

Misdirected antibody responses against an N-terminal epitope on human rhinovirus VP1 as explanation for recurrent RV infections

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ABSTRACT Rhinoviruses (RVs) are the primary cause of upper respiratory tract infections, generally known as the common cold. Moreover, RV infections can trigger severe exacerbations of asthma and chronic obstructive pulmonary disease (COPD). We expressed the 4 major RV capsid proteins, VP1–VP4, in *Escherichia coli* and used these proteins as well as recombinant and synthetic VP1 fragments to study and map antibody responses in RV-infected humans. VP1, which on infection binds to ICAM 1, was identified as a major target for the memory immune response, residing in the IgG1 subclass and IgA class. Interestingly, this response was mainly directed against an N-terminal 20mer peptide in VP1, P1a, which becomes exposed on intact RV only when it docks to its receptor ICAM 1. Molecular modeling using the 3-dimensional RV capsid structures revealed that P1a was localized inside the capsid and outside the areas involved in receptor binding or RV neutralization. Our results suggest misdirection of antibody responses against a nonprotective epitope as a mechanism how RV escapes immunity and causes recurrent infections. Based on these findings, it may be possible to design vaccines against RV infections and RV-induced respiratory diseases.—Niespodziana, K., Napora, K., Cabauatan, C., Focke-Tejkl, M., Keller, W., Niederberger, V., Tsoia, M., Christodoulou, I., Papadopoulos, N. G., Valenta, R. Misdirected antibody responses against an N-terminal epitope on human RV VP1 as explanation for recurrent RV infections. *FASEB J.* 26, 000–000 (2012). www.fasebj.org

Key Words: asthma • rhinovirus infection

RHINOVIRUS (RV) INFECTIONS are the major cause of the common cold (1). They also represent the most

frequent triggers of severe exacerbations of chronic respiratory diseases, such as asthma (2, 3) and chronic obstructive pulmonary disease (COPD; refs. 4, 5).

Rhinoviruses are small, nonenveloped, single-stranded RNA viruses that belong to the *Picornaviridae* family. The RV capsid is composed of 60 copies of each of the 4 structural capsid proteins, VP1–P4 (6). Three-dimensional crystal structures of several human picornaviruses have been solved at atomic resolution, revealing details of capsid architecture and the arrangements of these proteins in the capsid (7, 8). VP1, VP2, and VP3 are located on the external surface of the capsid and share a similar folding topology, whereas VP4 is located inside the virus (6, 9). VP1 is the most surface-exposed and immunodominant of all the RV capsid proteins. It contains most of the motifs known to interact with the cellular receptor ICAM-1 (10), which is the major binding site of major group RVs, whereas minor group RVs target the low-density lipoprotein receptor family (11).

The total duration of RV infection is usually very short (*i.e.*, ~7 d). RV-specific antibody responses develop in both serum and nasal secretions generally 1–2 wk after infection and may persist for 1 yr (12). However, unlike the case of many other viral infections that sometimes confer lifelong protection, there is no efficient protection against recurrent RV infections. RV infections can occur throughout the year with peaks in spring and autumn and may infect individuals several times each year (13).

Lack of immunological protection against viral infections may be due to many factors, such as the occurrence of a variety of different viral strains, the ability of

Abbreviations: COPD, chronic obstructive pulmonary disease; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HRP, horseradish peroxidase; HSA, human serum albumen; MBP, maltose-binding protein; NIIm-IA; neutralizing immunogenic site IA; RV, rhinovirus.

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viruses to change their antigenic properties, or the use of poorly immunogenic surface structures (*e.g.*, carbohydrates; ref. 14). One likely explanation for the lack of immunological protection against RVs and the difficulties to establish RV-specific vaccines may be huge sequence diversities among RVs. In fact, there are >100 serotypes showing high sequence variation, as exemplified by groups A and B (15) and the recently discovered group C (16).

So far, no detailed investigation of the natural humoral immune response against RVs, and in particular against the different capsid proteins and epitopes thereof, has been performed in humans. There are also no serological tests based on purified RV proteins or peptides available for the monitoring of immune responses (17).

Therefore, we expressed in *E. coli* and purified the 4 major RV capsid proteins VP1–VP4 as well as recombinant and synthetic VP1 fragments and peptides for studying the humoral immune responses and their fine specificities in RV infected humans. Our studies reveal that the natural VP1-specific humoral immune response is mainly directed against a highly conserved, N-terminal VP1 20mer peptide. Paradoxically, this peptide is located inside the RV capsid and does not include sequences involved in receptor binding or neutralization. Thus, our results suggest misdirection of immune responses as a possible explanation for the lack of protection against recurrent RV infections.

