Rhesus enteric calicivirus surrogate model for human norovirus gastroenteritis

Tibor Farkas¹,²

¹Division of Infectious Diseases, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH, USA
²University of Cincinnati College of Medicine, Cincinnati, OH, USA

Human noroviruses are one of the major causes of acute gastroenteritis worldwide. Due to the lack of an efficient human norovirus cell culture system coupled with an animal model, human norovirus research mainly relies on human volunteer studies and surrogate models. Current models either utilize human norovirus-infected animals including the gnotobiotic pig or calf and the chimpanzee models, or employ other members of the family Caliciviridae including cell culture propagable surrogate caliciviruses such as the feline calicivirus, murine norovirus and most recently the Tulane virus. One of the major features of human noroviruses is their extreme biological diversity, including genetic, antigenic and histo-blood group antigen binding diversity, and possible differences of virulence and environmental stability. This extreme biological diversity and its effect on intervention/prevention strategies cannot be modelled by uniform groups of surrogates, much less by single isolates. Tulane virus, the prototype recovirus strain, was discovered in 2008. Since then, several other novel recoviruses have been described and cell culture adapted. Recent studies indicate that the epidemiology, the biological features and diversity of recoviruses and the course of infection and clinical disease in recovirus-infected macaques more closely reflect those properties of human noroviruses than any of the current surrogates. This review aims to summarize what is currently known about recoviruses, highlight their biological similarities to human noroviruses and discuss applications of the model in addressing questions relevant for human norovirus research.

Introduction

Human noroviruses (NoVs) are one of the leading causes of acute gastroenteritis in both developed and developing countries. Annually worldwide, NoVs are responsible for more than one million hospitalizations and ~200 000 deaths in children less than 5 years of age (Patel et al., 2008). In the USA alone, an estimated 23 million cases of acute gastroenteritis, including >70 000 hospitalizations and 800 deaths are attributed to NoV infections each year (Hall et al., 2012; Lopman et al., 2011). The prototype Norwalk virus was discovered after 40 years ago (Kapikian et al., 1972). However, despite decades of efforts, a NoV vaccine or an effective antiviral is still not available. This is mainly due to the lack of a human NoV cell culture system or robust animal model. Human NoV research has relied on human volunteer studies or surrogate caliciviruses and/or hosts. However, these surrogate viruses or hosts often do not reflect essential biological features of the human NoVs and their host. One of these features is the extreme biological diversity of human NoVs, including genetic, antigenic and histo-blood group antigen (HBGA) binding heterogeneity.

Differences of virulence and environmental stability are also suspected among the different human NoV strains but evaluation of these features without an efficient cell culture system or animal model is extremely challenging (Friesema et al., 2009; Le Guyader et al., 2012).

The two major approaches for human NoV model development, besides human volunteers, include the experimental infection of animal hosts with human NoVs and the use of animal caliciviruses as surrogates in their natural hosts. The first approach is represented by the human NoV chimpanzee, gnotobiotic pig or gnotobiotic calf models (Bok et al., 2011; Cheetham et al., 2006; Souza et al., 2008). Recently, promising data on the development of a human NoV mouse model have also been reported (Taubet al., 2013). The advantage of these models is the use of human NoVs while the major common limitation is the lack of a cell culture system for multidisciplinary studies. Other limitations are variable among the different models and include the subclinical infection (Bok et al., 2011; Taube et al., 2013), the immunocompromised host (Cheetham et al., 2006; Souza et al., 2008; Taube et al., 2013), the unconventional route of inoculation (Bok et al., 2011; Taube et al., 2013), the NoV strain-specific infection...
Reovirus model

(Taube et al., 2013) and the high cost and restricted use of
the species (Bok et al., 2011).

The second approach relies on cell culture propagable surro-
gate caliciviruses, including the feline calicivirus (FCV),
murine NoV (MNV) and most recently the prototype
reovirus (ReCV) strain the Tulane virus (TV) (Farkas et al.,
2008; Fastier, 1957; Karst et al., 2003). MNV is the only NoV
with an efficient cell culture system and small animal model.
Due to these characteristics, since its discovery in 2003,
MNV has quickly become the most frequently used human
NoV surrogate (Wobus et al., 2006).

The TV was isolated from stool samples of rhesus macaques
in 2008. Since then, a large number of diverse ReCVs have
been described and/or isolated in cell culture. Although
ReCVs represent a novel genus within the family Calici-
viridae, their epidemiology, biological features, disease
spectrum and natural host are more closely related to those
of human NoVs than any of the other surrogate viruses. In
addition, the role of ReCVs as human pathogens has also
been indicated (Farkas & Wong Pin Lun, 2014; Farkas et al.,
2010a; Smits et al., 2012). The aim of this review is to
summarize our current knowledge about ReCVs, highlight
their biological similarities to human NoVs and discuss
applications of the model in addressing questions relevant
for human NoV research.

