METHODS IN MOLECULAR MEDICINE™

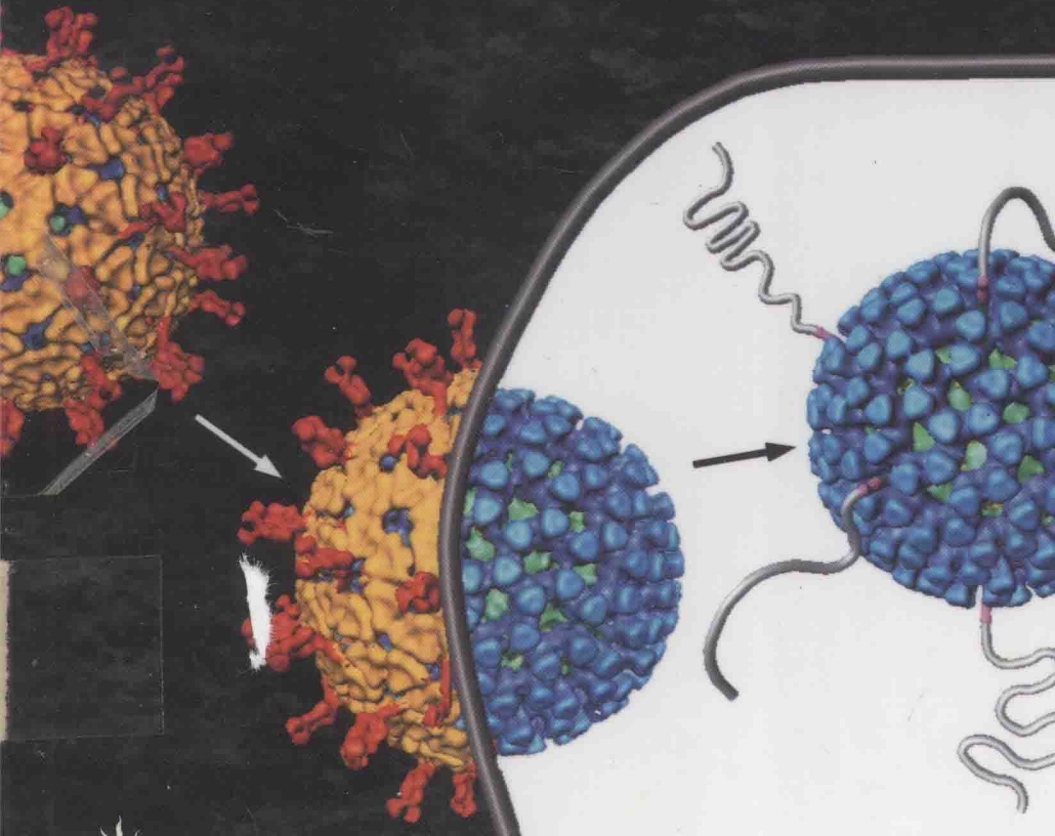
Rotaviruses

Methods and Protocols

Edited by

James Gray

Ulrich Desselberger



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Cover illustration: Structural transformation of rotavirus during its entry into the host cells. The mature triple-layered particle loses its outer layer (yellow) exposing the intermediate layer (blue) during the entry. The resulting double-layered particle (shown inside the cell) becomes transcriptionally active and extrudes the nascent transcripts through the channels at the five-fold vertices. The three-dimensional reconstructions of the rotavirus particles in their native and actively transcribing states were carried out using electron cryomicroscopy and computer image processing techniques. Courtesy of Drs. J. A. Lawton and B. V. V. Prasad, Baylor College of Medicine, Houston, TX. (For further details, *see* Chapter 2.)

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Preface

Rotaviruses: Methods and Protocols is among several volumes in the series *Methods in Molecular Medicine* that concentrate on a relatively specialized topic: Rotaviruses, one genus within the Reoviridae family. Rotaviruses are the most frequent cause of infantile gastroenteritis worldwide and a significant cause of death, following severe diarrhea and dehydration, in infants and young children of developing countries. Recently, a live attenuated tetravalent rotavirus vaccine has been licensed in the United States, and any widespread use of a rotavirus vaccine will be a further milestone in viral vaccinology. Structure, replication, and various functions of rotaviruses have been thoroughly investigated, and their medical importance clearly justifies and attracts interest to a detailed presentation of the modern methods and approaches used. In organizing this collection we considered it important to strike a balance of presentation among molecular and other modern techniques applied in rotavirus research, accompanied by the relevant background information and review material needed to render this collection attractive to the widest audience.