MATERIALS AND METHODS

Patients, antibodies, synthetic peptides

Fifty-nine children with fever and/or respiratory symptoms confirmed by physician diagnosis were enrolled in the study after informed consent was given. Viral RNA was extracted from nasopharyngeal aspirates, and RV detection was performed using RT-PCR (18, 19). Rabbit antibodies against VP1 were obtained by immunizing rabbits with recombinant VP1 (20), whereas RV89-specific guinea pig antiserum was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). VP1-derived peptides were synthesized on the CEM

microwave peptide synthesizer (CEM Corp., Matthews, NC, USA) using an Fmoc (9-fluorenylmethylmethoxycarbonyl) strategy with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation. The peptides shown in **Table 1** were purified to >90% purity by preparative HPLC, and their identity was verified by a Microflex mass spectrometer (Bruker Daltonics, Billerica, MA, USA).

Expression and purification of recombinant VP1–VP4 and recombinant VP1 fragments

cDNAs coding for VP1, VP2, VP3, and VP4 of RV89 and of VP1 from RV14 and RV group C (GenBank accession number YP_001552435.1) with codons optimized for expression in *E. coli* were synthesized with the addition of a cDNA coding for a hexahistidine tag at the 3' end and inserted into the *NdeI/XhoI* site of pET-27b (ATG:biosynthetics, Merzhausen, Germany). The cDNAs coding for the VP1 fragments (P1, P2, P3) were amplified by PCR using the VP1 cDNA as a template (20). PCR reactions were performed with GoTaq Green Master Mix (Promega, Madison, WI) using the following conditions: 1 cycle at 96°C, 2 min, 30 cycles at 96°C for 25 s, 60°C for 1 min, and 72°C for 2 min, and a final elongation step at 72°C for 5 min. The PCR products were extracted from a 1% agarose gel using the Wizard SV Gel and Clean-Up system (Promega). The primers used in the reactions are shown in **Table 2**. The PCR fragments were digested with *EcoRI* and *BamHI* (Roche, Mannheim, Germany) and cloned into the *EcoRI/BamHI* cut expression vector pMalC4X downstream of the *malE* gene of *E. coli* (New England Biolabs, Ipswich, MA). After transformation in *E. coli* XL1-Blue, positive clones were identified by restriction analysis and by automated nucleotide sequencing (Eurofins MWG Operon, Ebersberg, Germany).

Recombinant VP1–VP4 and VP1 fragments were expressed in cytoplasm of *E. coli* strain BL21 (DE3; Stratagene, La Jolla, CA). VP1–VP4 were purified by nickel affinity chromatography (20). Maltose-binding protein (MBP)-fusion proteins were purified from inclusion bodies by resuspending the *E. coli* pellet in buffer A (25 mM imidazole and 0.1% Triton X-100, pH 7.4), mechanical homogenization with an Ultra-Turrax (Janke & Kunkel-IKA Labortechnik, Staufen, Germany), and DNaseI digestion. After addition of buffer B (6% Triton X-100, 60 mM EDTA, and 1.5 M NaCl, pH 7.0), inclusion bodies were collected by centrifugation at 18,000 rpm for 20 min at 4°C and washed again with buffer B and once with buffer A. Inclusion bodies containing MBP-fusion proteins were then solubilized in 8 M urea, 100 mM NaH₂PO₄, and 10 mM Tris-HCl (pH 8.0) overnight at room

TABLE 1. Synthetic VP1-derived peptides used in this study

Peptide	Position	Length	Sequence
P1A	1–32	32	MNPVENYIDSVLNEVLVVPNIQPSTSVSSHAA
P1B	33–66	34	PALDAAETGHTSSVQPEDMIETRYVITDQTRDET
P1C	67–100	34	SIESFLGRSGCIAMIEFNTSSDKTEHDKIGKGFK
P1a	2–22	21	NPVENYIDSVLNEVLVVPNIQ
P1b	17–36	20	VVPNIQPSTSVSSHAAPALD
P1c	32–51	20	APALDAAETGHTSSVQPEDM
P1d	47–66	20	QPEDMIETRYVITDQTRDET
P1e	62–81	20	TRDETSIESFLGRSGCIAMI
P1f	77–96	20	CIAMIEFNTSSDKTEHDKIG
P1g	92–111	20	HDKIGKGFKTWKISLQEMAAQ

Position denotes the corresponding region of the native VP1 peptide. Length is in number of amino acids. Underscored amino acids denote overlapping sequences between peptides P1a and P1g.

