Acta virologica **55**: 273 – 278, 2011 doi:10.4149/av_2011_03_273

Evidence of conserved epitopes in variable region of VP8* subunit of VP4 protein of rotaviruses of P[8]-1 and P[8]-3 lineages

J.F. CONTRERAS[1], G.E. MENCHACA[1], R. INFANTE[2], C. E. HERNÁNDEZ[1], C. RODRÍGUEZ[1], R.S. TAMEZ[1]

[1]Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León. Cd. Universitaria, San Nicolás de los Garza, N.L. 66451 México; [2]Facultad de Ciencias Químicas, Universidad Autónoma de Chihuahua. Nuevo Campus Universitario, Chihuahua, Chih. 31125 México

Received February 15, 2011; accepted July 27, 2011

Summary. – Although antibody responses to the human rotavirus VP4 protein have been reported, few studies have analyzed the specificity of these responses to the VP8* subunit. This study investigated antibody responses generated against the variable region of the VP4 protein (VP8* subunit) in children infected with rotavirus genotype P[8]. Recombinant VP8* subunit (rVP8*) and truncations corresponding aa 1–102 (peptide A) and 84-180 (peptide B) of rotavirus strains P[8]-1 and P[8]-3 lineages were expressed in *Escherichia coli* and examined for antibody reactivity using ELISA and Western blot assays. Sera from infected children had IgG antibodies that reacted with full-length rVP8*, peptide A and B of both lineages, with stronger reactivity observed against peptide B. In addition, anti-strain Wa (P[8]-1) and anti-rVP8* (P[8]-3) rabbit polyclonal antiserum reacted against peptide B sequences of both lineages. These data indicate that the VP8* variable region of rotavirus belonging to P[8]-1 and P[8]-3 lineages have conserved epitopes recognized by antibodies elicited during natural infections.

Keywords: antibodies; conserved epitopes; VP8* subunit; VP4 protein; P[8] lineage; rotaviruses

Introduction

Rotaviruses (the species *Rotavirus* A, the genus *Rotavirus*, the subfamily *Sedoreovirinae*, and the family *Reoviridae*) express two outer proteins (VP4 and VP7) encoded by genes 4 and 9, respectively, which elicit the production of neutralizing antibodies (NtAb) that confer protection against subsequent infectious in children (Jiang *et al*., 2002). Nucleotide sequences of these two genes are used to classify rotavirus strains into genotypes. To date, 23 G genotypes (VP7) and 32 P genotypes (VP4) have been identified in humans and different animal species (Collins *et al*., 2010; Okitsu *et al*., 2011). Worldwide, 80% of human rotavirus strains result from infections with the P[8] genotype (Santos and Hoshino, 2005).

VP4 is an 88 kDa protein necessary for rotavirus penetration into target cells. During this process, VP4 is cleaved by trypsin, producing peptides VP8* and VP5*. Phylogenetic analysis of the VP8* region revealed four P[8] lineages. The amino acid sequence comparisons between lineages revealed 87–91% identity (Maunula and von Bonsdorff, 1998; Cunliffe *et al*., 2001; Arista *et al*., 2005). Each P[8] lineage contains a variable region between aa 71–204 that has serotype-specific epitopes (Larralde and Gorziglia, 1992). However, the relationship between serotype and the variable sequences in this region for different lineages is not clear. Therefore, determining the lineage-specificity of antibodies to this region might improve our understanding of humoral responses elicited against P[8] genotypes following natural infections.

To study the antibody response to the VP8* variable region of P[8]-1 and P[8]-3 lineages, truncated peptides corresponding to the VP8* subunit of each lineage were produced, and

E-mail: juan.contrerascr@uanl.edu.mx; contrerasjfco@gmail.com; fax: +52-8183524212.

Abbreviations: NtAb = neutralizing antibodies; rVP8* = recombinant VP8* subunit

antibody specificity present in the sera of rotavirus infected children was assessed by ELISA and Western blot analyses.

Materials and Methods

Stool and serum specimens. Fecal and serum samples (n = 10) were collected from patients <2-years of age during the acute phase of disease, admitted with gastroenteritis to hospitals in Chihuahua City, North Mexico between 2007–2008.

