Rotaviruses and rotavirus vaccines

Ulrich Desselberger*†, Emily Manktelow†, Wilson Li†, Winsome Cheung†, Miren Iturriza-Gómar‡, and Jim Gray‡

†Department of Medicine, University of Cambridge, Addenbrooke’s Hospital, Cambridge CB2 2QQ, UK and ‡Enteric Virus Unit, Virus Reference Department, Centre for Infections, Health Protection Agency, London NW9 5HT, UK

Background: Rotaviruses (RVs) are an important cause of acute gastroenteritis in infants and young children worldwide, resulting in more than 600 000 deaths per annum, mainly in developing countries. Since the 1980s, there has been intensive research on the development of RV vaccine candidates, and since 2006 two vaccines have been licensed in many countries.

Sources of data: The scientific literature since the 1970s has been consulted, and the results of original research carried out in authors’ laboratories were used.

Areas of agreement: There are firmly established data on virus particle structure, genome composition, gene–protein assignment, protein-function assignment (incomplete), virus classification, the mechanisms of several steps of the replication cycle (adsorption, primary transcription, virus maturation—all partial), several mechanisms of pathogenesis, aspects of the immune response, diagnosis, illness and treatment, epidemiology and vaccine development.

Areas of controversy: Research on the following areas is still in full flux and in part not generally accepted: several steps of the replication cycle (mechanism of viral entry into host cells, mechanisms of packaging and reassortment of viral RNAs, morphogenesis of subviral particles in viroplasms and maturation of virus particles in the rough endoplasmic reticulum (RER) with temporary acquisition and subsequent loss of an envelope), the true correlates of protection and the long-term effectiveness of RV vaccines.

Growing research: Recently, a system that allows carrying out reverse genetics with some of the RV genes has been established which, however, has limitations. There is intensive research ongoing, which is trying to develop better and universally applicable reverse genetics systems. There is broad research on the molecular mechanisms of the immune response and on which immunological parameter correlates best with lasting protection from severe RV disease. Research into other than live attenuated vaccines is growing.

Areas timely for developing research: The establishment of better reverse genetics systems for RVs is the most important research goal for both the understanding of the molecular biology of RVs and the development of new and safe RV vaccines. The black boxes of our knowledge on aspects of RV
replication (RNA packaging, RNA replication, control of reassortment and functions of the non-structural RV proteins) are under intensive research.

Keywords: rotavirus/rotavirus vaccines/rotavirus epidemiology/correlates of protection/rotavirus replication/rotavirus pathogenesis

Introduction

Rotaviruses (RVs) are a major cause of gastroenteritis among children of <5 years of age worldwide\textsuperscript{1,2} and of acute diarrhoea in the young of many other mammalian species (calves, piglets, lambs, rabbits, etc.) and of birds. In humans, they are responsible for more than 600 000 deaths each year, mostly of infants and young children in developing countries.\textsuperscript{2} RVs were found to cause human disease in 1973.\textsuperscript{3,4} Since the 1980s, research has focused on the development of RV vaccines, and since 2006 two vaccines have been licensed in many countries, in some with the aim of their inclusion in the expanded programme of immunization (EPI). Here RVs and their vaccines will be reviewed, and an outlook will be given on further likely developments within the field.