TABLE 2. PCR primers for the amplification of cDNAs coding for VP1-derived fragments

Primer	Sequence
P1 (F)	5'-CGGAATTCATGAACCCAGTTGAAAATTATATAGAT-3'
P1 (R)	5'-CGGGATCCTTATTTGAATCCTTTACCAATTTTATC-3'
P2 (F)	5'-CGGAATTCACATGGAAGGTTAGTCTTCAAGAAATG-3'
P2 (R)	5'-CGGGATCCTTAATAAAACATGTAATAGGCTGATGC-3'
P3 (F)	5'-CGGAATTCGATGGTTATGATGGTGATAGTGCAGCATC-3'
P3 (R)	5'-CGGGATCCTTAGACGTTTGTAAACGGTAAAAACATCAG-3'

Forward (F) and reverse (R) primers are indicated. *EcoRI* (F) and *BamHI* (R) restriction sites are underscored.

temperature and dialyzed in column buffer (20 mM Tris, 200 mM NaCl, and 1 mM EDTA, pH 7.4). The purification of the fusion proteins was performed using MBP's affinity for amylose (pMAL Protein Fusion and Purification System; New England Biolabs). The purity of recombinant proteins was analyzed by SDS-PAGE. Protein concentration was determined by the BCA Protein Assay Kit (Novagen, Darmstadt, Germany).

ELISA experiments

ELISA plates (Nunc, Langensfeld, Germany) were coated with 5 µg/ml of VP1–VP4, recombinant fragments and peptides of VP1, and human serum albumin (HSA) as a negative control, respectively. Human sera were diluted 1:50/1:100, rabbit sera 1:4000, and the HRV89-specific and normal guinea pig sera 1:1000. Bound human antibodies were detected with monoclonal mouse anti-human IgA, IgM, IgG₁, and IgG₂ (BD Pharmingen, San Diego, CA) diluted 1:1000, followed by detection with horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG antibodies (Amersham Bioscience, Freiburg, Germany) diluted 1:2000. Antigen-specific rabbit IgG antibodies were developed with a 1:2000 dilution of donkey anti-rabbit IgG peroxidase-coupled antibodies (Amersham Biosciences), whereas antigen-specific guinea pig IgG antibodies were detected with 1:2000 diluted goat antiguinea pig peroxidase-coupled antibodies (Jackson ImmunoResearch, Newmarket, UK). The optical density (OD) values corresponding to the level of antigen-specific antibodies were measured at 405 and 490 nm in an ELISA reader (Dynatech, Denkendorf, Germany).

Statistical analysis

Statistical significance between antibody levels was assessed by repeated measures using ANOVA or Friedman's test followed by pairwise comparisons of all data sets by Tukey's or Dunn's posttests, respectively, using Prism 5.04 (GraphPad Software, La Jolla, CA). Values of $P < 0.05$ were considered significant.

RESULTS

VP1 is a major target for RV-specific secondary antibody responses in humans

Recombinant capsid proteins (VP1–VP4) of RV strain 89 (major group RV belonging to species A) were expressed in *E. coli* and purified. Purified recombinant VP1–VP4 exhibited in Coomassie-stained SDS-PAGE

molecular masses of ~37, ~30, ~27, and ~10 kDa, respectively (Fig. 1). Serum samples from 48 patients with positive HRV-specific PCR results were tested for VP1–VP4-specific IgA, IgM, IgG_{1–4} antibodies (Fig. 2A). While VP3 and VP4 showed some IgM reactivity, VP1 was a major target recognized by IgG₁ and IgA antibodies, with IgG₁ levels being higher than IgA reactivity (median OD $\pm$ interquartile range, 2.29 ± 1.47 vs. 0.27 ± 0.67 , respectively). VP1-specific IgG₁ and IgA levels were significantly higher than those directed against VP3 and VP4 (Fig. 2A). VP1 also reacted with IgM and IgG2 antibodies.

