Fine mapping of sequential neutralization epitopes on the subunit protein VP8 of human rotavirus

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The epitopes of the HRV (human rotavirus), especially those involved in virus neutralization, have not been determined in their entirety, and would have significant implications for HRV vaccine development. In the present study, we report on the epitope mapping and identification of sequential neutralization epitopes, on the Wa strain HRV subunit protein VP8, using synthetic overlapping peptides. Polyclonal antibodies against recombinant Wa VP8 were produced previously in chicken, and purified from egg yolk, which showed neutralizing activity against HRV *in vitro*. Overlapping VP8 peptide fragments were synthesized and probed with the anti-VP8 antibodies, revealing five sequential epitopes on VP8. Further analysis suggested that three of the five epitopes detected, M1-L10, I55-D66 and L223-P234, were involved in virus neutralization, indicating that sequential epitopes may also be important for the HRV neutralization. The interactions of the antibodies with the five epitopes were characterized by an examination of the critical amino acids involved in antibody binding. Epitopes comprised primarily of hydrophobic amino acid residues, followed by polar and charged residues. The more critical amino acids appeared to be located near the centre of the epitopes, with proline, isoleucine, serine, glutamine and arginine playing an important role in the binding of antibody to the VP8 epitopes.

Key words: epitope characterization, immunoglobulin Y, peptide synthesis, virus neutralization, VP8, Wa strain.

INTRODUCTION

Until the 1970s, the important aetiological agents of diarrhoea were unknown [1]. In 1973, Bishop et al. [2] noticed a 70 nm virus particle while using electron microscopy to detect infection in the duodenum of young children hospitalized for treatment of acute diarrhoea. It soon became apparent that this 70 nm particle, subsequently designated *rotavirus* for its wheel-like appearance, was the major aetiologic agent of acute infantile gastroenteritis [1], infecting up to 90 % of children under the age of 3 [3] and resulting in approx. 600 000 infant deaths annually [4]. The HRV (human rotavirus), which is characteristically localized to the epithelial cells of the gastrointestinal tract [5], causes a shortening and atrophy of the villi of the small intestine [1]. This results in decreased water absorption leading to severe diarrhoea and vomiting, and eventually to death due to dehydration if left untreated [3,6]. Until now, no rotavirus vaccine has been available. Recently, a live, oral, attenuated rhesus-human reassortant tetravalent rotavirus vaccine was licensed in the Unites States for the universal immunization of healthy children [6]. However, in 1999, it was withdrawn following reports of intussusception (a type of bowel obstruction) in infants who received the vaccine [7,8].

Rotavirus is a member of the *Reoviridae* family, and is characterized by a segmented, double-stranded RNA genome, consisting of 11 gene segments and a non-enveloped, multilayered protein capsid. Of these 11 gene segments, six code for structural proteins and five code for non-structural proteins. The six structural proteins, designated VP1–VP4, VP6 and VP7, are arranged into three concentric layers surrounding the rotavirus genome [9].

The outermost layer is composed of two proteins, VP7 and VP4. VP7, a glycoprotein with a molecular mass of 34 kDa, is present as 780 molecules, which form the smooth external surface of the outer shell [1,10]. The minor component of the outer shell, VP4, is present as a series of 60 dimeric spikes, 10–12 nm in length, with a knob-like structure at the distal end, which projects outward from the VP7 shell [1,11]. VP4 is non-glycosylated and has a molecular mass of 88 kDa [1,10], and has been implicated in several important functions, including cell attachment and penetration, haemagglutination, neutralization and virulence [12–15]. Viral infectivity is enhanced by the cleavage of VP4 by trypsin to produce VP8 (the 28-kDa N-terminal segment) and VP5 (the 60-kDa C-terminal segment) [1,16]. The VP8 subunit, in particular, has been found to play a significant role in viral infectivity and neutralization of the virus, and studies with animal and HRVs have identified several conformational neutralization epitopes on VP8 [12,17–22]. It was found that monoclonal antibodies directed against VP8 not only functioned to inhibit the attachment of rotavirus to cells *in vitro* [5,16] but also were found to neutralize the virus *in vivo*, passively protecting mice against rotavirus challenge [23].

Whereas significant research has been performed on the neutralization epitopes of animal rotavirus and HRVs, complete amino acid sequences of neutralization sites are unknown. The identification of HRV neutralizing epitopes on VP8 would not only provide insight into the structure of VP8 and infectivity of HRV, but may also allow the production of increasingly specific HRV-neutralizing antibodies, raised against epitopes involved strictly in virus neutralization.