RV structure, genome and gene–protein assignment

The wheel-like structure is the characteristic feature of the RV particle in electron micrographs (Fig. 1) and has given the virus its name (\textit{Lat. rota} = wheel). The RV genome consists of 11 segments of double-stranded (ds) RNA encoding six structural proteins (VP1–VP4, VP6 and VP7) and six non-structural proteins (NSP1–NSP6) (Fig. 2A). The genomic RNA segments are between 667 (segment 11) and 3302 (segment 1) bp in size, and all share short, moderately conserved motifs within the 3’ and 5’ untranslated regions. The RV particles consist of an inner layer (‘the core’), containing VP1, VP2 and VP3 and enclosing the genome (Fig. 2B and C), an intermediate layer (‘the inner capsid’, made of VP6) and an outer layer (‘the outer capsid’, consisting of VP7 and projections of VP4) (Fig. 2C). The three-dimensional structure of RV particles has been studied by a number of groups.\textsuperscript{5–14} According to these findings, the three capsid layers are ordered along 5-, 3- and 2-fold symmetry axes (Fig. 2B–E) and are perforated by 132 aqueous channels of three different symmetry arrangements (‘classes’, Fig. 2B). The class I channels function as an outlet for mRNAs transcribed from subviral particles (see below).
Complete gene–protein assignments have been established for several RV strains (Fig. 2A); the protein-function correlations are only partially known. Details can be obtained from Table 1 of Estes and Kapikian.15

**Classification**

A classification scheme for RVs has been derived from genome composition and immunological reactivities of three of its components:15

1. The inner capsid protein, VP6, can be used to differentiate at least seven entities termed ‘groups’ (A–G). Within Group A RVs, two major subgroups (I, II) exist.16

2. Both surface proteins, VP4 and VP7, elicit neutralizing antibodies17,18 and are, therefore, considered to be involved in immune protection. Using these two proteins, a binary classification scheme has been established (similar to that developed for influenza viruses and so far only for Group A RVs) by which G types (VP7-specific, G for glycoprotein) and P types (VP4-specific, P for protease-sensitive protein) are distinguished. So far, at least 16 different G types and 27 P types have been detected, of which at least 11 G and 11 P types infect humans. G serotypes and genotypes carry identical numbers. Due to a lack of type-specific P antisera, it has been
agreed to designate the P serotype and genotype individually but jointly, the latter in square brackets: for example, the human Wa strain is classified as G1P1A[8], the human DS-1 strain as G2 P1B[4], the ovine strain Lp14 as G10P[15], etc.15

As RVs were found to reassort readily (see below) in doubly infected cells in vitro and in vivo and as VP4 and VP7 are encoded by different RNA segments, various combinations of VP4 and VP7 types have been observed in natural RV isolates.15 Virtually all RV genes are involved in reassortment events.19,20 The extensive genomic and antigenic diversity of RVs of both human and animal origins has led to the proposal to classify RVs according to the composition of the whole genome.20,21 Such a system will facilitate a full assessment of reassortment in nature,
of zoonotic transmission (see below) and probably of other, as yet undescribed, molecular events.

**Replication**

Viral replication occurs in the mature epithelial cells of the small intestine. Replication *in vitro* has been studied in some detail.\(^1\) The viral replication cycle is diagrammatically presented in Figure 3. Triple-layered particles (TLPs, i.e. the infectious virions) attach to the host cell and enter by receptor-mediated endocytosis or direct penetration. The cellular receptors of RVs have not been fully characterized, but viral uptake is characterized by a sequence of interactions with primary and secondary receptors, the latter acting as co-receptors in a post-attachment step.\(^{2,2a}\) Replication takes place exclusively in the cytoplasm. After removal of the outer capsid from TLPs by cellular
enzymes and a low intracellular Ca\(^{++}\) level, double-layered particles (DLPs) emerge, which become transcriptionally active, and large numbers of positive-stranded RNA molecules (capped but not polyadenylated) are transcribed from all 11 RNA segments, exiting the DLPs via the 12 aqueous class I channels located at the edges of the 5-fold axes of symmetry (Fig. 2F). The new RNA molecules either act as mRNAs, with their translation products accumulating in the cytoplasm, or undergo replication in intracytoplasmic inclusion bodies termed ‘viroplasms’. The two non-structural RV proteins, NSP2 and NSP5, are major components of viroplasms, which are essential for RNA replication and early morphogenesis.\(^{23-25}\) The viroplasms also contain VP1, VP2, VP3, VP6, NSP4 and, initially, mRNAs transcribed from all genomic segments. NSP5 interacts with both VP2\(^{26}\) and VP1.\(^{27}\) The exact order of events during early morphogenesis and the molecular interactions and control mechanisms by which packaging and reassortment of RNA segments into cores occur (Fig. 2C and E) are at present unknown. Cryo-EM studies have shown the arrangements of dsRNA inside the core as concentric rings.\(^{28,29}\) DLPs, formed in the viroplasms, consist of VP1, VP2, VP3 and VP6, and they contain one of each of the 11 RNA segments. The DLPs bud through the rough endoplasmic reticulum (RER), with NSP4 (at its C-terminal, cytoplasmic domain) acting as an intracellular receptor for VP6.\(^{30}\) An RER-derived envelope layer (which is lost before complete maturation) is transiently added to DLPs, and VP7 and VP4 are incorporated. VP4 may be added later in the process in ‘raft’-like structures near the plasma membrane.\(^{31}\) Triple-layered infectious virions are released by lysis of non-polarized cells (e.g. MA104), or by exit from polarized cells (e.g. Caco-2 cells), before a cytopathic effect becomes obvious.\(^{31}\) In faecal clinical specimens, TLPs and DLPs may often be seen side by side, and there may also be ‘empty particles’, which do not contain genomic RNA (Fig. 1). In immunodeficient hosts and under certain experimental conditions, RVs undergo genome rearrangements, which are intramolecular recombination events leading to partial gene duplication\(^{32}\).

