JOURNAL OF VIROLOGY, Jan. 1993, p. 617–620
0022-538X/93/010617-04$02.00/0

Vol. 67, No. 1

Human Rotavirus K8 Strain Represents a New VP4 Serotype

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Received 22 June 1992/Accepted 21 September 1992

The complete VP4 gene of the human rotavirus (HRV) K8 strain (G1 serotype) was cloned and inserted into the baculovirus transfer vector pVL941 under the control of the polyhedrin promoter. A K8VP4 recombinant baculovirus was obtained by cotransfection of *Spodoptera frugiperda* (Sf9) cells with transfer vector DNA containing the K8VP4 gene and wild-type baculovirus DNA. Infection of Sf9 cells with this VP4 recombinant baculovirus resulted in the production of a protein that is similar in size and antigenic activity to the authentic VP4 of the K8 strain. Guinea pigs immunized with the expressed VP4 developed antibodies that neutralized the infectivity of the K8 strain. This antiserum neutralized HRV strains belonging to VP4 serotypes 1A, 1B, and 2 with efficiency eightfold or lower than that of the homologous virus, indicating that the human rotavirus K8 strain represents a distinct VP4 serotype (P3). In addition, low levels of cross-immunoprecipitation of the K8VP4 and its VP5 and VP8 subunits with hyperimmune antisera to HRV strains representing different VP4 serotype specificities also suggested that the K8 strain possesses a unique VP4 with few epitopes in common with other P-serotype strains.

Rotaviruses are a major cause of severe diarrhea in infants and young children worldwide (10). Thus, intensive efforts are being made to develop an effective vaccine against human rotavirus (HRV) disease. In pursuit of this goal, the main focus of current studies is to understand the antigenic relationships among rotaviruses on the basis of both of their outer capsid proteins, VP7 and VP4 (8, 18, 19). The outer capsid protein VP7 induces neutralizing antibodies that have been previously shown to be associated with resistance to illness in experimental animals (7). On the basis of VP7 antigenic specificity, at least 13 different VP7 serotypes (G) among HRVs and animal rotaviruses have been established (1).

The other outer capsid protein, VP4, is also a major protective antigen (7, 19). On the basis of VP4 nucleotide and amino acid sequence homologies, HRVs have been classified into five genetic groups which are independent of the VP7 serotype (4, 20). Recent studies indicated that VP4s expressed in baculovirus retain their immunogenic capacities (6, 14, 16, 17). In a plaque reduction-neutralization assay employing antisera prepared with VP4 expressed in baculovirus of HRV strains KU, DS-1, and 1076, HRVs were classified into three VP4 serotypes and one subtype (6). In this serotype classification system, the HRV K8 strain, which belongs to VP4 genetic group 4, was tentatively classified as P3, because in one-way tests this strain was not neutralized by antisera with VP4 serotype 1A, 1B, or 2 specificity (6).

By sequence analysis, it was demonstrated that the HRV K8 strain, with VP7 serotype 1 specificity, possesses a unique VP4 with only 64 to 65% amino acid sequence homology with VP4s of strains in the other four genetic groups (24). These observations were consistent with the likelihood that the genetically distinct gene 4 of HRV strain K8 represents a distinct VP4 serotype.

In this study, the VP4 gene of the K8 strain was cloned and expressed in a baculovirus expression system. Neutralization assays using the antiserum produced against this expressed VP4 confirmed the classification of the K8 strain as a distinct VP4 serotype (P3).