In our previous studies, we have described the expression and purification of recombinant VP8 in *Escherichia coli*, and its use to induce anti-VP8 antibodies in chicken, which were found to be capable of neutralizing HRV *in vitro* [24]. In the present study, we report the use of these anti-VP8 polyclonal antibodies to identify and characterize sequential epitopes on VP8, from the Wa strain of HRV, as well as to determine which of these epitopes are involved in virus neutralization.

Abbreviations used: FCFU, fluorescent cell focus-forming units; HRV, human rotavirus; IgY, immunoglobulin Y; SPOTS, Simple Precise Original Test System; TBS, Tris-buffered saline; TBST, TBS containing Tween 20.

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EXPERIMENTAL

CD spectrum of VP8

The far-UV spectrum of recombinant VP8, derived from the Wa strain of HRV, which had been expressed in *E. coli* (JM 109) and purified as described previously [24], was measured using a JASCO J-600 spectropolarimeter (Easton, MD, U.S.A.) at 25 °C. A solution of purified VP8 (0.17 mg/ml) was prepared in 0.01 M phosphate buffer (pH 7.0). The buffer and sample were filtered through a 0.22 μm filter and degassed before use. The VP8 spectrum was obtained by scanning the sample four times, from 190 to 250 nm, using a quartz cuvette with a path length of 0.2 cm (Japan Spectroscopic, Tokyo, Japan). Phosphate buffer was used to establish a baseline, which was subtracted from the sample spectrum. The percentages of the secondary structures were estimated using the JASCO protein secondary-structure estimation program, based on the method of Yang et al. [25]. The mean molar residue ellipticities, ϑ ($\mathrm{deg \cdot cm^2 \cdot dmol^{-1}}$), were calculated using a mean residue mass of 0.110 kDa, as described by Mulkerrin [26].

Production of anti-VP8 IgY (immunoglobulin Y)

Polyclonal anti-VP8 IgY was produced in 25-week-old Shaver White Leghorn chicken (*Gallus domesticus*; Arkell Research Station, Poultry Unit, Guelph, ON, Canada) and isolated as described previously [24]. Briefly, preimmune egg yolk was screened by ELISA for the presence of antibodies which cross-reacted with the recombinant Wa VP8, and only those chickens which exhibited no cross-reactivity were used for antibody production. Chickens were injected with purified recombinant Wa VP8, and the IgY was isolated from the hens' egg yolk first by freeze–thawing, and then by saturated ammonium sulphate precipitation. The precipitated IgY was dialysed against water and then freeze-dried.

Affinity purification of anti-VP8 IgY

Cyanogen bromide-activated Sepharose 4B (0.5 g; Amersham Biosciences) was prepared according to the manufacturer's instructions, and added to 8 mg of the recombinant Wa VP8, which had been dissolved in coupling buffer (0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3) and filtered through a 0.45 μm syringe filter (Pall Gelman Sciences, Ann Arbor, MI, U.S.A.). The mixture was incubated overnight at 4 °C with gentle shaking, and excess ligand was washed away using 5 bed volumes of coupling buffer. Remaining active groups on the Sepharose 4B were blocked by incubation with 5 ml of 1 M ethanolamine (pH 8.0) for 2 h at room temperature. The Sepharose 4B was then transferred to a 2 ml column (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and washed with 5 bed volumes of alternatively 0.5 M NaCl in 0.1 M acetate buffer (pH 4.0) and 0.5 M NaCl in 0.1 M Tris/HCl (pH 8.0), repeated three times to remove any ionically bound ligand.

Freeze-dried anti-VP8 IgY (approx. 150 mg) was dissolved in 0.1 M Tris/HCl (pH 7.5) containing 0.15 M NaCl, and loaded on to the column. The column was washed with the Tris/HCl buffer until the absorbance of the flow through at 280 nm was < 0.01, and the bound antibodies were eluted with a 0.2 M glycine (pH 2.7) containing 0.5 M NaCl. The eluate was concentrated using a Microsep centrifugal filtration device, with a molecular mass cut-off of 100 kDa (Pall Gelman Sciences), and glycine and NaCl were removed by the repeated addition of TBS (Tris-buffered saline), pH 8.0. The column was regenerated according to the manufacturer's instructions.