Reovirus

Reovirus is a putative genus within the family Calici-
viridae that comprises small, non-enveloped, positive-sense RNA
viruses. The prototype ReCV strain (TV) was discovered in
stool samples collected from juvenile macaques housed at the
Tulane National Primate Research Center (TNPRC). TV
could be grown in LLC-MK2 monolayers and charac-
terization of cell culture propagated TV virions revealed
typical calicivirus morphology, size and buoyant density,
and one major structural protein of ~60 kDa. The TV
genome is 6714 nt long and contains three ORFs displaying
all the major amino acid motifs characteristic for calici-
viruses. Phylogenetic analysis revealed that TV roots with the genus Norovirus but with distances equal to that
between other established calicivirus genera. Based on the
acronym of rhesus enteric calicivirus a new genus name
Reovirus was proposed (Farkas et al., 2008).

Genetic diversity

Analysis of partial RNA-dependent RNA polymerase
(RdRp) sequences of ReCVs detected in 500 macaque stool
samples collected between May and October 2008 at the
TNPRC revealed two major ReCV genogroups (G1 and G2).
Moreover, the G1 strains further separated into three
genotypes (G1.1–G1.3) (Farkas et al., 2010a) (Fig. 1a).
Considering that the samples were collected during a short
period of time and from only one macaque colony, a much
greater, human NoV-like diversity of ReCVs can be expected.
Recently, capsid sequences for several G1.1 ReCVs and sequence information for two additional ReCV
strains were published (Handley et al., 2012; Smits et al., 2012;
Farkas et al., 2014). The WUHARV strain (Handley et al.,
2012) detected in macaque stool samples grouped with the
G1.2 ReCVs, while the ReCV Bangladesh/289 strain (Smits
et al., 2012) detected in human stool samples separated from
all the macaque strains with distances equivalent to those
between human NoV genogroups and was assigned to a third
genogroup (G3) (Farkas et al., 2014) (Fig. 1a). Comparison of
ReCV genomic sequences revealed that while all ReCVs
contain three ORFs, the distance or overlap and the frame
shifts between the ORFs exhibit significant variation among
the different ReCV genotypes (Farkas et al., 2014). Such
variation is not observed among human NoVs.

Antigenic diversity and serotypes

Because of the extreme genetic diversity of human NoVs,
information on the relationship between genetic types and
serotypes is important for vaccine development. However,
due to the lack of an efficient cell culture or animal model,
direct evaluation of human NoV serotypes remains challen-
ging. The most widely used MNV surrogate model exhibits
little genetic and antigenic variation and all laboratory strains
belong to the same serotype (Thackray et al., 2007).

Recently, the serotypic characterization of 10 cell culture
adapted G1 ReCVs, shown in bold type in Fig. 1(a), has
been reported (Farkas et al., 2014). Cross-neutralization
with hyperimmune sera raised in mice against one
representative of each of the three G1 genotypes revealed
the existence of at least four different neutralization types
(serotypes). All of the four G1.1 ReCV strains involved
in the study were neutralized only by the hyperimmune
serum raised against TV (G1.1), grouping these strains into
serotype 1 (S1). Similarly, all of the three G1.3 isolates
studied were neutralized by the hyperimmune serum raised
against the G1.3 representative strain (FT7) (S3). However,
one of the G1.3 strains (FT65) was also neutralized equally
by the anti-TV (S1) serum indicating that despite the 25 %
aa difference between the VP1 of FT65 and TV the two
strains still shared neutralization epitope(s). According to
these data, FT65 represents a strain with cross-neutraliza-
tion epitopes (S1 + S3). In contrast, the hyperimmune
serum raised against the G1.2 representative strain (FT285)
neutralized only FT285 (S2) and did not neutralize any of
the other strains including the two other G1.2 isolates.
According to these results, the two other G1.2 isolates
(FT499 and FT157), which shared 99 % aa identity in VP1
with each other, represent a fourth serotype (S4). The VP1
sequences of FT285 (S2) and the other G1.2 strains (S4)
exhibited 12 % aa difference. Considering the existence of
over 30 human NoV genotypes and the biological simi-
larities between ReCV and human NoVs, these observa-
tions have significant implications. In general, genotypic
classification seems to correlate well with serotypes.
Moreover, even strains that based on the currently used
NoV classification constraints are classified into the same
genotype can represent two novel serotypes (Zheng et al.,
2006; Kroneman et al., 2013). The role of ReCV virus

http://vir.sgmjournals.org
neutralization antibodies and serotypic differences in protection against infection or disease still needs to be evaluated in the macaque model but these recent data indicate that the number of human NoV serotypes could be an overwhelming challenge for human NoV vaccine development. Extended studies for understanding the mechanism of human NoV antibody neutralization will be important for the development of antibody protection based prevention and intervention strategies.