Majority of VP1-specific antibodies are directed against an N-terminal 20mer peptide

The binding sites of VP1-specific antibodies were mapped using 3 recombinant fragments comprising aa 1–100 (P1), aa 101–200 (P2), and aa 201–293 (P3) of the VP1 protein (Fig. 2B). The major IgA and IgG₁ epitopes were located at the N-terminal fragment, P1. P1-specific IgG₁ and IgA levels were significantly higher than those against P2 and P3 (Fig. 2B). None of the 3 fragments showed relevant reactivity with IgM and IgG2 (Fig. 2B). MBP, which was used as a

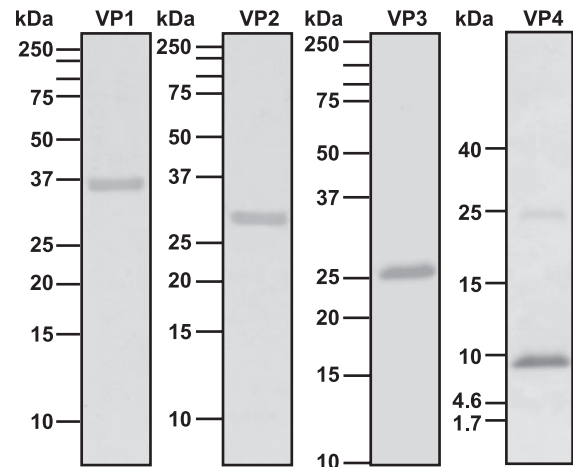


Figure 1. Coomassie-stained SDS-PAGE containing purified VP1–VP4 of HRV89. Molecular mass (kDa) is indicated at left.