Peptide synthesis

Using the amino acid sequence of the cloned Wa VP8 (GenBank® accession no. L34161), overlapping peptide segments, 12 amino acids in length, were synthesized using the SPOTS (Simple Precise Original Test System; Sigma-GenoSys, The Woodlands, TX, U.S.A.) synthesis method, as described by Frank and Overwin [27]. The peptides, each overlapping by six amino acids, were synthesized by coupling fluorenylmethoxycarbonyl amino acids (Sigma-GenoSys, Cambridge, U.K.) to a cellulose SPOTS membrane derivatized with a dimer of β-alanine–NH_2 groups (Sigma-GenoSys). Along with the VP8 peptides, spots having a single glycine residue served as negative controls, and a peptide corresponding to a known epitope on the human epidermal growth factor receptor (with the amino acid sequence CAHYID) was used as a positive control peptide. Membranes were dried and stored at − 30 °C until required.

Once the epitopes were identified, they were examined further by repeating the peptide synthesis, but shortening and extending the corresponding peptides to pinpoint the exact size of the epitopes.

Probing of synthetic peptides

The dried membrane was removed from − 30 °C, allowed to equilibrate to room temperature, and washed briefly with methanol. After three washings with TBS, the membrane was blocked overnight at room temperature with shaking, in 5 ml of SPOTS blocking buffer (Sigma-GenoSys), diluted ten times in TBST (TBS containing 0.05 % Tween 20) with 5 % (w/v) sucrose. The membrane was then washed once with TBST and incubated overnight at room temperature with shaking in 5 ml of pooled, affinity-purified anti-VP8 IgY, diluted to 0.5 μg/ml in SPOTS blocking buffer. The membrane was washed three times with TBST, and then incubated for 3 h at room temperature with shaking in alkaline phosphatase-conjugated rabbit anti-chicken IgG (Sigma), diluted 10 000 times in SPOTS blocking buffer. After thorough washing with TBST and rinsing in detection buffer (0.1 M NaCl in 0.1 M Tris/HCl, pH 9.5), the bound antibodies were detected using the chemiluminescent CDP-Star substrate (Boehringer Mannheim, Ingelheim, Germany) and Nitro-Block II (Tropix, Bedford, MA, U.S.A.), both diluted 100 times in detection buffer. The chemiluminescent reaction was visualized with a Molecular Light Imager (EG-G Berthold, Bad Wildbad, Germany). Image processing was performed using WinLight software (EG-G Berthold) to obtain the intensity of each spot, measured in counts/s.

The positive and negative control spots were probed similarly, by incubating the membrane for 4 h at room temperature with shaking in 2 ml of a mouse monoclonal antibody against the extracellular domain of human C-erb-1/epidermal growth factor receptor (Sigma-GenoSys), recognizing the epitope CAHYID, diluted 500 times in SPOTS blocking buffer, and detecting with alkaline phosphatase-conjugated anti-mouse IgG (Sigma), diluted 10 000 times in SPOTS blocking buffer.

Regeneration of SPOTS membrane

The membrane was regenerated according to the manufacturer's instructions (Sigma-GenoSys) by washing with regeneration buffer A [8 M urea/1 % (w/v) SDS/0.1 % (v/v) 2-mercaptoethanol], followed by washing with regeneration buffer B [50 % (v/v) ethanol/10 % (v/v) acetic acid]. The membrane was then washed with methanol, dried and stored at − 30 °C.

Identification of HRV sequential neutralization epitopes on VP8

Neutralization epitopes were identified by sequentially removing IgY directed against each of the five epitopes using a stepwise incubation of each epitope with a single pool of IgY. The SPOTS membrane used for epitope mapping was removed from − 30 °C, and the sections of the membrane corresponding to the five detected epitopes were each cut from the membrane. The first epitope was rinsed in methanol, washed with TBS and then blocked overnight at room temperature with shaking in SPOTS blocking buffer. The membrane was then washed with TBST and incubated overnight at room temperature with shaking in 1 ml of affinity-purified anti-VP8 IgY, diluted to 10 μg/ml in SPOTS blocking buffer. A 50 μl aliquot of the IgY solution was then removed for use in the virus neutralization assay, and the remaining solution was used for the next epitope. This was repeated for all the epitopes (in the following order: M1L10, I35R44, I55D66, V115G122 and L223P234), each time using the same IgY solution in which the previous epitope had been incubated. All IgY aliquots were stored at − 80 °C, until assayed for neutralization activity.

Virus neutralization assay

Virus neutralization titres were determined using a modified procedure of Brüssow et al. [28]. Briefly, serially diluted samples of the IgY were incubated with Wa strain HRV (2018-VR) (A.T.C.C., Rockville, MD, U.S.A.) at 37 °C for 1 h, then inoculated into African green monkey kidney cells (MA104) (A.T.C.C.) and incubated again. FCFU (fluorescent cell focus-forming units) were evaluated by an indirect immunofluorescent assay, and neutralization titres were expressed as the reciprocal value of the highest dilution showing a 50 % reduction of FCFU.