ReCV–HBGA interactions

HBGAs have been implicated as attachment molecules or receptors for both human NoVs and ReCVs. These viruses bind to synthetic HBGAs structures or HBGAs present in bodily secretions and on cell surfaces. At least eight different in vitro HBGa binding patterns have been identified among human NoVs, involving the ABO- and Lewis-type HBGAs (Huang et al., 2005). In addition, volunteer challenge studies have established an association between

Fig. 1. (a) ReCVs can be classified into three genogroups (G1–3). G1 and G2 contain strains detected in rhesus macaques, while G3 is represented by a strain detected in humans. The phylogenetic tree shown is based on analysis of 268 nt RdRp sequences. Cell culture adapted G1.1 strains used in subsequent studies are in bold type. The scale bars indicate the evolutionary distance represented by nucleotide or amino acid substitutions per site. The numbers at the nodes indicate the percentage of times that a node was supported during 1000 bootstrap analyses. (b) Saliva samples block ReCV replication in accordance with the HBGA binding properties (A, B, O types) of the isolates. Boiled human saliva samples were incubated with 100 TCID<sub>50</sub> of ReCVs for 1 h at 37 °C and transferred into 96-well tissue culture plates seeded with 10<sup>4</sup> LLC-MK2 cells per well. Virus- and mock-inoculated control wells were included. Plates were stained with crystal violet at 72 h post-inoculation. By this time all cells in the virus control wells were rounded and detached, resulting in clear unstained wells, while wells with attached cell monolayers stained blue. (c) While saliva samples block ReCV replication, BSA-conjugated type A and B oligosaccharides increase ReCV infectivity in a type-specific and concentration-dependent manner that corresponds to saliva blocking. The only exception is strain FT285, which is blocked by both the type B saliva and type B synthetic oligosaccharide.

ReCV–HBGA interactions

HBGAs have been implicated as attachment molecules or receptors for both human NoVs and ReCVs. These viruses bind to synthetic HBGAs structures or HBGAs present in bodily secretions and on cell surfaces. At least eight different in vitro HBGa binding patterns have been identified among human NoVs, involving the ABO- and Lewis-type HBGAs (Huang et al., 2005). In addition, volunteer challenge studies have established an association between
HBGA binding and susceptibility to infection for the Norwalk virus and more recently for a G2.4 human NoV, revealing a key role of the secretor status linked with function of the FUT2 gene (Frencik et al., 2012; Lindesmith et al., 2003). However, for the Snow Mountain virus, which binds to type B HBGA, an association between in vitro HBGA binding and susceptibility could not be established (Lindesmith et al., 2005).

In vitro, TV binds to type A and B salivary and synthetic HBGAs. An association between ReCV infections and particular HBGA type(s) could not be established by the assessment of 500 colony macaques because 98% of the animals phenotyped as type B (Farkas et al., 2010a). ReCV challenge studies to correlate in vitro HBGA binding to susceptibility to infection still remain to be performed.

Cell culture studies with TV also demonstrated that type A and B, but not type O boiled saliva samples block TV replication in vitro, most likely by blocking attachment of the virus to cell surface ligands (Farkas et al., 2010a). Evaluation of G1 ReCVs with a panel (n=35) of human saliva samples revealed the existence of three HBGA interaction types (unpublished data) (Fig. 1b). Replication of all G1.1 strains was blocked by the type A and B, but not by the type O saliva samples while the G1.2 strains were blocked only by the type B saliva samples. Interestingly, two of the three G1.3 isolates tested were blocked by both the type A and B saliva samples, while one isolate was not blocked by any of the saliva samples. Saliva samples blocked or neutralized ReCV replication at even higher dilutions (>1:2560) than convalescent serum samples obtained from animals after experimental challenge or natural infection (1:640–1280) (data not shown).

Very little is known about the mechanism of human NoV and ReCV infections, including the exact role of HBGAs and the target cell types in the natural host. However, the ability of salivary HBGAs to inhibit ReCV replication in cell culture indicates that secreted HBGAs and perhaps other oligosaccharides could act as natural antivirals (Farkas et al., 2010a). Since the route of human NoV and ReCV infections is faecal–oral, how can these pathogens remain infectious after the exposure to salivary/gastric HBGA? It was postulated that infectivity is regained after detachment of HBGAs under the low pH conditions in the stomach and/or by the help of digestive enzymes. However, incubating ReCV/saliva mixes at low pH (pH 1–4) did not result in any gain of infectivity (unpublished data). Moreover, we found that infectivity of ReCVs dropped significantly (>75%) at pH 2 and 9, and all isolates became completely inactivated at pH 1 and 10 even after 30 min exposure time (Fig. 2). The intragastric pH in healthy individuals is in the pH 1.3–2.5 range and the 50% emptying of stomach contents requires about 2.5–3 h (Degen & Phillips, 1996; Kong & Singh, 2008). Taken together, the infectivity of ReCVs and perhaps human NoVs would be significantly jeopardized in the stomach without some protective mechanism. Food intake temporarily increases the stomach pH to a pH 4.5–5.8 range that could explain virus survival. In contrast, obtaining a protective mucous layer by selective salivary/mucosal HBGA binding could be a mechanism that some enteric viruses employ for preserving infectivity under unfavourable conditions. In any case, the questions of how these pathogens regain their infectivity and how pH sensitive, non-binder strains pass through the stomach remain from to be answered. Alternatively, infection through bypassing the stomach by macrophage, dendritic cell or other immune cell uptake should also be considered. Detection of human NoVs or ReCVs in dendritic cells or and CD20+ B-cells in the lamina propria, but not in epithelial cells in intestinal biopsies has been reported (Bok et al., 2011; Chan et al., 2011; Lay et al., 2010; Sestak et al., 2012). Due to its similarities to human NoVs, the ReCV model could provide valuable insights to the mechanism of enteric calicivirus infections.