For the construction of VP4 cDNA of the K8 strain, methods described previously were followed (6). An established cell line of fetal rhesus monkey kidney cells, MA104, was used for rotavirus propagation. Rotavirus K8 mRNAs were produced from single capsid particles as described previously (6). The oligonucleotide 5′-CACGGATCCGGT CACATTTAAAATAGACAG-3′ (primer sequence representing a flanking *Bam*HI site and the complement of the 3′ end of gene 4 of the K8 strain) (24) was used to transcribe the first strand of DNA from viral mRNA by reverse transcriptase. After hydrolysis with NaOH (0.3 M, 37°C, 2 h), cDNA was hybridized with the oligonucleotide 5′-CACG GATCCGGCTATAAAATGGCTTCTTTA-3′ (primer sequence representing a flanking *Bam*HI site and the complement of the 5′ end of the VP4 gene of the K8 strain) (24) and transcribed with reverse transcriptase. After digestion with *Bam*HI, the cDNA was cloned into the *Bam*HI site of plasmid pTZ18R (pTZ18R-K8-G4) for amplification. The VP4 gene of the K8 strain was then excised with *Bam*HI and inserted into the unique *Bam*HI site of the intermediate baculovirus vector pVL941. Cotransfection of *Spodoptera frugiperda* (Sf9) cells with the DNA of the intermediate vector containing the K8VP4 gene and the DNA of wild-type *Autographa californica* nuclear polyhedrosis virus was performed as described previously (6, 17). The recombinant baculovirus expressing the VP4 protein of the K8 strain was screened by immunofluorescence with hyperimmune antiserum to the K8 strain and was purified by terminal dilution.

To confirm that the K8VP4 protein had been synthesized, [^{35}S]methionine-labeled protein extracts were prepared from Sf9 cells infected with the K8VP4 recombinant or wild-type baculovirus. As shown in Fig. 1A, the K8-G4 recombinant synthesized a protein with an estimated molecular size of 85 kDa which had an electrophoretic mobility indistinguishable

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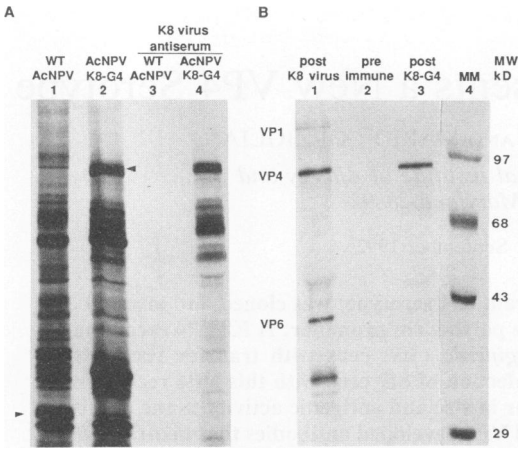

FIG. 1. (A) Expression of VP4 of HRV K8 strain by a recombinant baculovirus. Sf9 cells were infected with wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) or VP4 recombinant baculovirus and labeled for 2 h with 30 μCi of [^{35}S]methionine per ml at 48 h postinfection. VP4 recombinant baculovirus cell lysate (lane 2) and wild-type baculovirus cell lysate (lane 1) are shown. Polyhedrin and VP4 of the K8 strain are indicated by arrowheads. Lysates of Sf9 cells infected with VP4 recombinant baculovirus (lane 4) or with wild-type baculovirus (lane 3) were immunoprecipitated by hyperimmune antiserum to K8 virus. (B) Characterization of antiserum raised against a lysate-containing expressed VP4 of K8 strain. Guinea pig antiserum was tested for VP4-specific antibodies by immunoprecipitation of [^{35}S]methionine-labeled proteins present in K8 strain-infected MA104 cells. Total viral proteins immunoprecipitated with antiserum (1:400) to complete K8 virus (lane 1), immunoprecipitation with guinea pig serum (1:400) collected before (lane 2) or after (lane 3) immunization with a lysate containing K8VP4, and molecular size markers (in kilodaltons) (lane 4) are shown.

from that of VP4 produced in MA104 cells infected with the K8 strain. In addition, the identity of the expressed K8VP4 protein was confirmed by immunoprecipitation with guinea pig antiserum raised against the purified rotavirus K8 strain (Fig. 1A).