**Reverse genetics**

For RVs, a reverse genetics system has been recently developed,\(^{33,34}\) which permits the rescue of a viral RNA segment produced from cDNA in vitro into replication-competent progeny virus. However, the system requires helper virus, and despite intensive research a helper virus-free system is still proving elusive.
Pathogenesis

Upon infection with RV, extensive cellular necrosis of the epithelium of the small intestine develops, leading to villous atrophy, loss of digestive enzymes, reduction in absorption and increased osmotic pressure in the gut lumen and the onset of diarrhoea. This is followed by a reactive crypt cell hyperplasia accompanied by increased fluid secretion, which also contributes to the severity of diarrhoea.¹⁵

Viral factors determining the pathogenicity of RVs have been investigated in several animal models (piglets, mice, rabbits). The protein product of RNA segment 4, VP4, has been found to be a major determinant of pathogenicity in several systems, but products of other structural (VP3, VP7) and non-structural genes (NSP1, NSP2, NSP4) have also been implicated.

The discovery of NSP4 acting as a viral enterotoxin (the first of its kind) has explained the well-known observation that RV-infected animals exhibit profuse diarrhoea prior to detection of histologic damage. NSP4 or a peptide thereof (aa 114-135) induces dose- and age-dependent diarrhoea in laboratory animals (mice, rats) in the absence of histological changes. NSP4 produces an increase in intracellular Ca²⁺ concentration and perturbs cellular electrolyte homeostasis. A peptide of NSP4, an active enterotoxin, is secreted from infected cells. The secreted protein binds to recently discovered cellular receptors and initiates signalling cascades in uninfected cells. Silencing of NSP4 expression in RV-infected cells by specific siRNA affects mRNA production and the formation of viroplasms, suggesting a significant involvement of NSP4 in RV replication and morphogenesis as well. Immunization of mice with an NSP4-based vaccine, followed by specific antibody production, attenuates the symptoms of RV-induced diarrhoea. Whether NSP4 antibodies exert a protective function in humans remains to be explored. The enteric nervous system may be involved in RV diarrhoea (and diarrhoea of other causes) as drugs blocking this system were shown to alleviate the diarrhoea. RV RNA and antigen have been detected in the sera of children with acute RV gastroenteritis. While viremia in RV infection appears to be relatively frequent, systemic disease is rare (see below), suggesting that spread of RV may be coincidental with systemic disease caused by a different microorganism.