Substitution analysis

The critical amino acids in each of the Wa VP8 epitopes were examined by synthesizing multiple peptides with single amino acid substitutions at each position in the epitope, for each of the five epitopes. Peptides were synthesized on a SPOTS membrane, with each amino acid sequentially replaced by either glycine or alanine, depending on the amino acid composition of the epitope and probed twice as described above.

Statistical analysis

The spot intensities were measured using WinLight software (EG-G Berthold), and statistical analysis was performed using Student's *t* test with two independent samples.

RESULTS

CD analysis of VP8

CD was performed to examine the secondary structure of the recombinant Wa VP8. The observed spectrum showed an absorbance minimum at 216 nm and a maximum at 197 nm, characteristic of a protein rich in β-sheet structure (Figure 1). The recombinant Wa VP8 was found to contain 4.8 % α-helical structure and 47.9 % β-sheet structure, with 18.4 % β-turn and 28.9 % random coil.

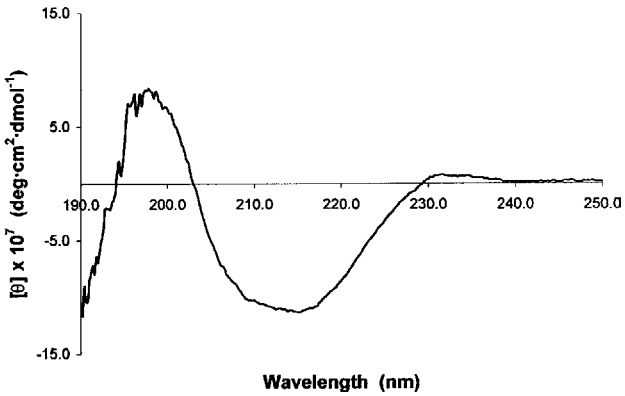

Figure 1 Far-UV spectrum of recombinant VP8

The CD spectrum of recombinant Wa VP8 was obtained by scanning the sample four times, from 190 to 250 nm. Phosphate buffer was used as the blank, and was subtracted from the VP8 spectrum.

Production and purification of IgY

The purified IgY was concentrated, and the concentration was estimated to be approx. 70 μg/ml, using the absorbance at 280 nm and a molar absorption coefficient (ε) of 1.51 ml $\cdot$ mg^{-1} $\cdot$ cm^{-1} for IgY [29].

Sequential epitope mapping of VP8

Epitope mapping was performed using arrays of synthetic peptides. Using the amino acid sequence of the recombinant Wa VP8, the peptides, 12 amino acids in length and overlapping by six amino acids, were synthesized. A positive and negative control peptide was probed first to ensure that the synthesis was successful (results not shown). Probing of the VP8 peptides revealed five distinct regions of the protein against which the IgY was directed (Figure 2). Analysis of the sequence of overlapping peptides in relation to the pattern of positive spots revealed that the epitopes corresponded to the amino acid sequences indicated in Figure 3. Some faint single spots were also detected, but were dismissed as false positives similar to the surrounding spots, which also shared some of the same amino acid sequence, but were not detected. Epitopes 1, 2, 3, 4 and 5 were designated as M1L10, I35R44, I55D66, V115G123 and L223P234 respectively.

Identification of HRV sequential neutralization epitopes on VP8

To identify which of the five detected epitopes were involved in the neutralization of HRV, the anti-VP8 IgY was sequentially adsorbed to each of the five epitopes to remove the corresponding antibodies. Adsorption was performed in the following order: M1L10, V115G123, I55D66, I35R44 and L223P234. The order in which each epitope was used for adsorption was determined using the intensity of the epitopes in the SPOTS analysis and their relative hydrophilicities, as determined by the method of Kyte and Doolittle [30] (Figure 4). This method predicts which segments of the protein are more likely to be exposed on the surface of the virus (hydrophilic) and therefore probably involved in virus neutralization. It follows from Figure 4 that M1L10 and I55D66 display hydrophilic character, whereas the others appear more hydrophobic. After each adsorption, an aliquot was removed and assayed for neutralization activity to determine the neutralization activity of the remaining IgY after the removal of antibodies