**Synthetic oligosaccharides**

In vitro binding assays demonstrated TV binding to type A and B saliva samples and BSA-conjugated type A and B trisaccharides. However, while type A and B saliva samples blocked TV replication, incubation of TV with BSA-conjugated type A and B trisaccharides resulted in a significant increase of plaque numbers. This increase of infectivity was concentration-dependent (Farkas et al., 2010a). Recent evaluation of other G1 ReCV isolates confirmed this observation, with one exception. While the replication of all A/B and B-binder ReCVs could be blocked by the corresponding type saliva samples, the BSA-conjugated type A and/or type B synthetic oligosaccharides increased infectivity in a concentration-dependent manner. The only exception was one of the G1.2 isolates. Replication of this strain (FT285) was inhibited by both the type B saliva samples and the BSA-conjugated type B oligosaccharide. Neither the saliva samples nor the synthetic oligosaccharides had an effect on the non-binder strains (Figs 1b, c). The opposite effect of saliva samples and synthetic oligosaccharides on ReCV replication in cell culture remains to be investigated. One possible explanation is an entry mechanism involving a yet unknown cell surface entry receptor and HBGA binding-dependent conformational activation of the viral entry receptor binding site. According to this hypothesis, interaction between the ReCV capsid and HBGAs leads to structural changes and activation of the entry receptor binding site that is necessary for binding and entry (Farkas et al., 2010a). When virus particles bind to cell surface HBGAs that are within an optimal distance from the entry receptor, virus entry will proceed and lead to successful infection (Fig. 3a), while attachment to HBGAs that are unable to recruit entry receptors will not support infection (Fig. 3b). In saliva, the large carrier molecules of HBGAs (mucins) block virus binding to cell surface HBGAs and/or entry receptors (Fig. 3c), while in the case of BSA-conjugated synthetic oligosaccharides, the relatively small BSA carriers have no interference with entry receptor binding. Moreover, due to the conformational activation of the entry receptor binding sites upon BSA-conjugated HBGA binding, virus
entry is supported through both entry receptors located at optimal distance from cell surface HBGAs (Fig. 3d) and entry receptors that are distant from cell surface HBGAs (Fig. 3e). This leads to increased efficiency of infection as evidenced by increased plaque numbers. It could be hypothesized that in the case of FT285, the HBGA binding and entry receptor binding sites are overlapping or are in very close proximity to each other, which could explain the blocking effect of BSA-conjugated type B oligosaccharides. While identification of the ReCV entry receptor and evaluation of this hypothesis in cell culture based and structural studies remains to be accomplished, previous work with other members of the family Caliciviridae points to a complex calicivirus cell entry mechanism. It was shown that cell lines expressing the necessary HBGAs bind the Norwalk virus (White et al., 1996), and when transfected with viral protein genome (VPg)-linked Norwalk virus RNA, assemble progeny virions, but remain non-permissive without cell-to-cell spread of the infection (Guix et al., 2007). Thus, cell surface expression of HBGAs alone does not confer susceptibility to human NoV infection and permissiveness of these cell lines is most likely hindered at the receptor/co-receptor binding, uncoating and/or budding stages of the replication cycle. For other caliciviruses a proteinaceous entry receptor has been already identified. FCV use α2,6-linked sialic acid for attachment and the feline junctional adhesion molecule 1 (fJAM-1) for internalization (Makino et al., 2006; Stuart & Brown, 2007). The role of annexin A2 was indicated in cellular entry of the rabbit vesivirus (González-Reyes at al., 2009). Moreover, supporting our hypothesis, recent studies also suggested receptor-induced conformational changes during FCV entry (Ossiboff et al., 2010).