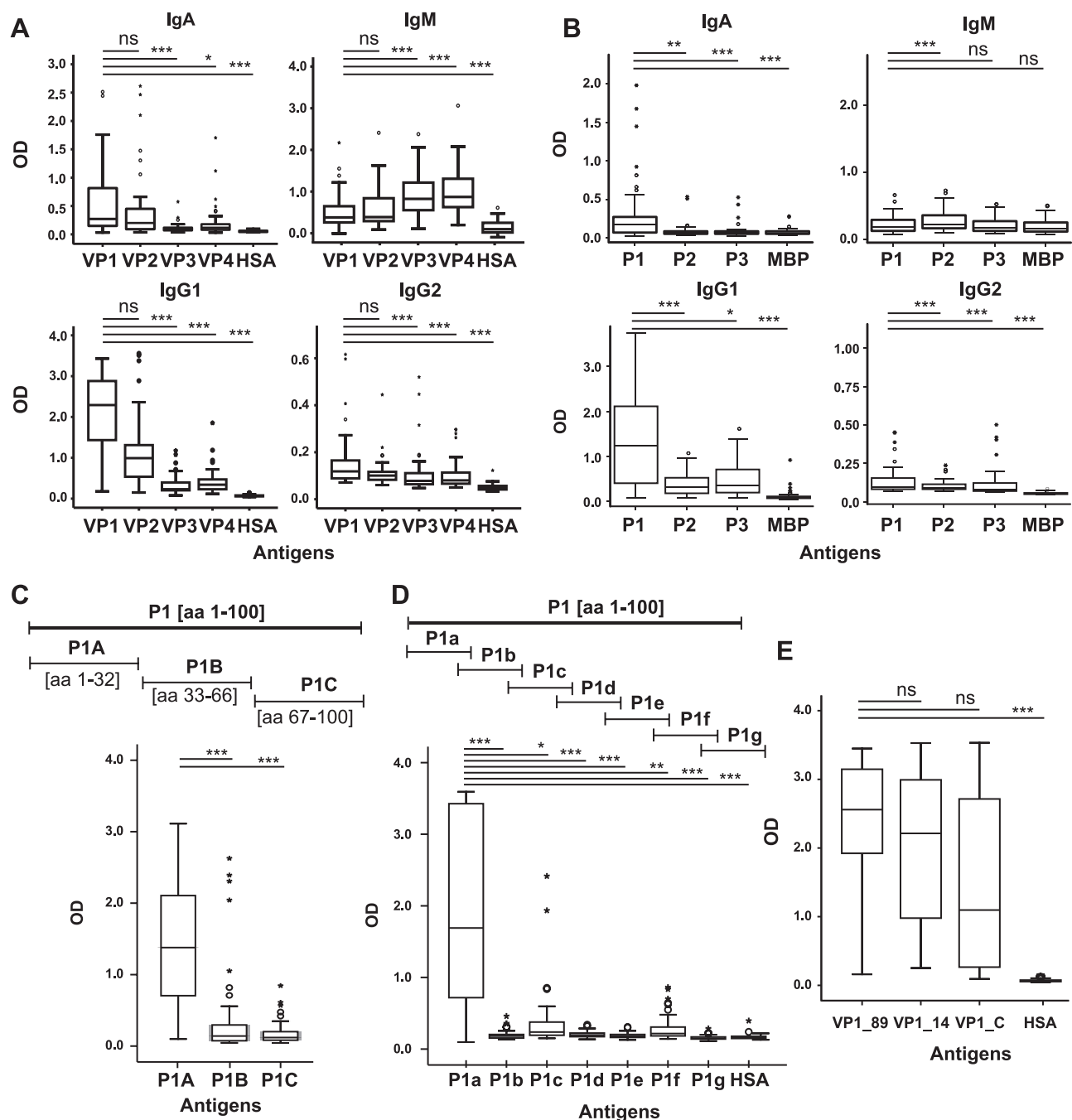


Figure 2. Human antibody responses to recombinant RV capsid proteins and recombinant VP1 fragments. *A, B*) VP1-, VP2- VP3-, VP4-, and HSA-specific antibody (*A*) and P1-, P2-, and P3-specific antibody (*B*) responses of blood samples from patients with positive RV-specific PCR results. *C*) Specificities of IgG₁ antibodies against synthetic P1 fractions P1A, P1B, and P1C. Location and length of the P1-derived synthetic peptides are also shown. *D*) High-resolution (20 aa) reactivities of IgG₁ antibodies against P1 synthetic peptides. *E*) IgG₁ reactivity to recombinant VP1 from RV89 (RV species A), RV14 (species B), and RV group C. Specific IgA, IgM, IgG₁, and IgG₂ levels determined by ELISA are expressed as optical density values (y axes: OD_{405 nm}). Results are displayed as box plots, where 50% of the values are within the boxes and nonoutliers between the bars. Lines within boxes indicate median values. All statistically significant and nonsignificant (ns) differences involving VP1 and its N-terminal regions (P1, P1A, or P1a) are indicated on the graphs. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

fusion partner for the recombinant fragments, also showed no relevant antibody reactivity (Fig. 2*B*). When synthetic VP1-derived peptides were used for mapping the fine specificities of antibody responses, peptide P1A, comprising aa 1–32, and peptide P1a, comprising aa 2–22, contained the major binding

sites for human IgG₁ antibodies (Fig. 2*C, D*). The other synthetic peptides and HSA showed no relevant antibody reactivities. Figure 2*E* shows that patient sera exhibited highest IgG₁ reactivities to VP1 from RV89 (RV species A), followed by RV14 (species B) and RV group C (median OD values of 2.56, 2.21,

and 1.096, respectively), although these differences were not statistically significant.

Paradox localization of the major epitope inside the viral capsid

To visualize the P1a-defined epitope on the 3-dimensional structure of human RV, the available structure of RV16, a closely related member of group A RV strains, was used. **Figure 3** shows a virus capsid in a surface representation when seen from the outer surface of the capsid along the 2-fold axis (left image, outside) and from the center onto the inner surface of the capsid in the same orientation (right image, inside). Portions of VP1, VP2, and VP3 are located at the outside, whereas VP4 is located completely in the inner part of the capsid (Fig. 3A). Surprisingly, the N-terminal epitope defined by P1a is located inside of the virus capsid, whereas the remaining amino acids of P1 (aa 34–100) are surface exposed (Fig. 3B).

P1a defines a non-neutralizing VP1 epitope

Figure 4 shows a multiple sequence alignment of the VP1 amino acid sequences of distantly related group A, B, and C strains. P1a, the most strongly antibody-reactive peptide, defines the N-terminal region of P1.