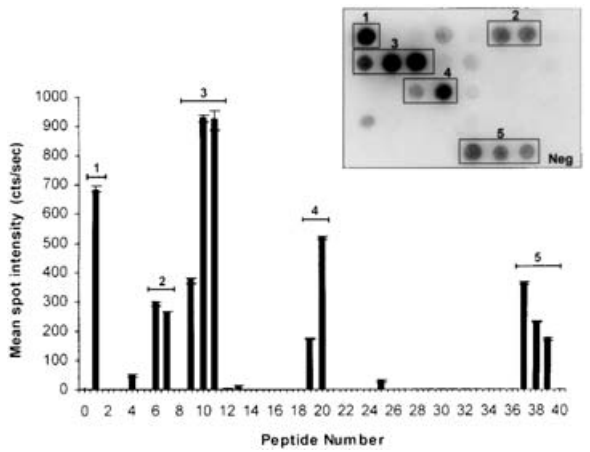

Figure 2 Sequential epitope mapping of VP8

Synthetic overlapping peptides, corresponding to the sequence of Wa strain VP8, were synthesized on a cellulose membrane (inset), and probed with the anti-VP8 IgY. The bound antibodies were detected with alkaline phosphatase-conjugated rabbit anti-chicken IgG, and visualized by the addition of chemiluminescent substrate. The anti-VP8 IgY was found to be directed against five distinct regions on the protein, numbered 1–5. The negative control spot, composed of a single glycine residue, is indicated in the bottom right corner of the membrane (Neg). Mean spot intensities are shown plotted for each peptide.

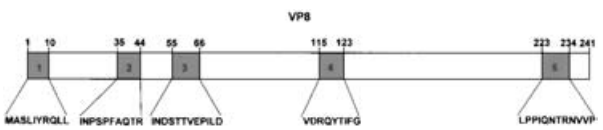

Figure 3 Sequential VP8 epitopes detected by epitope mapping

Regions on the protein detected by the epitope mapping were found to correspond to amino acids M^{1}–L^{10}, I^{35}–R^{44}, I^{55}–D^{66}, V^{115}–G^{123} and L^{223}–P^{234}. Shaded regions indicate the location of the epitope within the protein. These five regions of the protein (1–5) were subsequently designated M1L10, I35R44, I55D66, V115G123 and L223P234, respectively.

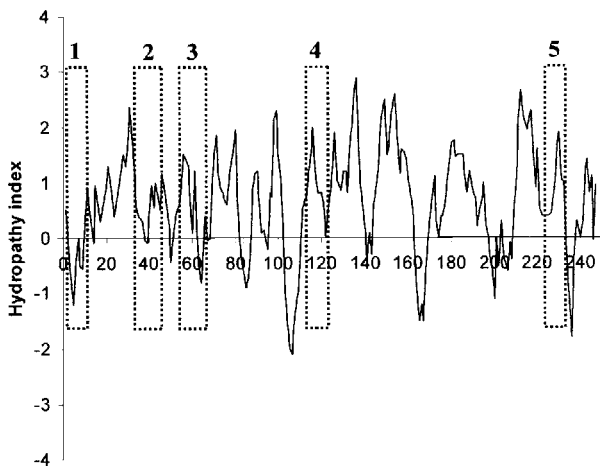

Figure 4 Hydropathy profile of VP8

A hydropathy plot was prepared using the Kyte–Doolittle method of calculating protein hydropathy [30]. Amino acids were scanned over a window length of seven amino acids, and the average hydropathy values were plotted. Hydrophilic regions are indicated by negative hydropathy values and hydrophobic regions by positive values. The amino acids corresponding to the VP8 sequence are numbered along the *x*-axis. Stretches corresponding to each of the VP8 epitopes are indicated by boxes. Numbers 1, 2, 3, 4 and 5 refer to epitopes M1L10, I35R44, I55D66, V115G123 and L223P234 respectively.

Table 1 Neutralization assay of adsorbed anti-VP8 IgY

Neutralization titres for the anti-VP8 IgY following adsorption with each of the epitopes are shown. To remove the corresponding antibodies, a solution of anti-VP8 IgY was incubated with each of the VP8 epitopes. The IgY solutions were serially diluted and incubated with HRV, and then inoculated on to MA104 cells. Neutralization titres had the highest dilutions showing a 50% reduction of FCFU. To verify the results, the neutralization assay was performed twice. Negative control is IgY from non-immunized chicken. Before adsorption, positive control is the anti-VP8 IgY, containing IgY against all epitopes. N/A, not applicable.