---

**Fig. 2.** Effect of pH on ReCV infectivity. (a) 25–100 p.f.u. of ReCVs were incubated at different pH values ranging from pH 1 to pH 10 for 30 min at 37 °C. After adjusting the pH to neutral, plaque assay was performed as described previously (Farkas et al., 2014). Aichi virus adapted to grow on LLC-MK2 cells was used as control. (b) Reduction of infectivity was calculated as percentage of p.f.u. at pH 7 by the following formula: 100 − [(p.f.u. at the corresponding pH/p.f.u. at pH 7)×100]. Values represent three independent experiments. Bars represent SD. Statistical significance was calculated by the unpaired t-test method with Welch correction. *P<0.05; ***P<0.0001. The following buffer systems were used: pH 1–2, KCl/HCl buffer; pH 3–6, citrate buffer; pH 7–8, phosphate buffer; pH 9–10, carbonate buffer.

---

**Fig. 3.** Hypothesized involvement of a putative entry receptor(s) in enteric CV replication based on observations of ReCV–HBGA interactions in cell culture. Detailed explanation of the figure can be found in the text.
During the preparation of this manuscript, Zhang et al., reported that TV tends to bind to the test tubes and since the presence of BSA inhibits virus binding to the plastic, the increased plaque numbers upon BSA-conjugated oligosaccharide treatment represent reduced binding to the test tube rather than a concentration-dependent increase of infectivity (Zhang et al., 2015). In our laboratory, including BSA at the same concentrations as the BSA-conjugated oligosaccharides in the assay did not increase the plaque numbers. In addition, no difference in plaque numbers was observed between TV plaqued directly from the virus stock or after incubation for 2 h in the assay tubes or 96-well tissue culture plates. The existence of non-binder strains, which are not affected by either the type B or type A BSA-conjugated oligosaccharides, or the strictly B-binder strains, which are affected only by the type B but not the type A BSA-conjugated oligosaccharides (Fig. 1c), respectively, further indicates that virus binding to the tubes was not an issue in our experiments. Furthermore, in correlation with our observations, a stimulative effect of HBGAs on G2.4 human NoV infectivity was recently also reported (Jones et al., 2014).

**Neutralization escape and HBGA binding**

The availability of unique ReCV isolates that cross-neutralize between different serotypes and the possibilities to generate neutralization escape mutants coupled with the animal model open up new ways for understanding the mechanism of enteric calicivirus antibody neutralization and the role of antibodies in protection against infection or disease. Based on studies with G2.4 human NoVs it was suggested that antigenic changes facilitating escape from herd immunity may also drive changes in HBGA binding affinities and lead to altered population susceptibility (Debbink et al., 2012; Lindesmith et al., 2012). To model this hypothesis we recently generated neutralization escape mutants by repeatedly subculturing ReCVs under constant serotype-specific polyclonal neutralizing antibody pressure (unpublished data). Neutralization escape was monitored in a plaque assay with 20–40 virus-neutralizing units (VNU) of serotype-specific hyperimmune sera as described in our previous studies (Farkas et al., 2014). The HBGA interaction pattern of variants showing neutralization escape was evaluated by analysing the effect of type-specific saliva samples and synthetic oligosaccharides on virus replication (Fig. 4). While TV variants exhibiting ~40% neutralization escape showed similar HBGA interaction patterns to the wild-type TV, the FT285 escape mutant exhibiting ~20% neutralization escape lost its interaction with type B HBGAs. These are only preliminary results and further analyses are needed, but they show that co-evolution of neutralization escape and HBGA interactions of enteric caliciviruses happens and can be modelled in vitro. Interestingly, the loss of type B HBGA interactions did not have an effect on the replication of the FT285 escape mutant in LLC-MK2 cells. This and the existence of ‘non-binder’ cell culture adapted strains indicate that ReCVs and possibly human NoVs are able to substitute HBGA binding with some other mechanism for attachment/entry. Analysis of these strains including the identification of mutations responsible for neutralization escape and/or HBGA interaction changes could greatly improve our understanding of enteric calicivirus biology and evolution. Availability of susceptible and non-susceptible cell lines, including those able to support first cycle ReCV replication after viral RNA transfection (unpublished data), cell culture adapted ReCV strains with different HBGA interaction patterns, including ‘non-binders’, and a simple ReCV reverse genetics system (Wei et al., 2008) make this model exceptionally fit for studying the role of HBGA binding in enteric calicivirus attachment, entry and susceptibility to infection. Study of virus–host interactions can be extended to other cell surface determinants and identification of the target cells in the animal model (Table 1).

**Pathogenicity differences**

Very little is known about human NoV virulence and whether significant differences exist among the different strains or genotypes. A retrospective study comparing clinical symptoms following experimental infection of volunteers with the Norwalk virus or Snow Mountain virus found that Snow Mountain virus infection was associated with more symptoms and a higher frequency of painful symptoms than Norwalk virus infection. In addition, all Snow Mountain virus infections were symptomatic, while both symptomatic and asymptomatic infections were observed with the Norwalk virus (Kirby et al., 2014). Such studies suggest the existence of pathogenicity differences among human NoVs. However, due to the lack of cell culture and animal models, and the unknown pre-exposure history of human volunteers, evaluation of human NoV virulence and its determinants remains challenging.