Immune responses and correlates of protection

Neonatal or primary RV infection elicits a mainly serotype-specific humoral immune response providing homotypic immunity. Partial
protection against subsequent RV infections by other serotypes also
develops and increases with the number of re-infections.51 The exact
correlates of protective immunity remain to be determined, but levels
of RV-specific copro-antibodies of the IgA class seem to correlate
best.52–55 Humoral antibodies directed towards the inner capsid pro-
teins VP2 and particularly VP6 develop to high titers.56–58 These anti-
bodies do not neutralize in vitro but protect in vivo, possibly by
interacting with DLPs intracellularly and preventing transcription or
maturation (‘intracellular neutralization’57,59). There is a RV-specific
cytotoxic T cell response, but its exact role in overcoming a primary
infection or in protection against subsequent infections is not
known.55,60 Natural infection or appropriate vaccination (see below)
seem to protect from severe disease in subsequent infections,51,52 even
if the serotype of the challenging virus differs from that of the previous
infections or those in a prior vaccine.

Illness, diagnosis and treatment

After a short incubation period of 1–2 days, the onset of the illness is
sudden. Symptoms include watery diarrhoea lasting 4–7 days, as well as
vomiting, fever and rapid dehydration. The degree of severity of disease
varies widely from inapparent infections by so-called ‘nursery strains’
(frequent61) to central nervous system infections (rare62) and chronic
infections and hepatitis in children with immunodeficiencies (rare63). In
SCID mice, rhesus rotavirus (RRV) causes hepatitis.64 Respiratory symp-
toms are not infrequently seen during RV disease; however, there is no
direct evidence for RVs actively replicating in lung tissue.50

Diagnosis of an RV infection is relatively easy as large numbers of
virus particles (up to 10\textsuperscript{11} particles/ml of faeces) are shed at the acute
stage of the disease (Fig. 1). The main techniques are enzyme-linked
immunoassays (ELISAs), passive particle agglutination tests and, when
searching comprehensively for diarrhoeagenic viruses, EM (at present
not widely used). G and P types of RV isolates can be determined sero-
logically, but molecular techniques are increasingly being applied for
both detection and genotyping. RV-specific oligonucleotide primers
complementary to common and type-specific regions of the VP6, VP7
and VP4 genes allow sensitive detection, subgroup determination and
typing for both G and P types, respectively, by RT-PCR.16,19–21,65–67

Treatment of RV disease is by oral, subcutaneous or intravenous
rehydration and pain relief as indicated. The formulae of oral rehydra-
tion treatment (ORT) and reduced osmolarity ‘light ORT’,
recommended by WHO, are widely used.68,69 Oral immunoglobulins
seem to have an effect on the duration of diarrhoea and virus shedding
but are not routinely used. Encephalinase inhibitors have been used to dampen the symptoms of severe diarrhoea caused by RVs and other microorganisms. For immunocompromised children, passive immunization with RV antibodies obtained from eggs or milk has been found to be beneficial, but their wider use is not practicable.

Epidemiology

RVs spread mainly via the faeco-oral route. Water, fomites and occasionally food may act as vehicles. RV particles are very resistant to environmental conditions. The high particle number in the faeces of children with acute RV disease and the very small 50% diarrhoea-causing dose [1 DD50 = 10 plaque forming units (pfu)] lead to wide and efficient spread to any susceptible host. While there are marked seasonal peaks (winter/spring) in countries with temperate climates, in tropical regions RV infections and disease occur throughout the year.

The epidemiology of Group A RV infections is complex, since RVs of different G and P types co-circulate within a geographical region at any one time. The relative incidence of different G types also changes over time in the same location. Approximately 95% of co-circulating strains are types G1–G4 in most regions of temperate climate, typically G1P1A[8], G2P1B[4], G3P1A[8] and G4P1A[8], but other G types may be represented at high frequencies, particularly in tropical areas.