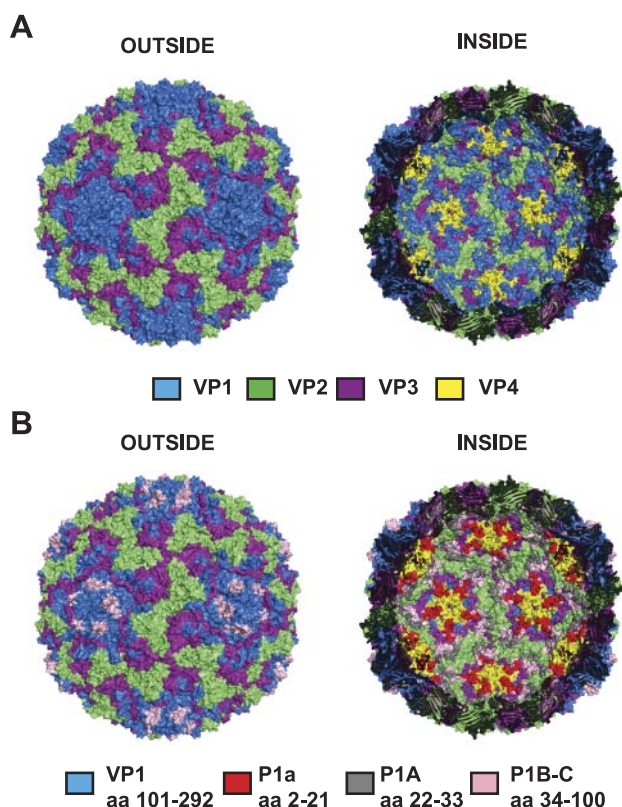


Figure 3. Localization of VP1-derived peptides in the context of the RV16-derived model of the 3-dimensional structure of the viral capsid. Outer (left image) and inner (right image) surface representation with VP1–VP4-derived (A) and VP1-derived peptides (B) highlighted in different colors.

Interestingly, P2, representing the middle 100-aa segment of VP1, and not P1, the N-terminal 100-aa fragment of VP1, shows the highest sequence identity among RV strains (65 and 52% overall sequence identity for P2 and P1, respectively). As far as P1 is concerned, P1B, representing aa 33–66 of the N-terminal 100 aa of VP1, and not P1A is the most conserved portion among RV A, B, and C strains, with an overall sequence identity of 68%. Amino acids involved in ICAM-1 binding and binding sites of previously described neutralizing antibodies are located outside the P1a-defined peptide (Fig. 4). **Table 3** shows the epitope specificity of neutralizing rabbit and guinea pig antibodies. These antibodies recognize P1a but also strongly react with the C-terminal portion of VP1, as defined by fragments P2 and P3, each containing neutralizing epitopes (20).

Seasonal variation of VP1-specific secondary IgG₁ and IgA responses in humans

RV infections typically occur in spring and autumn (13). We therefore investigated whether VP1-specific antibody responses vary over a period of 1 yr. **Figure 5** shows the median VP1-specific IgA and IgG₁ antibody responses in serum samples obtained from 25 RV-positive patients at 5 different time points across a 1-yr period (February 1997, April/May 1997, June/July 1997, September/October 1997, January 1998). Levels of both VP1-specific IgG₁ and IgA antibodies in patient sera obtained in winter 1998 were significantly higher ($P < 0.001$) than those in sera from winter and summer of the previous year, indicating a boosting of these antibody responses by RV infections.

DISCUSSION

In this study, recombinant major RV capsid proteins VP1–VP4 from RV89 as well as VP1 fragments and peptides thereof were used to investigate the natural antibody response in humans who have experienced recurrent RV infections. The RV-specific antibody responses belonged mainly to the IgG₁ subclass and IgA class and were primarily directed against 2 of the 4 RV coat proteins, that is, VP1, the protein interacting with its human receptor ICAM 1 and VP2.

Recombinant VP1 from RV89 seemed to be useful as a diagnostic reagent to detect changes in RV-specific antibody responses that correspond to reported peak periods of RV infections because it showed higher antibody reactivity than VP1 from RV14 and RV group C (13).

A fine mapping of the epitope specificity with recombinant VP1 fragments and synthetic VP1 peptides revealed that the majority of the VP1-specific antibody responses focused onto a N-terminal 20mer peptide of VP1. Unexpectedly, this peptide, designated P1a, is entirely located inside the intact viral