		Neutralization	
Sample	Epitopes adsorbed	Titre 1	Titre 2
Negative control	N/A	< 8	< 8
Positive control	None	2048	4096
1	M1L10	512	512
2	M1L10, V115G123	256	512
3	M1L10, V115G123, I55D66	32	64
4	M1L10, V115G123, I55D66, I35R44	32	32
5	M1L10, V115G123, I55D66, I35R44, L223P234	< 8	< 4

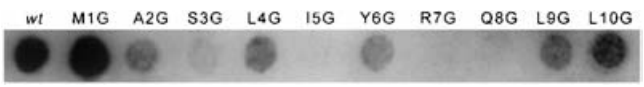

Figure 5 Substitution analysis of sequential VP8 epitopes

Results shown represent substitution analysis of epitope M1L10. The epitope was characterized by synthesizing the corresponding peptide and sequentially substituting each amino acid with glycine. Peptides were then re-probed using the anti-VP8 IgY, and visualized by the addition of chemiluminescent substrate. Amino acid substitutions are indicated in the Figure. The number refers to the amino acid position at which substitution was performed, the letter on the left refers to the amino acid that was substituted, and the letter on the right is the amino acid used for substitution. The native epitope sequence, which was shown in Figure 3, is indicated by *wt*.

corresponding to each epitope. Each epitope was regenerated and re-probed with the IgY solution to confirm that there was no reactivity of the residual IgY with each epitope previously used for adsorption (results not shown). Removal of IgY due to non-specific binding to the membrane was assumed to be negligible due to the low background observed on chemiluminescent detection. Serial dilutions of the IgY aliquots were prepared and assayed in the virus neutralization assay. The resulting neutralization titres are shown in Table 1. The positive control corresponds to the IgY solution before adsorption (IgY against all five epitopes), which was previously shown to be capable of neutralizing HRV [24]. IgY from non-immunized chicken was used as a negative control. From the significant decrease in the neutralization titre of the antibody solution after the removal of IgY against M1L10, I55D66 and L223P234, it is evident that these epitopes are involved in neutralization of the virus.

Substitution analysis

To characterize further the binding of the anti-VP8 IgY to each of the Wa VP8 epitopes, and to identify those amino acids integral for IgY binding, a substitution analysis was performed. Each amino acid in each of the five epitopes was sequentially replaced with a simple, non-polar and hydrophobic amino acid, either glycine or alanine, and re-probed with the anti-VP8 IgY. The substitution analysis of M1L10 is shown in Figure 5. Differences in mean spot intensities were evaluated using a Student's *t* test (Table 2). In some cases, the amino acid substitutions were found to reduce significantly or eliminate completely IgY binding to the epitope,

indicating that the amino acid replaced is critical for IgY binding to the epitope. These amino acids are indicated in Table 3. Some substitutions were also found to enhance IgY binding to the epitopes, particularly in epitopes M1L10 and I55D66. The amino acids of all five epitopes were then classified as either hydrophobic, polar or charged residues, and enumerated (Figure 6), and the properties of those amino acids considered to be critical were examined.

Table 2 Statistical analysis of critical amino acids

Peptides corresponding to each epitope were synthesized and each amino acid was substituted with glycine or alanine. The peptides were probed with anti-VP8 IgY. The intensities of the resulting spots were measured and analysed for significant differences in IgY binding from the wt, or non-substituted peptide.

Peptide	Mean spot intensity (counts/s)
M1L10	
wt	4558.6 ± 165
M1G*	7513.0 ± 165†
A2G	1797.8 ± 81.7
S3G	456.6 ± 26.7‡
L4G	1254.4 ± 83.4
I5G	0.0‡
Y6G	971.5 ± 44.8
R7G	0.0‡
Q8G	0.0‡
L9G	2148.8 ± 25.5
L10G	3195.8 ± 31.6
I35R44	
wt	2510.4 ± 5.02
I35G	2618.7 ± 93.3
N36G	1339.4 ± 75.9
P37G	0.0‡
S38G	0.0‡
P39G	0.0‡
F40G	1382.8 ± 55.4
A41G	880.8 ± 12.5‡
Q42G	542.0 ± 11.0‡
T43G	1126.2 ± 121
R44G	183.2 ± 17.3‡
I55D66	
wt	5001.5 ± 34.2
I55G	3986.1 ± 0.424
N56G	5235.8 ± 176
D57G	2835.4 ± 269
S58G	3519.4 ± 83.1
T59G	0.0‡
T60G	0.0‡
V61G	0.0‡
E62G	0.0‡
P63G	0.0‡
I64G	3841.5 ± 87.4
L65G	6139.6 ± 28.4†
D66G	4755.4 ± 171
V115G123	
wt	2063.8 ± 60.1
V115A	1679.0 ± 102
D116A	1507.2 ± 16.6
R117A	0.0‡
Q118A	633.5 ± 4.73‡
Y119A	0.0‡
T120A	1726.0 ± 24.0
I121A	0.0‡
F122A	0.0‡
G123A	1520.5 ± 73.4
L223P234	
wt	1433 ± 5.66
L223G	0.0‡
P224G	0.0‡
P225G	0.0‡
I226G	0.0‡
Q227G	578.6 ± 9.28‡
N228G	470.6 ± 24.4‡
T229G	568.6 ± 6.36‡
R230G	968.0 ± 30.7
N231G	648.4 ± 38.4
V232G	974.5 ± 20.7
V233G	1846.0 ± 14.2†
P234G	1304.0 ± 28.1