Titration of neutralizing antibodies. Human rotavirus strain Wa (kindly provided by Carlos F. Arias, IBT-UNAM, Mexico) was propagated in MA104 cell as described (Taniguchi *et al.*, 1998). The presence of NtAb to rotavirus strain Wa in the sera of children was determined using the immunochemical focus reduction neutralization test (Arias *et al.*, 1987). The NtAb titer for each serum sample examined was defined as the highest serum dilution where the number of infected cells was reduced by at least 60% compared to infected cells not incubated in the presence of serum.

RT-PCR. RNA was extracted from rotavirus Wa-infected cells and from 20% fecal suspensions obtained from 10 field isolates using the TRI-REAGENT (MRS). A 762 bp amplicon corresponding to the gene 4 product encoding the VP8* subunit of the VP4 protein was amplified by RT-PCR. The primers used were PA1 (5'-CACGGATCCGGCTATAAAATGGCTTCACTC-3´, nt 1–21) and PC2 (5´-CACGTCGACTTCATTAACTTGTGCTCT-3´, nt 745–762) (Larralde and Gorziglia, 1992). To obtain VP8* cDNA, viral RNA and 0.1 µmol/l PC2 primer were denatured at 94°C for 5 mins and then added to the reaction mixture containing 1X RT buffer (Bioline), 2.5 mmol/l $MgCl_2$, 0.8 mmol/l of each dNTP and 40 U of MMLV Reverse Transcriptase® (Bioline). Reverse transcription was conducted at 42°C for 30 mins. The PCR reaction mixture (12.5 µl) contained 1 µl cDNA-VP8*, 1X NH_4 buffer (Bioline), 1.5 mmol/l $MgCl_2$, 0.4 mmol/l of each dNTP, 0.5µmol/l of each primer and 250 U of Red™ DNA Polymerase (Bioline). The reaction conditions were as follows: 95°C/2 mins followed by 30 cycles at 95°C/30 secs, 42°C/30 secs, and 72°C/40 secs with a final extension at 72°C/15 mins.

Cloning and sequencing of the VP8 subunit.* VP8* amplicons (762 bp) of strain Wa and the 10 field isolates were purified from agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega) and cloned into the pGEM-T plasmid using the pGEM®-T Vector System I Kit (Promega). The nucleotide sequence was determined at IBT-UNAM, Mexico using an automated system (Perkin Elmer, Applied Biosystems). Sequencing and phylogenetic analysis of the VP8* subunit was performed using CLUSTAL W/BioEdit v7.0 (Hall, 1999) and the neighbor-joining method using the MEGA v4.1 software, respectively (Tamura *et al.*, 2007). The sequences were deposited at GenBank database under Acc. Nos. FJ665385 to FJ665391 and HQ585864 to HQ585866.

Cloning, expression, and purification of His-tagged VP8 peptides A and B.* The primers used to generate full-length rVP8*, peptide A (aa 1–102) and peptide B (aa 84–180) of rotavirus strain Wa (P[8]-1 lineage) and isolate MX08-659 (P[8]-3 lineage) were modified to match strain Wa (Larralde and Gorziglia, 1992). Each PCR product was cloned into the pGEM-T plasmid (Promega) according to the manufacturer's instructions and later subcloned into the *Bam*HI and *Sal*I sites of the pET28a(+) vector (Novagen). Protein expression was performed in *Escherichia coli* BL21(DE3) induced with 1 mmol/l IPTG (isopropyl-beta-D-thiogalactopyranoside) (Dowling *et al.*, 2005). The His-tagged recombinant proteins were purified using HisTrap FF Crude columns (GE Healthcare) under denaturing conditions. Each recombinant protein was characterized by SDS-PAGE and Western blot.

ELISA assays. We used a modification of the procedure previously described (Hyser *et al.,* 2008). Briefly, 96-well microtiter plates were coated with 10 µg/ml of P[8]-1 and P[8]-3 rVP8* in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing with PBS-0.1% Tween 20 the plates were blocked with 5% nonfat dry milk in PBS at 4°C for 12 hrs. After 4 washes, 2-fold serial dilutions of respective serum samples were added and incubated at 37°C for 1 hr. FCS was used as a negative control. After washing four times, peroxidase-conjugated protein A (Amersham) was added and incubated at 37°C for 1 hr. After 4 washes, ABTS peroxidase substrate (Zymed) was added at 37°C for 30 mins. Absorbance at 405 nm was measured using an automatic plate reader (Digital and Analog Systems S.R.L.). The IgG-titer was defined as the highest dilution of serum giving the absorbance at least two SD greater than the negative control.