The only ReCV challenge study demonstrated that TV-infected macaques develop clinical disease, including diarrhoea and fever (Sestak et al., 2012). However, whether there are differences among ReCV isolates in their virulence, and the existence of highly pathogenic strains causing severe, distressing disease remains to be investigated. In vitro evaluation of G1 ReCVs indicates significant differences of growth kinetics, end titre and plaque morphology among the different isolates (unpublished data). These differences are unlikely the result of selection of cell culture adapted variants, since working stocks for all ReCV strains were made equally at low passage numbers (>3). In vitro growth differences of pathogens are often indicative of in vivo virulence differences. For example, it was reported that virulent systemic (VS) FCV isolates produce infectious progeny virus and display cytopathic effects earlier, and grow to substantially higher titres in cell culture than vaccine/non-VS isolates (Ossiboff et al., 2007).

Based on the availability of diverse ReCV strains, the close relationships between human NoVs and ReCVs and their natural hosts, this surrogate model represents an exceptional
tool for our understanding of enteric calicivirus pathogenicity and virulence.

ReCV animal model

In this review, the comparison between the ReCV model and other surrogate models will focus on the MNV model. MNV is the only cell culture propagable NoV and it also is the only small animal model for human NoV research. Due to several advantages compared to other surrogates, MNV became the most widely used human NoV model and contributed significantly to our understanding of NoV biology, pathogenesis and immunity (Wobus et al., 2006).

However, there are also major differences between MNV and human NoVs that should not be overlooked. MNV infection is asymptomatic and often persistent in normal mice. In immunocompromised mice, MNV infection leads to systemic, lethal disease, while human NoV gastroenteritis is an acute disease followed by self-clearance of the infection. MNVs infect haematopoietic cells and several murine macrophage and dendritic cell lines efficiently support in vitro MNV replication. The cell tropism of human NoVs is not yet known. It was shown that macrophages or dendritic cells derived from the peripheral blood of susceptible humans do not support Norwalk virus replication (Lay et al., 2010). The presence of an additional open reading frame (ORF4) in the MNV genomes may indicate differences in replication strategy. MNV exhibits little genetic and antigenic variation and does not utilize HBGAs for attachment. In addition, classical inbred laboratory mice strains have a limited level of genetic variation compared with humans and there are significant physiological differences between the two hosts. While one of the major clinical signs of human NoV gastroenteritis is vomiting, mice are physiologically unable to vomit. These differences

Fig. 4. (a) ReCVs cultured under continuous type-specific polyclonal neutralizing antibody pressure develop partial neutralization escape. (b) Neutralization escape had no effect on the saliva-blocking pattern of TV. Replication of both the wild-type and escape mutant TVs was blocked by type A and B saliva samples. In contrast, while the wild-type FT285 was blocked by type B saliva samples, the escape mutant FT285 (E) also escaped type B saliva blocking. (c) The effect of synthetic oligosaccharides on TV replication remained unchanged after neutralization escape. Replication efficiency of both the wild-type and escape mutant TVs increased after incubation with BSA-conjugated type A and B trisaccharides (10 μg ml⁻¹). In contrast, while the wild-type FT285 was blocked by the BSA-conjugated type B trisaccharide, the escape mutant FT285 (E) also escaped oligosaccharide blocking. Means and SD of three independent experiments are shown.
may represent limitations of the model in specific areas of research or raise reservations about the direct applicability of research outcomes obtained with the MNV model to human NoVs (Vashist et al., 2009).

Compared to MNV, very little is known about the epidemiology and clinical manifestation of ReCV infections. Molecular detection and/or seroprevalence studies established that ReCVs are endemic among rhesus macaques housed at National Primate Research Centers in the USA and are also present at variable levels in other captive non-human primate species (Farkas et al., 2010a, b, 2012). ReCV infections in primates living in the wild have not yet been studied. In a recent study, enteric viruses including caliciviruses were not detected in free-ranging New World monkeys in Brazil (Souza et al., 2012).

The only ReCV challenge study to date reported the development of serotype-specific seroresponses and virus shedding, indicating infection of the three rhesus macaques challenged with TV (Sestak et al., 2012). In addition, two of the infected macaques developed clinical disease characterized by diarrhoea and fever. Vomiting was not observed. Duodenum biopsies showed moderate villous blunting and lymphocytic infiltration in the lamina propria. TV-positive enterocytes were not detected. However, TV-positive cells, including CD20+ B-cells, were identified in the lamina propria. In addition, when in vitro cultures of peripheral blood mononuclear cells collected from healthy macaques were inoculated with TV, a 2-log increase of viral RNA by 6 days post-inoculation suggested TV replication in cultured lymphocytes. The presence of TV antigen was confirmed predominantly in CD20+ HLA-DR+ B-cells in these cultures. Recently, it was reported that G2.4 human NoVs infect B-cells in culture as indicated by increased viral copy number and viral protein synthesis. Moreover, bacteria that express the appropriate HBGA