Recently, G9 RVs have been isolated as the predominant outbreak strains in several locations in the USA and in Europe. Besides being acquired in the community, RV infections are increasingly recognized as the cause of a significant proportion of nosocomial infections and diarrhoeal disease. Group B RVs have caused outbreaks of diarrhoea in children and adults in China and have been isolated from sporadic cases of gastroenteritis in Calcutta, India, and Bangladesh. Group C RVs are associated with small outbreaks in humans. Apart from the accumulation of point mutations (genomic drift), gene reassortment (genomic shift) plays a major role in generating the high diversity of RVs. Animals of different mammalian species are increasingly recognized as significant reservoirs for human RV infections as animal RVs have been found to infect humans directly and to form reassortants with human RVs.

Vaccine development

The burden of RV disease is high worldwide. According to the estimates based on studies carried out worldwide during 1986–2000, RVs
cause 100 million episodes of acute gastroenteritis, 25 million clinic
visits, 2 million hospitalizations and approximately 600 000 deaths per annum.\textsuperscript{1,2} While the incidence of RV disease is similar for developed and developing countries, death from RV disease is most frequent in developing countries of sub-Saharan Africa and Asia.\textsuperscript{1} Thus, the development of vaccines is urgent and started in the early 1980s.\textsuperscript{104} The early efficacy results were variable, owing to the enormous genomic and antigenic diversity of RVs.\textsuperscript{105–110} Animal RVs (of simian or bovine origin) were used as live attenuated vaccines for humans. In many cases, protection from infection and/or mild disease was only modest (40–50%), but 80–90% protection from severe disease including dehydration was achieved when a cocktail of different viruses was applied.\textsuperscript{111–113} A tetravalent vaccine contained a rhesus rotavirus (RRV) of G3 type and three mono-reassortants, which individually carry the VP7 gene of human serotypes G1, G2 and G4 in the RRV genetic background (Rotashield\textsuperscript{\textregistered}, developed by Wyeth) and received Food and Drug Administration approval as a universal vaccine in the USA in August 1998.\textsuperscript{114} More than 1.5 million doses were administered in the following 10 months. During that time, several cases of intussusception (IS) were observed in vaccinees, particularly within 3–7 days after the first dose (odds ratio of 27.9, 95% confidence interval [CI] 10.8 to 72.1) in a case–control analysis of IS.\textsuperscript{115} These observations led the CDC and the American Academy of Pediatrics\textsuperscript{116,117} to withdraw the recommendation of Rotashield for use in infants, and vaccine production ceased. Since IS also occurs spontaneously, there has been considerable controversy about the vaccine-attributable risk of the occurrence of IS, which has been estimated to be approximately 1:10 000.\textsuperscript{118} IS was not found to be epidemiologically linked with natural RV infection,\textsuperscript{119,120} although abnormal gut anatomy has been observed in children with acute RV disease.\textsuperscript{121} A recent re-analysis of the data indicated that the age of vaccinees is a critical factor for IS, as a disproportionately high number (>80%) of children who developed IS after the first dose of RV vaccine were older than 90 days.\textsuperscript{122} On the basis on these observations, ‘catch-up vaccinations’ of children older than 3 months are considered as contraindicated, and the same arguments apply when using the most recently developed vaccines (see below).

Despite this major setback, work on alternative live attenuated RV vaccines has continued.\textsuperscript{106–109} In particular, two new vaccines have recently emerged. A pentavalent RV vaccine (RotaTeq\textsuperscript{\textregistered} developed by Merck) is based on the attenuated bovine RV WC3 strain (G6P7[5]).\textsuperscript{123} It consists of a mixture of five mono-reassortants, in which the VP7 gene of the WC3 strain is individually replaced by the VP7 genes of human G1, G2, G3 and G4 strains, and the VP4 gene by that of a human P[8] strain, the other 10 genomic RNA segments in all
mono-reassortants being provided by the WC3 strain. This vaccine was found to be highly efficacious in preventing severe RV gastroenteritis (98%) caused by G1, G2, G3, G4 and G9 strains. No link with IS was found in >32,000 vaccinees when compared with the same number of matched, double-blinded placebo controls. The vaccine was licensed for use in the USA, has been recommended for universal use by the Advisory Committee on Immunization Practices and has now been licensed by the European Medicines Agency and in various other countries worldwide.