To assay the immunogenicity of baculovirus-expressed K8VP4, guinea pigs were immunized with a lysate of K8-G4 recombinant baculovirus-infected Sf9 cells prepared as previously described (17). Guinea pig serum was screened prior to immunization for preexisting antibody to rotavirus. The preimmunization serum neutralized the homologous strain at a titer of 1:≤30. In addition, this preinoculation serum failed to immunoprecipitate the VP4 in lysates of K8 strain-infected MA104 cells. Immunization of a guinea pig with a lysate of Sf9 cells expressing K8VP4 induced antibodies which immunoprecipitated the homotypic VP4 (Fig. 1B).

The result of a plaque reduction-neutralization assay (9) demonstrated that baculovirus-expressed K8VP4 antiserum possessed marked neutralizing activity to the homologous K8 strain, achieving a titer of 1:2,560 (Table 1). This serum was then used to measure neutralizing activity against HRV strains belonging to VP4 serotype 1A, 1B, or 2 (Table 1). Although various degrees of cross-reactivity in neutralization with heterotypic strains, ranging from 1:80 to 1:320, were observed, an eightfold or greater difference in neutralization was found between the homotypic and heterotypic strains. Hyperimmune antisera to baculovirus-expressed VP4 of HRVs of VP4 serotype specificity 1A, 1B, or 2 failed to neutralize the K8 strain. Since the VP4 serotyping system is based on a ≥eightfold difference in reciprocal neutralizing antibody titers, the results in this study confirmed that HRV strain K8 is a distinct VP4 serotype.

In the presence of trypsin, VP4 is cleaved into two polypeptides (VP5 and VP8), resulting in an enhancement of rotavirus infectivity (2, 3). Previous studies indicated that amino acid homology of VP4 among rotavirus strains was greater in the VP5 cleavage subunit of VP4 than in the VP8 subunit (5). Furthermore, sequence analysis of escape mutants selected with neutralizing monoclonal antibodies

TABLE 1. Antigenic relationships among K8 strain and other HRVs observed in neutralization assays employing hyperimmune antiserum to recombinant baculovirus-expressed VP4 protein of K8 strain

HRV VP4 serotype	Symptomatic infection	VP7 serotype	Rotavirus strain	Reciprocal of 60% antibody titer of hyperimmune antisera against expressed VP4 protein of strain:			
				K8	KU	DS-1	1076
1A	+	1	KU	320	5,120		
		1	Wa	320			
		3	P	240			
		3	Yo	120			
		3	Mo	120			
		4	Hochi	80			
		4	VA70	80			
		4	Hosokawa	80			
		9	WI61	120			
		9	F45	120			
1B	+	2	DS-1	320		640	
		2	S2	240			
2	−	1	M37	320			2,560
		2	1076	120			
		3	McN	320			
		4	ST3	80			
3	+	1	K8	2,560	80	80	80

