound on a "spring-like structure" on the virus particle that was connected across to an epitope known to cause particle disassembly (Koromyslova and Hansman, 2015).

Results and discussion

EM of citrate-treated VLPs

In order to visualize the effects of citrate buffer, we treated VLPs with different concentrations of citrate buffer for 30 min at room temperature (Fig. 1). Between 0.49 and 7.85 mM of citrate buffer, the VLPs appeared homogenous and indistinguishable from untreated VLPs. At

* Correspondence to: CHS Foundation, University of Heidelberg, and DKFZ. Norovirus Study Group, Im Neuenheimer Feld 242, Heidelberg 69120, Germany.
E-mail address: g.hansman@dkfz.de (G.S. Hansman).

http://dx.doi.org/10.1016/j.virol.2015.07.009
0042-6822/

Table 1
Data collection and refinement statistics of GII.10 P domain citrate complex structures.

	GII.10-lemon juice (5BSX)	GII.10-Puregreen24 (5BSY)
Data collection		
Space group	$P12_11$	$P12_11$
Cell dimensions		
a, *b*, *c* (Å)	65.08, 79.75, 69.47	65.28, 80.36, 70.15
α, β, γ (°)	90, 100.98, 90	90, 101.42, 90
Resolution range (Å)	43.45–1.60 (1.65–1.60)[a]	43.83–1.78 (1.84–1.78)[a]
R_{sym}	5.64 (64.47)[a]	4.76 (38.08)[a]
$I/\sigma I$	14.98 (1.84)[a]	15.17 (2.62)[a]
Completeness (%)	99.58 (98.80)[a]	96.77 (96.68)[a]
Redundancy	4.3 (4.3)[a]	2.7 (2.7)[a]
CC1/2	0.999 (0.708)[a]	0.999 (0.821)[a]
CC[a]	1 (0.91)[a]	1 (0.95)[a]
Refinement		
Resolution range (Å)	43.45–1.60	43.83–1.78
No. of reflections	91,737	65,865
R_{work}/R_{free}	16.00/18.86	17.18/19.52
No. of atoms	5548	5388
Protein	4843	4798
Ligand/ion	37	25
Water	668	565
Average *B* factors (Å^2)		
Protein	25.60	24.80
Ligand/ion	40.10	38.40
Water	37.80	33.10
RMSD		
Bond length (Å)	0.007	0.006
Bond angle (°)	1.06	1.08

Each data set was collected from single crystals, respectively.

[a] Values in parentheses are for highest-resolution shell.

15.63 mM, a small number of the VLPs had slightly altered morphology, i.e., the outer spikes of the VLPs were surrounded by a new ring-like structure (Fig. 1). The morphological changes appeared in most VLPs at the higher concentrations of citrate buffer, where at 62.50 mM of citrate buffer the majority of VLPs showed the ring-like structure.

The diameters of the VLPs (length and width) were manually measured at 0, 0.95, 7.81, 62.50, and 125 mM of citrate buffer. At 0, 1, and 7.81 mM of citrate buffer, the diameter of the VLPs were ~42 to 44 nm, while at 62.50 and 125 mM of citrate buffer, the diameter of the VLPs were ~46 to 49 nm (Fig. 2). The diameter of the cryo-EM VLP structure was ~42 nm (Hansman et al., 2012b), which not only indicated that, the citrate buffer enlarged the VLPs, but also altered their morphology. However, the stability of these citrate treated-particles was not determined and particle disassembly may have resulted from the citrate buffer treatment.

ELISA of citrate-treated VLPs

The VLPs were mixed with citrate buffer and the effects were analyzed using an antigen ELISA with polyclonal and monoclonal antibodies directed against the VLPs. At low concentrations of citrate buffer (0.25–7.81 mM), the polyclonal antibody weakly detected the VLPs at OD_{490} = ~0.15 (Fig. 3A), which was similar to untreated VLPs (~0.15). At 15.63 mM of citrate buffer, there was a slight increase in the OD_{490} value (~0.51). The OD_{490} values continued to rise with the increasing concentrations of citrate buffer and reached a plateau at 62.50 mM of citrate buffer, where the OD_{490} = ~2.20. A similar result was observed with the monoclonal antibody. Between 0.25 and 7.85 mM of citrate buffer, the OD_{450} = ~0.11 (Fig. 3B). At 15.63 mM of citrate buffer, the OD_{450} increased to ~0.65 and then reached a peak at 62.50 mM of citrate buffer, where the OD_{450} = ~3.60. These ELISA results suggested that with increasing concentrations of citrate buffer additional antibodies were capable of binding to the VLPs.