A short introductory chapter (U. Desselberger) sets the scene. The enormous progress made in elucidating the detailed structure of rotaviruses using cryoelectron microscopy and complex computer imaging techniques is presented in the chapter by B. V. Prasad and M. K. Estes. Owing to easy propagation of some rotaviruses in tissue culture and to the application of molecular labeling, blotting, and specialized electrophoretic techniques, details of rotavirus replication, some in common with other Reoviridae and others specific to themselves, have been unraveled and are described in the chapter by J. T. Patton, V. Chizhikov, Z. Taraporewala, and D. Chen., J. M. Gilbert, and H. B. Greenberg contribute some recently developed methods to study the still evolving mechanisms of interaction of viral receptor(s) with the host cell and of viral penetration as the initial steps of viral replication. Because of the segmented nature of their genomes, rotaviruses (like other segmented RNA viruses: reo-, influenza-, bunyaviruses, etc.) have, from the very beginning of their identification, elicited the interest of viral geneticists, and R. F. Ramig's chapter describes some of the methods used in this context.

Rotaviruses have a very wide animal reservoir, and animal models (gnotobiotic piglets, calves, rabbits, mice) have significantly contributed to our understanding of pathogenesis, the immune response, and the study of the

most relevant correlates of protection. Three chapters are devoted to these important issues: L. S. Saif and L. A. Ward review pathogenesis models; K. K. Macartney and P. A Offit, the application of immunological techniques; and M. A. Franco and H. B. Greenberg, the application of mouse genetics to the study and recognition of the significance of different branches of the immune response for protection. Properly controlled animal models have also been crucial for studying and dissecting the immune responses to rotavirus vaccine candidates of various kinds, and M. Ciarlet and M. E. Conner provide a comprehensive overview of the methods applied in this context in smaller animal models. We considered inclusion of a chapter on methods of human rotavirus vaccinology, but abstained since it would lead readers too far away from the framework of this book series.

In both humans and various mammals, rotaviruses exhibit a high degree of diversity of cocirculating strains, and reliable methods for detection, typing (by serological and, increasingly, molecular techniques), and phylogenetic grouping (based on genomic nucleotide sequence information) are prerequisite to any understanding of the detailed epidemiology and also to carrying out implementation studies on widely used vaccines. M. Iturriza Gómara, J. Green, and J. J. Gray review and describe the techniques applied for this purpose. Some of the epidemiological tools used in rotavirus surveillance are discussed by D. Brown and M. Ramsay. In a final chapter U. Desselberger and M. Estes have attempted to identify topics of future research and have come up with a number of relevant items, hoping that readers may be stimulated.

The editors have made an effort to produce a standard layout in all chapters, to convey better the application of the methods. Introductory remarks are followed by sections on Materials needed and the Methods proper; added Notes often reflect personal experience of the authors with the methods conveyed and are worth reading and considering; and Reference lists were intended to be up-to-date.

The editors wish to convey their sincere thanks to all contributors for providing their chapters in time and in smooth interaction. The Publishers have been understanding and very helpful, and we wish to thank Tom Lanigan, Craig Adams, Fran Lipton, and John Morgan. We are confident that the contributions speak for themselves and hope that readers will have some gain from and enjoy reading them.

J. J. Gray
U. Desselberger

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Contents

1

Rotaviruses: Basic Facts

Ulrich Desselberger

1. Introduction

Rotaviruses (RVs) are the chief etiologic agent of viral gastroenteritis in infants and young children, and in the young of a large variety of animal species. Since the discovery of RVs in man 25 yr ago, much has been learned about their genome and protein composition; their three-dimensional structure; their replication, pathogenesis and clinical pattern; the host's immune response; and the epidemiology. Measures of individual treatment have recently been complemented by the licensure in the United States of a tetravalent (TV), live attenuated rhesus rotaviruses (RRV)-based, human reassortant vaccine which may to be universally applied.