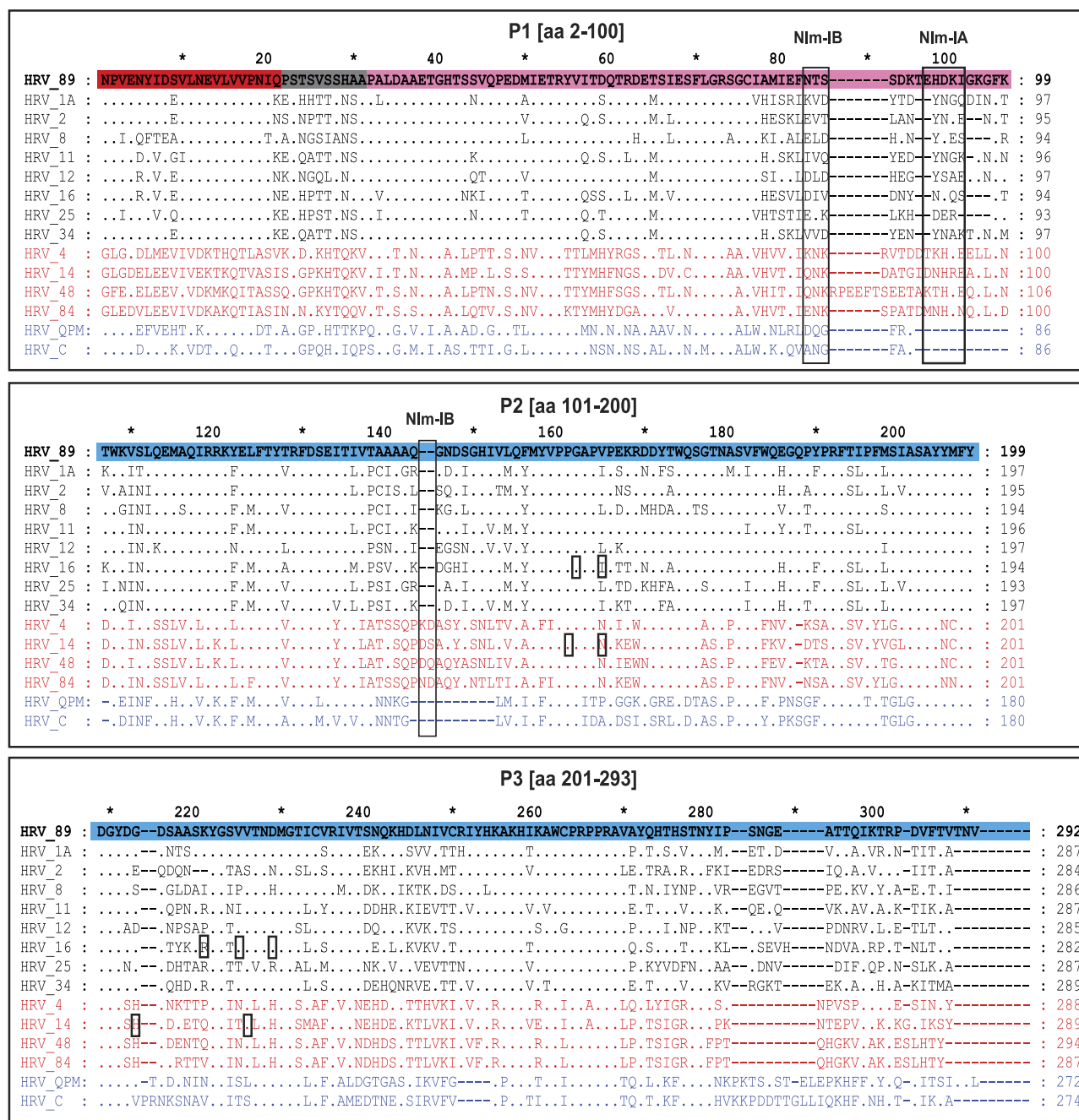


Figure 4. Multiple sequence alignment of VP1 proteins. Amino acid sequences of representative group A (black), B (red), and C (blue) RV strains (left margin) are aligned with VP1 from RV89 (group A RV, top line). Dots and dashes represent identical amino acids and gaps, respectively. P1 fragments and peptides are colored as in Fig. 3; binding sites for neutralizing antibodies (Nlm-IA, B) and amino acids involved in ICAM 1 binding are boxed.

capsid, as demonstrated by molecular modeling. For modeling, the 3-dimensional structure of the RV16 capsid was used. In fact, the sequence of RV89-derived VP1 studied by us shows a 66% sequence identity (78% sequence homology) with the VP1 from RV16, and both strains belong to group A RVs. A possible explanation for the highly selective recognition of the P1a, which is buried in the capsid of intact RV, is that antibody responses are generated to RV when it is bound to its cellular receptor, ICAM-1. During this process, RV undergoes uncoating, which

leads to externalization of the N terminus of VP1 (21). Lewis *et al.* (22) have observed that RV14 may also externalize the N terminus of VP1 in a spontaneous and reversible manner, called “breathing.” This process can be inhibited by antiviral compounds or antibodies raised against the neutralizing immunogenic site IA (Nlm-IA), which stabilize the virus and interfere with uncoating (23).