<table>
<thead>
<tr>
<th>Area of research</th>
<th>Research question</th>
<th>Research approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus–host interaction</td>
<td>What is the role of HBGAs in susceptibility to infection?</td>
<td>In vitro and in vivo studies utilizing susceptible and non-susceptible cell lines and animals</td>
</tr>
<tr>
<td></td>
<td>What other molecules are involved in cellular attachment/entry for strains that do not bind to HBGAs?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Is there an entry receptor involved in ReCV infection?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Are these elements shared between non-human primates and humans or other species that would promote the zoonotic transmission of ReCV?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>What are the target cells of ReCVs?</td>
<td></td>
</tr>
<tr>
<td>Virulence</td>
<td>Are there differences in virulence among the different genogroups/types or strains?</td>
<td>In vitro and in vivo studies utilizing the challenge model and reverse genetics system</td>
</tr>
<tr>
<td></td>
<td>What are the viral determinants of ReCV virulence?</td>
<td></td>
</tr>
<tr>
<td>Immunity</td>
<td>Is the immunity against ReCVs long-term or short-term?</td>
<td>In vitro and in vivo studies utilizing the challenge model</td>
</tr>
<tr>
<td></td>
<td>Is protection driven by neutralizing antibodies and is it type specific?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>What is the protective level of virus-neutralizing antibodies?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Do ReCVs target CD20+ B-cells causing a perturbed immune response by affecting the development of long-lived plasma cells?</td>
<td></td>
</tr>
<tr>
<td>Antibody neutralization/evolution</td>
<td>What is the mechanism of ReCV antibody neutralization?</td>
<td>In vitro studies including neutralization escape, reverse genetics, cryo-electron microscopy and X-ray crystallography</td>
</tr>
<tr>
<td></td>
<td>Do antibody neutralization sites overlap with HBGA binding?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Will antibody neutralization escape alter HBGA binding?</td>
<td></td>
</tr>
<tr>
<td>Recombination</td>
<td>What is the frequency of recombination events?</td>
<td>In vitro studies utilizing the cell culture and reverse genetics systems</td>
</tr>
<tr>
<td></td>
<td>Is there inter-genogroup recombination?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Are macaque/human ReCV recombinants viable?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Are there recombination hotspots in the ReCV genome?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Can recombination lead to sudden changes in virulence?</td>
<td></td>
</tr>
<tr>
<td>Environmental stability</td>
<td>Are there differences among the different isolates?</td>
<td>In vitro studies relying on diverse isolates and cell culture techniques measuring infectivity</td>
</tr>
<tr>
<td></td>
<td>Can the ReCV model be used for evaluation of disinfectants, food processing techniques, shellfish de contamination?</td>
<td></td>
</tr>
<tr>
<td>Antiviral development</td>
<td>Are there biologically important conservations (structural or others) among human NoVs and ReCVs that could be exploited for broad antiviral strategies?</td>
<td>In vitro and in vivo studies</td>
</tr>
</tbody>
</table>

These applications of the ReCV model have been proposed in detail to the NIH in grant applications R21OD011933, R24OD11884, RO1AI068104, RO1AI084998, RO1AI08707 and RO1AI089587.
and free HBGA stimulate G2.4 human NoV infectivity, while non-HBGA-expressing bacteria or LPS have no effect (Jones et al., 2014). These are striking similarities to our earlier reports with TV (Farkas et al., 2010a; Sestak et al., 2012).

In the TV challenge study, serotype-specific virus-neutralizing antibody responses could be detected within 7 days post-challenge and neutralizing antibodies persisted at peak levels for 5–6 weeks, and then started to decline to almost pre-inoculation levels by post-inoculation day 80. The protective efficacy of virus-neutralizing antibodies was not assessed in rechallenge studies. Nevertheless, the kinetics of virus-neutralizing antibody responses suggests a short-term antibody protection against TV infection, which might be linked to perturbed B-cell functions, especially long-lived plasma cell functions.

Even though the low number of animals involved in this study did not allow proper statistical evaluation of the clinical symptoms, the outcomes of TV infection resembled observations obtained in human NoV volunteer challenge studies. These included the clinical signs of gastroenteritis, mononuclear infiltrates in the lamina propria, villus blunting in the small intestine, and the possible short-term, strain-specific immunity (Green, 2013). Furthermore, it is important to highlight the presence of low level pre-challenge TV-neutralizing antibodies and the rapid serotype-specific neutralizing antibody response at post-challenge days 5–7, indicating prior exposure and existing immune memory of the animals. The presence of low level neutralizing antibodies at challenge and the rapid memory response could have prevented the development of more severe symptoms, possibly including vomiting. Follow-up studies with a higher number of animals, also including other ReCV strains and macaques representing the different HBGA types will be necessary to fully establish the novelty of the ReCV animal model for human NoV research.