The brief introductory description mostly follows recent reviews *(1–4)* in which more special references can be found.

2. The RV Genome

The genome of RVs consists of 11 segments of double-stranded RNA (dsRNA) with conserved 5' and 3' ends, ranging from 667 bp (segment 11) to 3302 bp (segment 1) in size (SA11 simian RV strain), and totaling 6120 kDa, or 18,555 bp.

3. Gene-Protein Assignment

This is complete for several RV strains, and is shown for the SA11 strain in **Table 1**. With the exception of two genes (RNA 9 and 11), all genes are monocistronic, and the untranslated 5' and 3' regions are very small.

4. RV Proteins

There are six structural viral proteins (VPs: termed VP1, VP2, VP3, VP4, VP6, and VP7) and five nonstructural proteins (termed NSP1–NSP5). The

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Table 1
Gene-Protein Assignments, Protein Location, and Function of Group A RVs (SA11 RV/strain)

RNA segment	Size (bp)	Protein product: Designation	Protein product: Deduced mol wt (kDa)	Posttranslational modification	Location and function
1	3302	VP1	125.0	–	Inner core protein; RNA polymerase
2	2690	VP2	102.7	Myristylation	Inner core protein; RNA binding; leucine zipper
3	2591	VP3	88.0	–	Inner core protein; guanylyl transferase; methalyse
4	2362	VP4	86.7	Proteolytic cleavage (VP5* + VP8*)	Surface protein (dimer) Hemagglutinin Neutralization antigen (serotype specific) Fusogenic protein Virulence Pathogenicity
5	1611	VP5 (NS53)	58.6	–	Nonstructural (?) Zinc fingers; assembly
6	1356	VP6	44.8	Myristylation	Inner capsid protein (trimer); group and subgroup antigen
7[a]	1104	VP8 (NS35)	36.7	–	Nonstructural; RNA replication?
8[a]	1059	VP9 (NS34)	34.6	–	Nonstructural; RNA binding
9[a]	1062	VP7 (1)	37.4	Cleavage of signal sequence; glycosylation	Surface glycoprotein
		VP7 (2)	33.9		Neutralization antigen (serotype specific); Ca^{2+} binding site?
10	751	VP10 (NS29)	20.3	Glycosylation	Nonstructural; intracellular receptor; morphogenesis; enterotoxin
11	667	VP11 (NS26)	21.7	Phosphorylation	Nonstructural

[a]Gene protein coding assignment for SA11 RV strain; assignment different in other strains.
Adapted with permission from **ref. *4***.

functions of all proteins are summarized in **Table 1** and reviewed below (as far as known):

1. VP2 provides a scaffolding function of the inner core (*see* **Subheading 5.**).
2. VP6 carries group- and subgroup-specifying determinants.
3. VP7 and VP4 both carry neutralizable, and thus type-specifying, epitopes.
4. VP7 is a glycoprotein of 326 amino acids (aa) (a second in-frame initiation codon lies 30 codons downstream), with nine variable regions contributing to type specificity at varying degrees (*see* **Subheading 6.**).
5. VP4 is a nonglycosylated protein of 776 aa, and has a large number of functions: It is the viral hemagglutinin; it is posttranslationally cleaved (in aa positions 241 and 247, the latter being the preferred cleavage site) into the larger VP5* and the smaller VP8* subunits, and cleavage of VP4 enhances infectivity; it is the determinant for protease-enhanced plaque formation and growth restriction; it interacts with the cellular receptor; it has a fusion domain (of still unclear function); it is a virulence determinant.
6. The NSPs have various functions in replication (*see* **Subheading 7.**). NSP4 has been found to act as a viral enterotoxin.

5. RV Particle Structure

The particle is of icosahedral symmetry, measures 75 nm in diameter, and consists of three layers:

1. The core layer, formed by VP2, and containing the viral genome and the proteins VP1 (the RNA-dependent RNA polymerase) and VP3 (a guanylyltransferase and methylase) (these proteins may have other enzyme functions);
2. The inner capsid (intermediate layer), consisting of 260 VP6 trimers, which are interrupted by 132 aqueous channels of three different kinds in relation to the capsid's symmetry;
3. The outer capsid (third layer), consisting of 260 VP7 trimers and 60 spike-like VP4 dimers. VP4 interacts with VP7 and VP6.