For poliovirus, it has been reported that antibodies raised against a synthetic peptide, spanning amino acid residues 24 to 40 of the VP1 protein, were able

to neutralize viral infectivity (24). However, no neutralization of infectivity was observed with antibodies specific for a peptide corresponding to the first 30 residues of VP1 from RV14 (25). The known binding sites for neutralizing antibodies (*i.e.*, Nlm-IB, Nlm-IA), as well as amino acids that are thought to be relevant for the ICAM-1 interaction (Fig. 4), do not involve the VP1 N terminus but are found in the VP1 portions with the highest sequence homology among RV strains. In accordance with the latter data, we found that neutralizing antibodies that prevent virus infection (rabbit anti-VP1 and guinea pig anti-RV antibodies) recognized VP1 fragments P2 and P3, representing the C-terminal 200 aa of VP1 and peptides located in P1 containing Nlm-IB and Nlm-IA binding sites.

Thus, it appears that the majority of the VP1-specific antibody response in humans who have experienced repeated RV infections is misdirected against a non-neutralizing epitope that becomes exposed during uncoating or breathing but is not accessible on the intact viral coat. In addition to strain variability, which may prevent protection against RV infections, we propose misdirection of antibody responses against RV as a potential novel mechanism of how RV may escape protective immunity in humans. Based on our results, it should be possible to engineer vaccines that allow the redirecting of antibody responses against neutralizing RV epitopes and to treat RV-related diseases, such as common cold and exacerbations of asthma and COPD.

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TABLE 3. Epitope specificity of RV-neutralizing antibodies

Peptide	Rabbit IgG, IS	Guinea pig IgG	
		NS	IS
VP1	2.29	0.60	3.27
P1	1.83	1.36	3.48
P2	1.52	ND	0.13
P3	2.17	ND	3.17
P1a	1.63	0.06	1.21
P1b	0.11	0.07	0.22
P1c	0.20	0.10	0.29
P1d	1.09	0.08	0.27
P1e	0.03	0.09	0.25
P1f	0.05	0.14	1.59
P1g	0.00	0.05	0.08

Data are optical density values (y axes: OD_{405 nm}) corresponding to antibody levels specific for VP1 and VP1 fragments and peptides for mouse IgG₁ and rabbit and guinea pig IgG. NS, normal serum from guinea pigs; IS, immune serum from guinea pigs; ND, not detected.

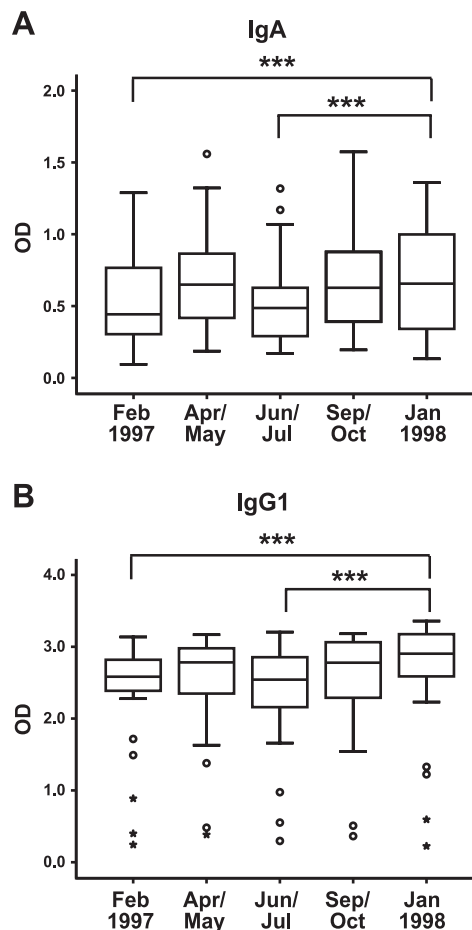


Figure 5. Seasonal fluctuations of VP1-specific IgA (A) and IgG₁ (B) antibody responses across 5 seasons (February 1997–January 1998). Displayed are optical density values (y axes: OD_{405 nm}) corresponding to VP1-specific specific IgA and IgG₁ antibody reactivity in sera from 25 RV-positive individuals. Results are shown in box plots, where 50% of the values are within boxes and nonoutliers between bars. Line within boxes indicates median values. ****P* < 0.001.

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