A monovalent vaccine (Rotarix® developed by GlaxoSmithKline) is derived from an attenuated human RV isolate, 89-12, and is of the G1P1A[8] type. It was found to be highly effective (85%) in preventing severe RV gastroenteritis and hospitalization and did not present a risk of IS above background in over 30,000 vaccinees when compared with the same number of matched, double-blinded placebo controls. The monovalent vaccine provided good heterologous protection against the G3P[8], G4P[8] and G9P[8] strains and also against disease after infection with the G2P[4] strains. The vaccine has now been licensed in Mexico, Brazil, Venezuela and numerous European countries, and has been incorporated into EPI schemes for universal mass vaccination of birth cohorts in Mexico, Brazil, Venezuela, Belgium, Luxembourg and Austria. European Guidelines for usage of both vaccines have been published recently.

In Europe, each year, more than 90,000 infants and young children are admitted to the hospital with severe, RV-associated gastroenteritis, which represents 28–52% of all hospitalizations for acute gastroenteritis in children under 5 years of age. The medical (direct) and societal (indirect) costs of RV disease have been estimated for several European countries. The benefits of the RV vaccination outweigh the costs in all countries with high mortality from RV disease. In developed countries, RV vaccination is at present considered to be less cost-effective, even when the societal costs are taken into account. Acceptance of RV vaccination is highly sensitive to diarrhoea incidence rate, proportion of severe cases, mortality rate and vaccine price. Lack of awareness of RV disease among parents and health care professionals is considered to be a major barrier in Europe. The WHO strongly recommends inclusion of RV vaccination into national immunization programmes, where vaccine efficacy has been proven, the vaccination programme can be sustained and adequate sentinel surveillance is in place.

The impact of the new RV vaccines, particularly in countries where it has been decided to administer them universally to the annual birth cohorts, remains to be seen. Although one of the vaccines has been tested in some developing countries, the real test of vaccinating...
populations of infants in the poorest parts of the world is still lacking. The risk of undesirable effects of live attenuated vaccines [clinical complications, reversion to virulence, genetic interaction (reassortment) with co-circulating wildtype RV strains, etc.] is considered to be low but not zero and may only become apparent, or be excluded, once millions of children have been vaccinated. Cases of IS may occur in temporal association with vaccination, although the overall background level of IS may not be increased. Thus far, post-marketing investigations have not detected a gross increase of IS with either of the two vaccines. However, intensive post-marketing surveillance is required during the introduction of the new vaccines in many countries, and is in fact in place. There is a good case for including surveillance of animal RVs in these exercises as well. With regard to the efficacy of a schedule of universal mass vaccination against RV disease, there are first encouraging initial data from the USA showing a delayed onset and diminished magnitude of RV disease during the 2007/08 winter season. Clinical trials with the new RV vaccines in developing countries, where they are most needed, are ongoing.

A lamb RV strain LLR (G10P[12]) has been licensed as a live attenuated vaccine for human use in China, but no clinical trial data have become available so far. A tetravalent vaccine, based on monoreassortants of human VP7 genes with the genes of the attenuated bovine UK Compton RV strain, has proven to be effective and safe and has been expanded into a hexavalent vaccine candidate. Asymptomatic neonatal human RV strains have also been developed as live attenuated candidate vaccines. RV vaccine candidates other than live attenuated virus are being explored in animal models: virus-like particles originating from baculovirus recombinants expressing structural proteins (VP2, VP6, VP4 and VP7), preparations enhancing RV immunogenicity by microencapsulation and DNA-based vaccines. None of the non-replicating RV vaccine candidates has reached the stage of extensive clinical trials.

**Supplementary material**

Supplementary material is available at BRIMED online.

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References
(The complete list of references is available online)