* The number refers to the amino acid position at which substitution was performed, the letter on the left refers to the amino acid which was substituted and the letter on the right is the amino acid used for substitution.

† Means are significantly higher ($P < 0.005$) than wt (Student's t test).

‡ Means are significantly lower ($P < 0.0005$) than wt (Student's t test).

Table 3 Critical amino acids of sequential VP8 epitopes

The substitutions which resulted in a significant decrease in anti-VP8 IgY binding to the epitope were considered to be critical amino acids. The critical amino acids in each epitope are shown by boldface and underlined.

Epitope	Critical amino acids
M1L10	M^{1}A**S**L**I**Y**RQ**LL^{10}
I35R44	I^{35}N**PSP**F**AQ**T**R**^{44}
I55D66	I^{55}NDS**TTVEP**ILD^{66}
V115G123	V^{115}D**RQY**T**IF**G^{123}
L223P234	**L**^{223}**PPIQNT**RNVVP^{234}

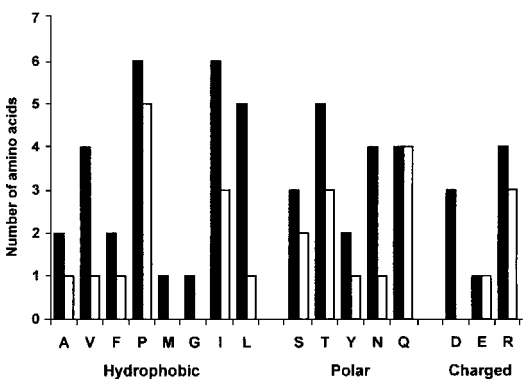

Figure 6 Classification of amino acids in the sequential VP8 epitopes

The amino acids making up all five sequential Wa VP8 epitopes were classified as hydrophobic, polar and charged residues. The frequency with which each amino acid residue occurred was totalled and graphed. Black bars represent the total number of amino acids of each type found in the epitopes, whereas the white bars indicate the number of amino acids which were found to be critical.

DISCUSSION

In the present study, we have mapped and characterized sequential epitopes on the VP8 subunit of the Wa strain of HRV using polyclonal antibodies produced against recombinant Wa VP8. In previous studies, we found that these antibodies, which were produced in chicken, displayed significant neutralizing activity against the Wa strain of HRV *in vitro* and, therefore, were ideally suited for identifying neutralization epitopes on Wa VP8. Chickens were used for antibody production since chicken IgY is the functional equivalent of IgG found in mammals; however, it is transferred to the egg yolk [31,32] and can easily be purified from the yolk in large quantities.

CD analysis was performed to examine the secondary structure of the recombinant Wa VP8, and indicated that the recombinant VP8 contained a very small amount of α-helical structure and was largely composed of β-sheet structure. This is similar to previous CD results of VP8 [33] and secondary-structural analyses of VP8 [34,35], which indicated that VP8 is made up of 11 β strands, separated by loops, and flanked by two small α-helices. This would also suggest that the conformation of the recombinant Wa VP8 is similar to the native form of VP8 found in the virus [36].