Western blot analysis. Full-length rVP8*, peptide A and B of rotavirus P[8]-1 and P[8]-3 lineages were subjected to 12.5% SDS-PAGE and blotted at 20 volts for 2 hrs onto 0.45 µm nitrocellulose membranes (Sigma-Aldrich). The membranes were then blocked with 2% Tween in TBS (Tris Buffered Saline) and then incubated overnight at 4°C in the presence of respective serum samples. Antibody reactivity was detected using the ProtoBlot® II AP System with the Stabilizing Substrate Kit (Promega).

Results

VP8 subunit sequencing analysis*

Amplicons (762 bp) corresponding to the VP8* subunit encoded by gene 4 of prototype rotavirus strain Wa and 10 rotavirus strains collected from infected children were cloned and sequenced. Alignment of the VP8* amino acid sequences of the field isolates showed that they shared 97.2–100% identity and belonged to the P[8] genotype. These isolates, compared to rotavirus strain Wa, had 19–22 amino acid substitutions (91.2–92.4% identity). Sixteen substitutions were localized in the variable region (aa 71–204). Phylogenetic analysis revealed that all field strains clustered to the P[8]-3 lineage whereas rotavirus Wa clustered to the P[8]-1 lineage (Fig. 1).

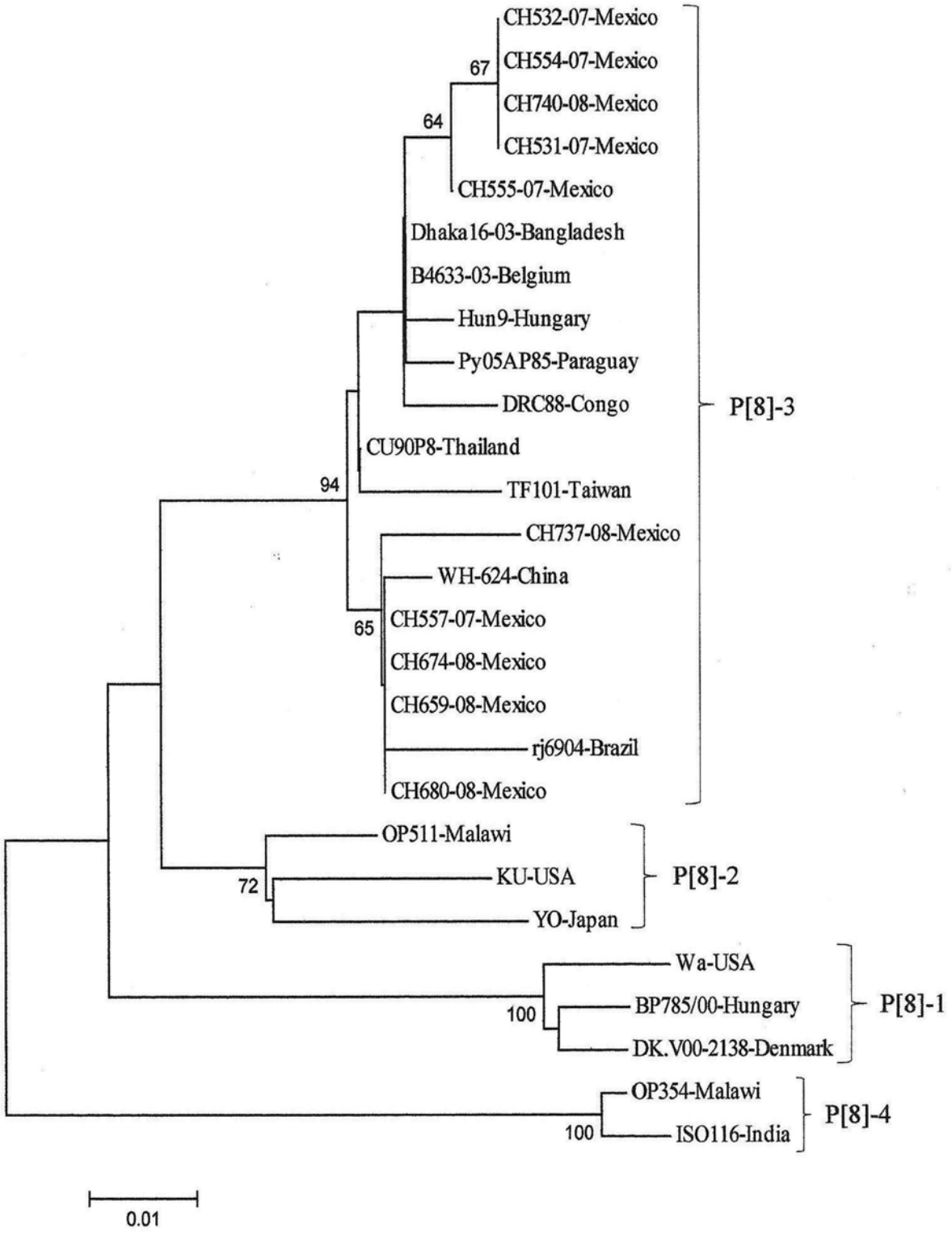

Fig. 1

Phylogenetic analysis of the deduced VP8* subunit amino acid sequences of P[8] human rotavirus strains

The tree was constructed using the neighbor joining method; bootstrap of 1,000 replications (MEGA v4.1).

Assessment of the antibody responses to the VP8 variable region*

To measure the antibody response to the VP8* subunit, sera from infected children were first screened for neutralization activity against rotavirus strain Wa followed by ELISA and Western blot analyses to detect reactivity against the VP8* His-tagged recombinant constructs. We found that 9/10 serum samples examined had detectable levels of NtAb. Specific IgG antibodies reactive against the full-length rVP8* of P[8]-1 and P[8]-3 lineages were also detected in sera with titers ranging between 100-1,600 (Table 1).

Based on these binding data, we focused our analyses to antibody responses recognizing the VP8* variable region (peptide B) and then compared this response to peptide A reactivity (which is the most conserved VP8* subunit). Each serum sample was then analyzed by Western blot for reactivity against peptides A and B of rotavirus strain Wa (P[8]-1 lineage) and rotavirus strain MX08-659 (P[8]-3 lineage). Although peptides from both lineages were detected in all serum samples tested, the reactivity with peptide B was higher than reactivity observed against peptide A (Fig. 2).

Finally, to determine if the serum reactivity observed against peptide B was lineage specific, rabbit hyperimmune antiserum raised against rotavirus strain Wa or against MX08-659 full-length rVP8* was tested by Western blot. These experiments demonstrated that rabbit anti-sera recognized both peptide B of P[8]-1 and P[8]-3 lineages (Fig. 2).