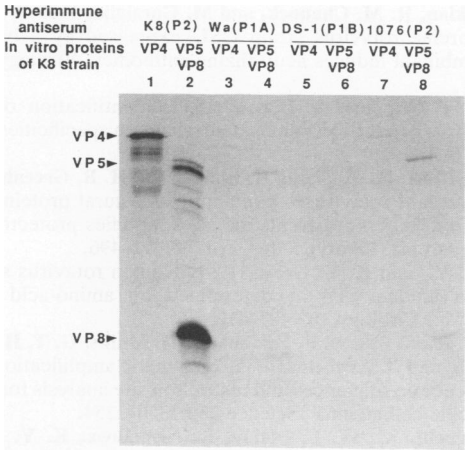

FIG. 2. Immunoprecipitation of K8VP4 and its subunits VP5 and VP8 with antisera to strains of different VP4 serotype specificities. The two primers used to amplify the VP8 subunit were the plus sense (see text) and the minus sense 5′-CACGGATCCTTTAATC TGTCTAGATGTAAT-3′ (sequences 741 to 712, containing an extra *Bam*HI site). The two primers used to amplify the VP5 subunit were a primer complementary to the 3′ end of K8 gene 4 (see text) and the plus sense 5′-CACGGATCCGCTCAGATGAATGAAGACATA-3′ (sequences 753 to 773, containing an extra *Bam*HI site). The resulting products were cloned in the pTZ18R vector and selected for the correct T7 promoter orientation. Plasmids were transcribed into mRNAs by RNA T7 polymerase, and the mRNAs were translated into protein by a rabbit reticulocyte lysate in the presence of [^{35}S]methionine (1 mCi/ml). VP4, VP5, and VP8 proteins were immunoprecipitated and assayed directly by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis followed by fluorography. Lanes: 1, 3, 5, and 7, immunoprecipitation of K8VP4; 2, 4, 6, and 8, immunoprecipitation of a mixture of K8 VP5 and K8 VP8 proteins (all sera were used at a dilution of 1:400); 1 and 2, hyperimmune antiserum to K8 virus; 3 and 4, hyperimmune antiserum to Wa (VP4 serotype 1A); 5 and 6, hyperimmune antiserum to strain DS-1 (VP4 serotype 1B); 7 and 8, hyperimmune antiserum to strain 1076 (VP4 serotype 2).

(NMAbs) indicated that the cross-reactivity was related to a site on the VP5 subunit (22). In contrast, NMAbs and polyclonal antisera directed at a site on VP8 were observed to contain serotype-specific and cross-reactive epitopes (12, 13, 15).

In an attempt to identify the locations of specific and cross-reactive domains on VP4 of the K8 strain, in vitro-translated VP4, VP5, and VP8 proteins of K8 were produced. For in vitro transcription of VP8 and VP5 subunits, sequences encoding both fragments were generated by polymerase chain reaction (PCR) (21) with the cloned K8VP4 gene as a template. Transcripts of VP4 and its subunits were produced by using a Riboprobe system kit (Promega), following the manufacturer's specifications. In vitro translation was performed with rabbit reticulocyte lysate treated with nuclease (Promega). After incubation at 37°C for 1 h, the samples were immunoprecipitated by guinea pig hyperimmune antisera to HRVs of different VP4 serotype specificities.

In vitro-translated VP4 and VP8 proteins of the K8 strain were more efficiently immunoprecipitated by the homologous serum than by antisera to HRVs of VP4 serotype 1A, 1B, or 2 specificity (Fig. 2). Although the cross-immunoprecipitation of VP8 of K8 with heterologous antisera was weak, this result indicates that this subunit contains epitopes that are conserved among rotavirus strains of different VP4 serotypes. Similarly, immunoprecipitation of the in vitro-translated VP5 subunit showed low levels of cross-reactivity. This result is consistent with previous findings demonstrating that cross-reactive VP4 NMAbs were unable to react with strain K8 (23) and that K8-VP4 NMAbs only neutralized the homologous K8 strain (11). Sequence analysis of escape mutants selected with these VP4 NMAbs indicated that amino acids at residues 305, 392, 394, 433, and 439 are involved in antigenic sites on the VP5 subunit (22, 23). This low level of immunoprecipitation of VP4 and its subunits suggested that the K8 strain possesses a unique VP4 with few epitopes in common with other HRVs of different VP4 serotypes.

Classification of the HRV K8 strain as a new VP4 serotype will facilitate studies of antigenic relationships among HRVs. The establishment of a dual VP7 and VP4 serotyping system will aid in the analysis of serological and epidemiological data obtained from infants and young children with naturally occurring rotavirus infection or following rotavirus vaccination. For example, HRV strains KU and K8 both belong to VP7 serotype G1, but each possesses a different VP4 serotype specificity. In addition, studies of the serologic response of expressed VP4 may be important for evaluating the feasibility of developing an effective subunit vaccine for protection against rotavirus gastroenteritis.

We thank Robert M. Chanock and Albert Z. Kapikian for helpful discussions, Ron Jones and Mitzi Sereno for technical assistance, and T. Heishman for editorial assistance.

G.L. was the recipient of a grant from the World Health Organization.

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