6. Virus Classification

According to VP6 reactivities, there are at least seven different groups (groups A–E are confirmed by complete crossreactivities; groups F and G are likely to be new groups). Within group A, subgroups I, II, I + II, and non-I, non-II are distinguished (according to reactivities of VP6 with two monoclonal antibodies).

Because there are two neutralizable outer capsid proteins (VP4 and VP7), a dual classification system has emerged ***(1)***, similar to the dual classification established for influenzaviruses (distinguishing different hemagglutinins and neuraminidases):

1. There are at least 14 different VP7-specific types, termed G-types (derived from *g*lycoprotein);

2. There are at least 19 different VP4-specific types, termed P-types (derived from *p*rotease-sensitive protein).

All G- and P-types can be unambiguously distinguished by sequencing of the relevant genes (genotypes). All G-genotypes have been characterized as serotypes; however, this is not the case for all P-genotypes. Therefore, the following nomenclature has been agreed upon: Each virus has a P-type, indicated by an order number for the serotype and by an order number in square brackets for the genotype, and a G-serotype, indicated by an order number (coinciding with genotype number). Thus, the human Wa strain is defined as P1A[8]G1 (P-serotype 1A, P-genotype 8; G-sero/genotype 1); the equine RV L338 is P[18]G13 (P-genotype 18, P-serotype not determined; G-sero/genotype 13); and so on.

Because VP4 and VP7 are coded for by different RNA segments (RNA4, and RNA7–9, respectively), various combinations of G- and P-types can be observed in vivo and in vitro, both in man and in animals ***(4)***.

7. RV Replication

The major features of RV replication are shown in **Fig. 1**, and can be described as a sequence of the following steps (all replication takes place in the cytoplasm):

1. Adsorption to cellular receptor(s) and receptor-mediated endocytosis, or direct penetration.
2. Messenger RNA (mRNA) production in the cytoplasm from single-shelled (bilayered) subviral particles.
3. mRNA translation to synthesize six structural and five nonstructural proteins (*see* **Subheading 4.**).
4. Assembly of single-shelled particles containing VP2, VP1, VP3, and VP6, and a full complement of 11 single-stranded RNAs (ssRNAs); involvement of NSP2 and NSP5; formation of dsRNA (replication) within particle precursors (no free dsRNA or negative ssRNA in cytoplasm); formation of aggregates of bilayered particles (pseudocrystals termed "viroplasm");
5. Particle maturation to double-shelled (triple-layered) particles:
   a. Glycosylation of VP7 in rough endoplasmic reticulum (RER), NSP4 acting as an intracelluar receptor for bilayered particles.
   b. Transiently enveloped particles in RER containing VP4 and VP7.
   c. Envelope removal.
6. Liberation of double-shelled (triple-layered) infectious particles (virions) by cell lysis.

For further details, *see* **ref. *1***; for questions to be investigated further, *see* Chapter 12.

Double infection of cells with two different RV strains leads to simultaneous replication of the genes and synthesis of proteins of both viruses, and, at