Discussion

Previous studies have described the world-wide circulation of four lineages corresponding to P[8] genotype rotavirus strains. There is an 8.6–13% amino acid sequence divergence between lineages (Maunula and von Bonsdorff, 1998; Cunliffe *et al*., 2001). Therefore, it is important to determine if amino acid differences between lineages can affect the reactivity of antibodies generated following natural rotavirus infections. In this report, all field isolates grouped to the P[8]-3 lineage, which is considered the most prominent lineage throughout the world (Ansaldi *et al*., 2007; Araujo *et al*., 2007; Rahman *et al*., 2007; Espínola *et al*., 2008). We also tested serum from rotavirus-infected children for reactivity against the VP8* subunit of the prototype human rotavirus strain Wa (P[8]-1 lineage) that is similar to the Rotarix vaccine licensed in Mexico since 2004 (Cheuvart *et al*., 2009). To determine the genetic relationship between these two strains, we analyzed the amino acid sequence of the VP8* subunit of VP4 of the Rotarix vaccine (data not shown). Five substitutions were detected following comparison of the vaccine strain sequence to the non-vaccine Wa strain sequence. This analysis revealed three substitutions, in addition to those reported by Ward *et al.* (2006). For this reason, we used full-length rVP8* corresponding to the P[8]-1 and P[8]-3 lineages to determine if the antibodies elicited following natural infections reacted against these recombinant proteins.

Table 1. ELISA and neutralizing antibody titers of sera from rotavirus-infected children

Children serum samples	IgG ELISA titers to the VP8* subunit		NtAb titers to strain Wa
	Strain Wa P[8]-1 lineage	MX08-659 isolate P[8]-3 lineage	
MX07-531	200	400	200
MX07-532	100	100	50
MX07-554	200	400	50
MX07-555	200	400	50
MX07-557	200	800	50
MX08-659	200	200	200
MX08-674	>1,600	>1,600	100
MX08-680	>1,600	>1,600	>3,200
MX08-737	>1,600	>1,600	25
MX08-740	400	800	<25

We detected comparable IgG-titers against both P[8]-1 and P[8]-3 rVP8*, and titers were similar to those reported by Padilla-Noriega *et al.,* who measured the antibody responses to rVP8* from the rhesus rotavirus (RRV) strain using sera from children vaccinated with RRV (Padilla-Noriega *et al*., 1992). Our results suggested that children infected with P[8] rotavirus strains mounted a homologous immune response to this genotype. In addition, these results were in agreement with our previous findings that demonstrated that a homologous antibody response could be mounted to the P1A serotype VP4 protein following natural infection (Menchaca *et al*., 1998).