X-ray crystallography of GII.10 P domain and lemon juice and disinfectant complexes

Following from our previous study (Hansman et al., 2012a), we proceeded to determine if citrate from lemon juice and a norovirus disinfectant containing citric acid could also bind to the P domain. A single crystal of the GII.10 P domain citrate-lemon juice complex diffracted to 1.6 Å resolution (Table 1). The P1 subdomain comprised of β-sheets and one α-helix, while the P2 subdomain contained six antiparallel β-strands that formed a barrel-like structure (Fig. 4A), as previously described (Hansman et al., 2011). A large patch of electron density clearly representing the citrate molecule was observed at one HBGA pocket (Fig. 4B), whereas the opposite pocket only contained a small patch of electron density that could not be identified. The binding interaction of citrate from the lemon juice was similar to the previously solved GII.10 P domain citrate complex, except for several water-mediated interactions (Hansman et al., 2012a). Direct hydrogen bonds were provided from the side-chains of Arg356 and the main chain of Gly451 and Asn355 (Fig. 4C). Water-mediated hydrogen bonds with the citrate were provided with the side chains of Asn342, Asp385, and Ser387. The GII.10 P domain citrate-disinfectant complex diffracted to 1.78 Å resolution (Table 1). A similar set of binding interaction of the citrate from lemon juice was also observed with the citrate from the disinfectant (data not shown). However, the disinfectant was composed of silver dihydrogen citrate, yet the silver ion was not observed on the GII.10 P domain, although this may be due to the low concentration of the silver ions in the solution (~0.003%). Of importance, citrate was also found to bind a GII.4 norovirus P domain using NMR (Mallagaray et al., 2015), indicating that citrate likely has a broad reactivity among diverse GII noroviruses. Taken together, these results showed that citrate from different sources (e.g., lemon juice, disinfectant, and citric acid in solution) was capable of binding to the norovirus P domain.

We previously showed that the 5B18 Fab monoclonal antibody bound to the lower region of the P domain (Hansman et al., 2012b). In the context of the intact VLPs, the antibody-binding site was occluded. The 5B18 antibody was used in a diagnostic sandwich ELISA kit and likely detects intact particles (Hansman et al., 2012b). However, the relative large size of the IgG suggested that not all antibody-binding sites on the particles might be accessible for binding, due to steric clashes on the intact particles (Hansman et al., 2012b). Our EM data revealed that the VLPs treated with citrate buffer increased in size, ~2 nm in radius, and the ELISA data showed that the VLPs retained their antibody binding capabilities. These results suggested, that the larger particles might allow additional IgGs to bind. Therefore, the disinfectant could work by "opening" or dissembling the particles and in both cases – render the particles inactive or expose the vulnerable RNA.

We recently discovered that a norovirus specific Nanobody (termed Nano-85) bound to norovirus VLPs and the binding interaction caused the VLPs to disassemble (Koromyslova and Hansman, 2015). The Nanobody bound to mainly conserved residues, underneath the P1 subdomain, termed the trigger region. Interestingly, the trigger region was linked with contiguous amino acids to the P1 interface loop, which interacted with the citrate molecule (Fig. 5A). In this view, it is tempting to speculate that the citrate binding to intact VLPs causes a chain of events through this "interface spring" to the Nanobody trigger region, which in turn, causes the particles conformational change and/or disassembly. Indeed, conformational changes in the feline calicivirus particles upon receptor binding were reported and the particle morphological changes appeared in the S and P domains (Bhella et al., 2008). Compared to other calicivirus P domain structures, lagovirus and murine norovirus also contained