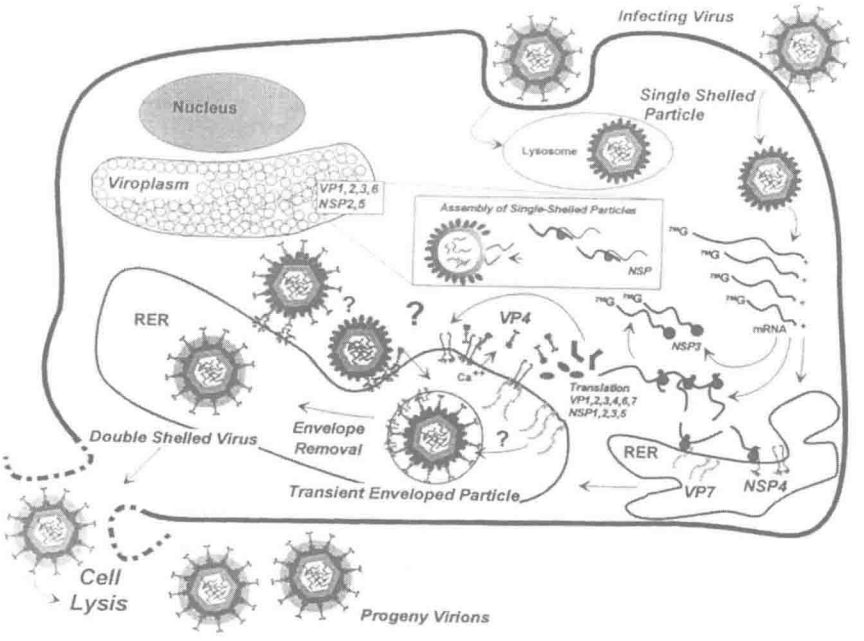

Fig. 1. Major features of the RV replication cycle. Adapted with permission from **ref. *1***.

the stage of assembly, the formation of reassortants of various gene segment combinations; those also include the emergence of different G/P reassortants (*see* **Subheading 6.**).

8. Pathogenesis and Animal Models

RVs infect the apical cells of the villi of the small intestine, causing cell death and desquamation. At the zenith of the disease, up to 10^{11} virus particles/mL stool have been counted, concomitant with infection of all susceptible cells in a very short period. The necrosis of the apical villi reduces digestion, causing diarrhea because of primary maladsorption, and leads to villous atrophy (**Fig. 2**). This is compensated for by a reactive crypt cell hyperplasia accompanied by increased secretion, which also contributes to the diarrhea. Recovery is by replacement of villous epithelium by enteroblasts ascending from crypts. The disease process takes 5–7 d ***(5)***.

The viral factors determining pathogenicity of RVs have been investigated in several animal models. The product of RNA segment 4, VP4 (**Table 1**), is likely to be a major determinant, but products of other structural genes (RNA3 coding for VP3; RNAs 8 or 9 coding VP7) and of some of the nonstructural genes (RNA5 coding for NSP1; RNA 8 coding for NSP2, and RNA 10 coding for NSP4) have also

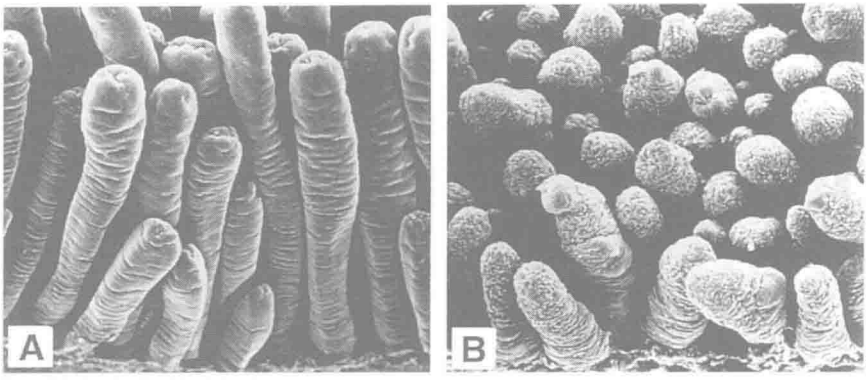

Fig. 2. Mid-small intestine of gnotobiotic calves. **(A)** Healthy control animal. **(B)** animal inoculated experimentally with bovine RV. The normally extended finger-like villi (A) have become stunted and misshapen (atrophy), and the enterocytes of the upper part of the villi are disarranged and swollen. Adapted with permission from **ref. *5***.

been associated with pathogenicity (for review, *see* **ref. *6***). Host factors, such as age and host restriction of viral replication, are involved as well in determining pathogenicity, but are less clearly defined.

9. Clinical Symptoms and Treatment

After a short incubation period of 24–48 h, the onset of illness is sudden, with watery diarrhea, vomiting, and rapid dehydration. Untreated RV infection is a major cause of infantile death in developing countries.

It should be noted, though, that clinical symptoms after RV infection vary widely, and asymptomatic infections in neonates with so-called "nursery" strains have been described.

Treatment is by oral or parenteral rehydration with oral rehydration solution (ORS) formulae, which have been approved by the World Health Organization for worldwide treatment in developing countries, and by some drugs *(7)*.