The major disadvantage of a non-human primate model is the associated cost that substantially exceeds the cost of a small animal model. However, the genetic, immunological, anatomical and physiological characteristics of non-human primates are the closest to human. These and the biological similarities between ReCVs and human NoVs are important.

ReCV infection in humans

The first indication of ReCV infections in humans was the detection of TV-neutralizing antibodies in archived serum samples collected from animal caretakers at the TNPRC (Farkas et al., 2010a). TV was not neutralized by mouse or rabbit hyperimmune sera raised against human NoV VLPs, indicating that the possibility of cross-neutralization by anti-NoV antibodies in human serum samples was unlikely. On the basis of these data, it was suggested that TV-neutralizing antibodies in human serum samples are most likely the result of zoonotic ReCV infections. Direct evidence for human ReCV infection has been provided by the molecular detection of a novel ReCV strain in stool samples of six Bangladeshi patients exhibiting clinical symptoms of gastroenteritis (Smits et al., 2012). In a more recent serology study, significantly higher prevalence of neutralizing antibodies was found against three ReCV serotypes in serum samples collected from zookeepers (28–100 %) than in the general population (3–18 %), which further supports the zoonotic origin of human ReCV infections (Farkas & Wong Ping Lun, 2014). In contrast, genetic analysis of the ReCV/Bangladesh/289 strain revealed that its capsid protein (VP1) shares only 38–41 % amino acid identity with the macaque isolates and it represents a new genogroup (G3) of the genus Recovirus (Farkas et al., 2014; Smits et al., 2012). Several animal NoVs, including swine NoVs within G2 or canine NoVs within G4, are more closely related to human NoVs than the human and macaque ReCVs to each other. Recently the binding of canine G4.2 NoVs to HBGAs has been demonstrated (Caddy et al., 2014). Nevertheless, despite decades of worldwide NoV surveillance, detection of these animal NoVs in human clinical samples has not yet been reported. These data and the observation that the titre of ReCV-neutralizing antibodies in the human samples was significantly lower than in macaques call for further investigations, including the isolation of strains circulating in both non-human primate and human populations, before ReCV zoonosis can be clearly established. Regardless, based on the close biological features of ReCVs and human NoVs and their natural hosts, the zoonotic potential of ReCVs is evident. Since the aetiology of a large proportion of human diarrhoea cases cannot be identified and the emergence of new viral infections that are often linked to zoonosis (e.g. HIV, SARS, avian flu, Nipah virus, Ebola) cannot be predicted, studies to further investigate the role of ReCVs as human pathogens are important.

Closing remarks

Due to the lack of an efficient human NoV cell culture system, human NoV research is highly dependent on surrogate models. However, the ability of the different surrogates to accurately represent human NoVs is frequently questioned (Richards, 2012). While the use of human subjects is clearly optimal for at least certain areas of human NoV research, it has its own limitations including the time-consuming and highly regulated processes associated with human subject research, the unknown pre-exposure history of the volunteers, the restricted sampling procedures, the associated cost and facilities, and the lack of cell culture system for the analysis of clinical samples.

Continuous development of new surrogate models is important for overcoming the limitations of the current systems. An optimal human NoV surrogate model should provide capabilities for both in vitro and in vivo studies and represent the biological features of human NoVs and their host. Even though the progress in ReCV research is behind that of other surrogate systems, this research has clearly
demonstrated that the ReCV/macaque cell culture and animal model reflects the biological features and diversity of human NoVs and their host more closely than any of the other current surrogate models. In addition, use of ReCVs in macaques provides a disease model that is able to reproduce most of the clinical symptoms of human NoV gastroenteritis. Recent work sponsored by the USDA-NIFA Food Virology Collaborative (NoroCORE) has shed some light on TV as a valuable human NoV surrogate for the food industry (Cromeans et al., 2014; Wang et al., 2013). However, the major value of the ReCV/macaque model over the other surrogate models is its human NoV-like diversity and this point should not be ignored.

As summarized in Table 1, its unique features discussed in this review make the ReCV/macaque model highly applicable for addressing important questions about human NoV virus-host interactions, virulence, immunity, antibody neutralization, evolution, environmental stability and antiviral development that cannot be easily studied by the other models (Table 1).

Due to limited support, our knowledge on ReCVs is much behind that of the other surrogates. In this review I have tried to summarize work that was mainly performed in my laboratory or in collaborations with others, and highlight some possible applications of this novel model. I truly hope that this effort will lead to increased awareness and support of the ReCV surrogate system to human NoV research.

Acknowledgements

I thank Monica M. McNeal for critical reading of the manuscript and Dr Teruo Yamashita for providing the Aichi virus. I am grateful to my family for their support during my involvement in recovirus research.

References