Neutralization epitope studies have been performed previously on VP4 and its cleavage products, VP5 and VP8, primarily in simian [12,17,18,20,22], bovine [37] and human [19,21,38,39] rotaviruses. However, these studies have focused on the identification of single amino acids or groupings of amino acids, rather than full epitopes, using neutralization escape mutants of rotavirus and antibody competition experiments. In the present study, we demonstrate that sequential epitopes are also important for HRV neutralization. Our study revealed five sequential epitopes on the Wa strain HRV VP8. To identify which of these five epitopes were involved in the neutralization of HRV, the anti-VP8 IgY was sequentially adsorbed to each of the epitopes, removing the corresponding antibodies from the solution. The neutralizing activity of the remaining IgY was then measured. Since the tertiary structure of the protein was not known, a Kyte–Doolittle hydropathy scale, a method of antigenicity prediction relying on the predicted accessibility based on amino acid sequence [30], was used to estimate which of the epitopes were more likely to be exposed on the surface of the virus. These epitopes should then show a greater effect on neutralizing activity when removed from the solution and were thus adsorbed accordingly. The IgY was removed sequentially from a single pool of IgY, rather than individually from separate pools of IgY, to avoid the masking of potential neutralizing activity by the other antibodies. From the neutralization assay of the adsorbed anti-VP8 IgY solutions it follows that epitopes M1L10, I55D66, and possibly L223P234, appear to be involved in HRV neutralization. Epitopes I35R44 and V115G123 are either not involved in virus neutralization and, therefore, do not produce a significant change in neutralization titre, or these epitopes are located at a site which is not accessible to antibodies in the native VP8 structure on the virus. From the results obtained, it would appear that these particular antibodies were primarily directed against sequential epitopes, as the neutralization titre decreased significantly after removal of IgY against all five of the linear epitopes.

Recently, laying chickens have attracted considerable attention as an alternative source of antibodies for the prevention and treatment of infectious gastrointestinal diseases. The use of chickens for the production of polyclonal antibodies provides several advantages (only for the IgG class antibody, less invasive, more hygienic, low cost and suitable for large-scale production etc.) [40]. In the present study, we have detected sequential, rather than conformational, epitopes using polyclonal antibodies generated in chickens. Chicken antibodies might recognize more sequential epitopes rather than conformational structure. However, the differences in antigen recognition between mammalian hosts and chickens have not been well studied. It is obvious that the polyclonal anti-VP8 IgY raised in chickens has strong neutralization activity against HRV *in vitro*, as demonstrated previously [24]. Furthermore, it has been suggested that variations in the amino acid sequences of animal rotavirus and HRV VP4, most notably the lack of a cysteine residue at position 203 of HRV VP4, may result in structural differences between animal rotavirus and HRVs, making comparisons difficult [41]. Comparative studies on differences of antigen recognition between mammals and other species are of great interest and further research is necessary in this direction.

The binding of the IgY to each of the epitopes was characterized using substitution analysis, where each amino acid was replaced with glycine or alanine, to examine the effect of the substitution of each amino acid on antibody binding. Small amino acids, such as alanine and glycine, were used because they have been found to have low propensities for being critical amino acids in epitopes [42]. As expected, some amino acid substitutions in each epitope resulted in a decrease or complete elimination of IgY binding, and were considered to be critical amino acids. Substitution had no effect on those amino acids which were not critical for IgY binding. There were a total of 53 amino acids in all of the Wa VP8 epitopes that were classified as hydrophobic, polar or charged. Hydrophobic residues were found to be the most frequently occurring (27/53), followed by polar residues (19/53) and then charged residues (8/53), and of these charged residues, aspartate and arginine residues appeared to play an important role. The critical amino acids followed a similar pattern, with most of the critical residues belonging to the hydrophobic group, followed by polar and charged. The amino acids proline and isoleucine (hydrophobic residues), serine and glutamine (polar residues) and arginine (charged residue) seemed to be most sensitive to mutation, as a significant number of the substitutions in these amino acids resulted in a decrease in IgY binding. It should also be mentioned that, generally, the critical amino acids appeared to be located near the centre of the epitopes (except for L223P234). However, some substitutions were found to enhance IgY binding, most notably in epitopes M1L10 and I55D66. The substitution of M1 with glycine in epitope M1L10, and N^{56} and L^{65} with glycine in epitope I55D66 produced noticeably darker spots than the native epitope sequences, indicating an increase in IgY binding. This apparent increase in binding may be due to an increased similarity between the structure/conformation of the substituted peptide and the epitope on the native Wa VP8, against which the antibodies were raised. In other words, these particular substitutions may produce peptides that closely mimic the conformation of the recombinant Wa VP8, and provide valuable information regarding the conformation of these epitopes.

Our previous research suggested that the recombinant Wa VP8, which was capable of eliciting specific neutralizing antibodies against HRV, had potential as a vaccine candidate. The results presented in the present study, will allow the isolation of amino acid sequences responsible for eliciting neutralizing antibodies and the production of highly specific HRV-neutralizing antibodies, which have significant implications for vaccine research. This information may also aid in determining whether these anti-VP8 antibodies can bind and neutralize heterologous strains of HRV, rather than the Wa strain, despite amino acid sequence variations in other strains, and therefore be useful for the widespread prevention of HRV.

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