10. Diagnosis

Because of the high number of virus particles in feces during the acute illness, diagnosis is easy, using electron microscopy, passive particle agglutination tests, or enzyme-linked immunosorbent assays.

11. Immune Response and Correlates of Protection

Acute RV infection is followed by a virus-specific, humoral immune response comprising immunoglobulin (Ig)M, IgG, and IgA antibodies, and by

a cell-mediated immune response of RV-specific cytotoxic T-cells in the lamina propria of gut tissue. After prolonged research and discussions, it has become increasingly clear that RV-specific, local secretory IgA antibodies (copro-IgA) represent the best correlate of protection ***(8–10)***.

12. Epidemiology

RVs are the main etiologic agents of serious diarrheal disease in infants and young children under 2 yr of age throughout the world. For developing countries, approx 125 million cases of RV infection occur annually in children under 5 yr of age, of which 18 million are moderately severe to severe; almost 900,000 children die annually from RV infections in these countries. For the USA it is estimated that RV infections cause an estimated 1 million cases of severe diarrhea and approx 150 deaths per annum.

RVs are transmitted mostly by the fecal–oral route. A high degree of resistance to physical inactivation, the large number of virus particles shed, and the very low diarrhea dose 50% ensure that infection is also easily taken up from environmental sources, as demonstrated by tenacious nosocomial infections once a clinical ward has been contaminated. Animals infected by various RV types may act as a reservoir for human RV infections.

The epidemiology of RVs in complex. Group A RVs are the major cause of human infections. Outbreaks with a strict seasonal winter pattern occur in temperate climates, in tropical regions infections are spread more evenly throughout the year. At any one time and site, there is cocirculation of RVs of different G- and P-types. Viruses of multiple different G/P-type combinations have been isolated. However, G1–G4 viruses represent over 95% of the human strains co-circulating worldwide, and G1 viruses approx 50%. The P/G combinations found are P1A[8]G1, P1B[4]G2, P1A[8]G3, and P1A[8]G4. Within one country, the relative incidence figures for the different types show regional differences, as well as changes over time, and changes in relative incidences of different types are unpredictable.

13. Prevention and Control

Since August 1998, a TV, RRV based human reassortant vaccine has been licensed in the United States for universal use, and a decision of licensure for Europe is pending. The vaccine does not prevent infection to a significant degree (i.e., does not produce sterilizing immunity), but has been shown to prevent severe disease with an efficacy of 80%. The vaccine carries G1–G4 epitopes of human RV strains (G1, G2, and G4 on RRV monoreassortants, G3 on RRV). It remains to be seen to what extent the vaccine will produce heterotypic immunity, and whether, upon extensive use of the vaccine, new RV types emerge in man (for review, *see* **refs.** ***11,12***).

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2

Electron Cryomicroscopy and Computer Image Processing Techniques

Use in Structure–Function Studies of Rotavirus

B. V. Venkataram Prasad and Mary K. Estes

1. Introduction

Rotavirus (RV), a double-stranded (ds)RNA virus in the family Reoviridae, is a complex, relatively large (diameter, including spikes = 1000 Å), nonenveloped icosahedral virus. Once RV was recognized as a major human pathogen, it was extensively studied using modern molecular genetic and biological techniques, as discussed elsewhere in this book. These studies provided basic information about gene-coding assignments, protein processing, genome expression and replication, viral morphogenesis, and pathogenesis *(1)*. In addition, molecular epidemiological studies, coupled with the characterization of neutralizing monoclonal antibodies (MAbs) and sequencing of the genes that encode the neutralizing antigens, provided an understanding at the molecular level of the antigenic and genetic variability of the RVs.

Medical relevance, intriguing structural complexity, and several unique strategies in the morphogenesis of RVs have provoked extensive structural studies on these viruses in recent years *(2–10)*. A detailed architectural description of these complex viruses, including the topographical locations of the various structural proteins and their stoichiometric proportions, was obtained as the resolution of these techniques improved. Together with molecular biological studies, structural studies are permitting a dissection of the molecular mechanisms that underlie biological processes of the virus, such as cell entry, neutralization, transcription, gene expression, and virus assembly *(8)*. This chapter reviews current knowledge of RV structure and the methods used in structural analysis.

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