A previous report described that the variable region within the VP8* subunit had serotype-specific epitopes and that this region could be recognized by rabbit antibodies specific to whole human rotavirus (Larralde and Gorziglia, 1992). Therefore, we determined if the sera from infected children contained antibodies specific to the VP8* variable region. This was carried out by examining the reactivity against rVP8* truncated peptides (peptides A and B) of rotavirus strain Wa (P[8]-1 lineage) and MX08-659 isolate (P[8]-3 lineage). Western blot results showed that sera from the infected children examined in this report recognized epitopes present in peptides of both lineages, but that the reactivity against peptide B was stronger than reactivity against peptide A. These results suggested that during a natural rotavirus infection, an immune response against the immunodominant epitopes in the variable region of VP8* subunit is mounted.

To verify that the reactivity against peptide B corresponding to P[8]-1 and P[8]-3 lineages was to conserved epitopes, rabbit polyclonal antiserum generated against either whole

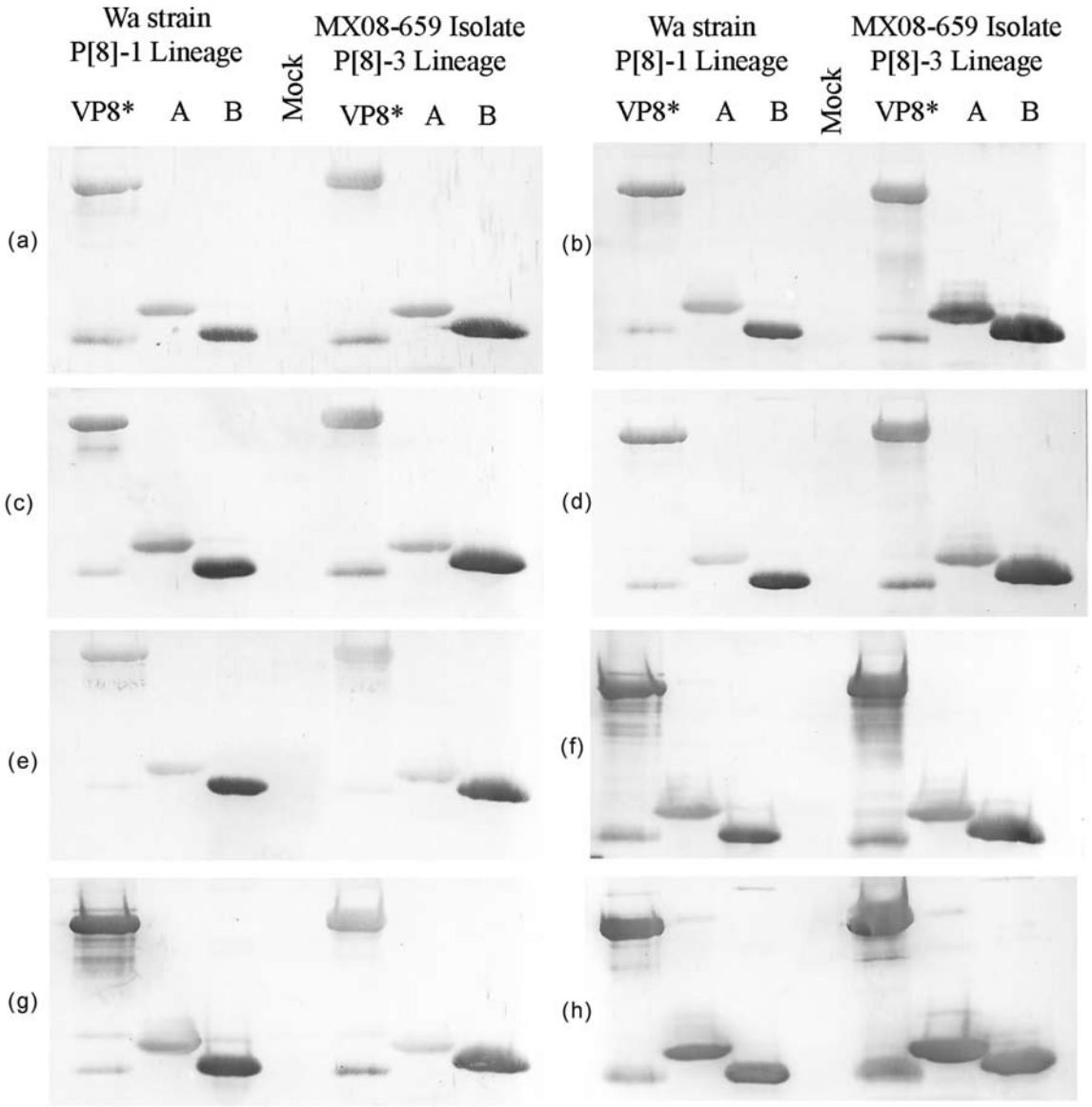

Fig. 2

Reactivities of IgG antibodies in the sera from rotavirus-infected children with VP8* and peptides A and B of P[8]-1 and P[8]-3 lineages

Western blot analysis. The blotted antigens were reacted with the children's sera (a-f), antiserum to Wa strain (P[8]-1) (g) and antiserum to VP8 of isolate MX08-659 (P[8]-3) (h), respectively.

rotavirus Wa (P[8]-1 lineage) or against recombinant MX08-659 VP8* (P[8]-3 lineage) were tested in Western blot analyses. Both rabbit serum samples recognized peptide B of P[8]-1 and P[8]-3 lineages, confirming the presence of conserved epitopes in the VP8* variable region across lineages. A possible explanation for the reactivity of the rabbit antisera is that within peptide B (aa 84-180), we found 13 amino acid substitutions between lineages P[8]-1 and P[8]-3, but the positions 106 (V/I), 108 (I/V), 120 (T/N), 121 (I/V), 150 (E/D) and 173 (V/I) had similar biochemical characteristics, suggesting that the antigenicity was not likely to have been significantly altered in the VP8* variable region.

To our knowledge, this is the first report describing the reactivity of antibodies present in the serum from infected children against the variable VP8* region corresponding to different P[8] human rotavirus lineages. Extended studies using recombinant proteins of the four P[8] lineages may be of value in monitoring vaccine efficacy against circulating rotavirus strains.

Acknowledgements. This work was supported in part by grant No. 52036 from the National Council for Science and Technology, Mexico (CONACYT) and grant SA1683-07-PAICYT from the Universidad Autónoma de Nuevo León, México. Griselda E. Menchaca was recipient of a fellowship from the CONACYT, México.

References

Ansaldi F, Pastorino B, Valle L, Durando P, Sticchi L, Tucci P, Biasci P, Lai P, Gasparini R, Icardi G (2007): Molecular characterization of a new variant of rotavirus P[8]G9 predominant in a sentinel–based survey in central Italy. J. Clin. Microbiol. 45, 1011–1015. doi.org/10.1128/JCM.02054–06

Araujo IT, Assis RM, Fialho AM, Mascarenhas JD, Heinemann MB, Leite JP (2007): Brazilian P[8], G1, P[8], G5, P[8], G9, and P[4], G2 rotavirus strains: nucleotide sequence and phylogenetic analysis. J. Med. Virol. 79, 995–1001. doi.org/10.1002/jmv.20918

Arias CF, Lizano M, López S (1987): Synthesis in Escherichia coli and immunological characterization of a polypeptide containing the cleavage sites associated with trypsin enhancement of rotavirus SA11 infectivity. J. Gen. Virol. 68, 633–642. doi.org/10.1099/0022–1317–68–3–633

Arista S, Giammanco GM, De Grazia S, Colomba C, Martella V (2005): Genetic variability among serotype G4 Italian human rotaviruses. J. Clin. Microbiol. 43, 1420–1425. doi.org/10.1128/JCM.43.3.1420–1425.2005

Cheuvart B, Friedland LR, Abu-Elyazeed R, Han HH, Guerra Y, Verstraeten T (2009): The Human Rotavirus Vaccine RIX4414 in Infants A Review of Safety and Tolerability. Pediatr. Infect. Dis. J. 28, 225–232. doi.org/10.1097/INF.0b013e31819715fa

Collins PJ, Martella V, Buonavoglia C, O´Shea H (2010): Identification of a G2-like porcine rotavirus bearing a novel VP4 type, P[32]. Vet. Res. 41, 73–84. doi.org/10.1051/vetres/2010045

Cunliffe NA, Gondwe JS, Graham SM, Thindwa BDM, Dove W, Broadhead RL, Molyneux ME, Hart CA (2001): Rotavirus strain diversity in Blantyre, Malawi, from 1997 to 1999. J. Clin. Microbiol. 39, 836–843. doi.org/10.1128/JCM.39.3.836–843.2001

Dowling W, Denisova E, LaMonica R, Mackow ER (2005): Selective membrane permeabilization by the rotavirus VP5* protein is abrogated by mutations in an internal hydrophobic domain. J. Virol. 74, 6368–6376. doi.org/10.1128/JVI.74.14.6368–6376.2000

Espínola E, Amarilla E, Arbiza J, Parra GI (2008): Sequence and phylogenetic analysis of the VP4 gene of human rotaviruses isolated in Paraguay. Arch. Virol. 153, 1067–1073. doi.org/10.1007/s00705–008–0096–8

Hall TA (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hyser JM, Zeng CQY, Beharry Z, Palzkill T, Estes MK (2008): Epitope mapping and use of epitope-specific antisera to characterize the VP5* binding site in rotavirus SA11 NSP4. Virology 373, 211–228. doi.org/10.1016/j.virol.2007.11.021

Jiang B, Gentsch JR, Glass RI (2002): The role of serum antibodies in the protection against rotavirus disease: an overview. Clin. Infect. Dis. 34, 1351–1361. doi.org/10.1086/340103

Larralde G, Gorziglia M (1992): Distribution of conserved and specific epitopes on the VP8 subunit of rotavirus VP4. J. Virol. 66, 7438–7443.

Maunula L, Von Bonsdorff CH (1998): Short sequences define genetic lineages: phylogenetic analysis of group A rotaviruses based on partial sequences of genome segments 4 and 9. J. Gen. Virol. 79, 321–332.

Menchaca G, Padilla-Noriega L, Méndez-Toss M, Contreras JF, Puerto FI, Guiscafré H, Mota F, Herrera I, Cedillo R, Muñoz O, Ward R, Hoshino Y, López S, Arias CF (1998): Serotype specificity of the neutralizing-antibody response induced by the individual surface proteins of rotavirus in natural infections of young children. Clin. Diagn. Lab. Immunol. 5, 328–334.

Okitsu S, Khamrin P, Thongprachum A, Maneekarn N, Mizuguchi M, Ushijima H (2011): Predominance of porcine P[23] genotype rotaviruses in piglets with diarrhea in Northern Thailand. J. Clin. Microbiol. 49, 442–445. doiorg/10.1128/JCM.02263–10

Padilla-Noriega L, Fiore L, Rennels MB, Losonsky GA, Mackow ER, Greenberg HB (1992): Humoral immune responses to VP4 and its cleavage products VP5* and VP8* in infants vaccinated with rhesus rotavirus. J. Clin. Microbiol. 30, 1392–1397.

Rahman M, Matthijnssens J, Yang X, Delbeke T, Arijs I, Taniguchi K, Iturriza-Gomara M, Iftekharuddin N, Azim T, Van Ranst M (2007): Evolutionary history and global spread of the emerging G12 human rotaviruses. J. Virol. 8, 2382–2390. doi.org/10.1128/JVI.01622–06

Santos N, Hoshino Y (2005): Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. Rev. Med. Virol. 15, 29–56. doi.org/10.1002/rmv.448

Tamura K, Dudley J, Nei M, Kumar S (2007): MEGA 4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599. doi.org/10.1093/molbev/msm092

Taniguchi K, Maloy WL, Nishikawa K, Green KY, Hoshino H, Urasawa S, Kapikian AZ, Chanock RM, Gorziglia M (1998): Identification of cross-reactive and serotype 2-specific neutralization epitopes on VP3 of human rotavirus. J. Virol. 62, 2421–2426.

Ward RL, Kirkwood CD, Sander DS, Smith VE, Shao M, Bean JA, Sack DA, Bernstein DI (2006): Reductions in cross-neutralizing antibody responses in infants after attenuation of the human rotavirus vaccine candidate 89–12. J. Infect. Dis. 194, 1729–1736. doi.org/10.1086/509623