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Antibodies induced with recombinant VP1 from Human Rhinovirus exhibit cross-neutralization

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Human rhinoviruses (HRVs) are the major cause of the common cold and account for 30-50% of all acute respiratory illnesses. Although HRV infections are usually harmless and

invade only the upper respiratory tract, several studies demonstrate that HRV is involved in the exacerbation of asthma. VP1 is one of the surface-exposed proteins of the viral capsid that is important for the binding of rhinoviruses to the corresponding receptors on human cells. Here we investigated its potential usefulness for vaccination against the common cold. We expressed in *Escherichia coli* VP1 proteins from two distantly related HRV strains, HRV89 and HRV14. Mice and rabbits were immunized with the purified recombinant proteins. The induced antibodies reacted with natural VP1 and with whole virus particles as shown by immunoblotting and immunogold electron microscopy. They exhibited strong cross-neutralizing activity for different HRV strains. Therefore, recombinant VP1 may be considered a candidate HRV vaccine to prevent HRV-induced asthma exacerbations.

Human rhinoviruses (HRVs), generally known as the primary cause of acute upper respiratory infections, have recently emerged as important triggers for exacerbations of asthma, chronic pulmonary disease (COPD) and cystic fibrosis [1-5]. Furthermore, the pathogenic role of HRVs in lower respiratory tract infections of infants, elderly and immunocompromised patients has been reported [6-8].

HRVs are non-enveloped, single-stranded RNA viruses that belong to the *Picornaviridae* family [9-10]. Currently, more than 100 distinct serotypes are primarily assigned to two genetic species, HRV-A and HRV-B, with >70% amino acid identity and a similar antiviral susceptibility pattern within each species [11]. Recently, a third clade, HRV-C, including a number of previously unrecognized non-cultivable HRVs has been identified [12-13]. In addition, the serotypes have also been classified into major and minor groups according to the receptor they use to enter epithelial cells of the respiratory tract. Members of the major receptor group bind to the intracellular adhesion molecule-1 (ICAM-1) and the representatives of the minor receptor group use members of the low-density lipoprotein receptor (LDL-R) family [14-15]. The capsid of HRVs is composed of 60 copies of each of the four capsid proteins: VP1, VP2, VP3, and VP4 [16-17]. Three of these proteins are located on the surface of the capsid and are responsible for the antigenic diversity of HRV, whereas the fourth, VP4, is located inside the virion [17-18]. Of the four viral capsid proteins, VP1 is the most exposed and immuno-dominant surface protein. It is critically involved in the infection of respiratory cells and is predominantly recognized by HRV neutralizing antibodies [19-23].

The ability to identify rhinoviruses using highly sensitive and specific PCR-based technology has dramatically increased the frequency of HRV detection and has provided the basis for several studies indicating the involvement of HRVs in triggering asthma [1-2, 24-27]. HRVs have been found to be the most frequent pathogen present in 50% to 80% of asthma exacerbations in both children and adults with peak levels in spring and autumn when rhinovirus infections usually occur [24-25]. Furthermore, rhinovirus infections during the infancy are the most significant risk factors for the subsequent development of asthma [26]. Finally, rhinoviruses may also contribute to the persistence and severity of the disease when they are present in the lower airway [2, 28].

Several avenues have been followed towards the development of preventive and therapeutic strategies against rhinovirus infections [18, 29-32]. However, due to the occurrence of more than 100 HRV serotypes with high sequence variability in the antigenic sites [10], no rhinovirus vaccine and no HRV-specific drug has become available.

In this study we have expressed, in *E. coli,* VP1 proteins from two very distantly related HRV strains, HRV89 and HRV14, belonging to genus A and B, respectively, and studied whether their recombinant VP1 proteins can be used to induce cross-protective antibody responses in mice and rabbits.

MATERIALS AND METHODS

Construction of expression vectors harboring the VP1 cDNA of HRV14 or HRV89

The plasmid containing the whole genome of HRV14 [33] was used as a template for the amplification of 14VP1 (VP1 of HRV14) by PCR. The following primers were used:

5' CGGAATTCCCATGGGCTTAGGTGATGAATTAGAAGAAGTCATCGTTGAGA 3'

5' GATGGAATTCTCAGTGGTGGTGGTGGTGATAGGATTTAATGTCAC 3'

The restriction sites (NcoI, EcoRI) are underlined. The cDNA coding for the 14VP1 region (data base # AY355195) was inserted into the NcoI and EcoRI sites of plasmid pET23d (Novagen, Merck Bioscience, Darmstadt, Germany). Virus stocks of HRV89 were obtained from the collection of the Institute of Virology, Medical University of Vienna. Viral RNA was prepared from cell culture supernatants using the QIAamp viral RNA kit (Qiagen, Hilden, Germany) and RNase inhibitor (Boehringer GmbH, Germany) was added to a final concentration of 0.01 U/µl. The 89VP1 cDNA (VP1 of HRV89) was amplified by RT-PCR using the SuperScript One Step RT PCR Kit from Invitrogen (Carlsbad, California) using the following primers:

5’ CGGAATTCATTAATATGAACCCAGTTGAAAATTATATAGATAGTGTATTA 3’

5’ CGATTAATTCAGTGGTGGTGGTGGTGGTGGACGTTTGTAACGGTAA 3’

The restriction sites (EcoRI, AseI) are underlined. The cDNA encoding the complete 89VP1 region (data base # AY355270) was subcloned into the NdeI and EcoRI site of a pET17b (Novagen, Merck Bioscience, Darmstadt, Germany).

Expression and purification of recombinant 89VP1 and 14VP1

Recombinant 89VP1 and 14VP1 were expressed in *E. coli* BL21(DE3) (Stratagene, La Jolla, CA). Protein synthesis was induced for 5 hours at 37°C with 1 mM IPTG and the recombinant proteins were purified from the inclusion body fraction after solubilization in 6 M guanidinium hydrochloride, 100 mM NaH_2PO_4, 10 mM Tris (pH 8) using a Ni-NTA affinity matrix (Qiagen, Hilden, Germany). The proteins were adsorbed, the column washed with washing buffer (8M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl (pH 5.9)) and the proteins were eluted with the same buffer adjusted with HCl to pH 3.5. Protein preparations were dialyzed against buffers with decreasing urea concentration and finally against H_2O_{dd}. Protein purity and concentration were checked by SDS-PAGE followed by Coomassie blue staining [34].

Recombinant 89VP1 fragments comprising aa 1-100, aa 101-200 and aa 201-293 were expressed as fusions to maltose binding protein using plasmid pMalC4X in *E. coli* BL21 (DE3) and purified by amylose affinity chromatography (New England Biolabs, Ipswich, MA, USA).

Synthetic peptides and peptide conjugates

HRV14-derived peptides (PVP1A, PVP1B and PVP3A) were synthesized on the Applied Biosystems (Foster City, CA) peptide synthesizer Model 433A using Fmoc (9-fluorenyl-methoxy-carbonyl) strategy with HBTU [2-(1H-Benzotriazol-1-yl)1,1,3,3 tetramethyluronium

hexafluorophosphat] activation. The following peptides were purified to >90% purity by preparative HPLC (piChem, Graz, Austria) and their identity was verified by mass spectrometry:

PVP1A: VVQAMYVPPGAPNPKEC amino acids 147-162 of VP1 [18];

PVP1B: CRAPRALPYTSIGRTNYPKNTEPVIKKRKGDIKSY amino acids 256-289 of VP1 (i.e. C-terminal residues);

PVP3A: KLILAYTPPGARGPQDC amino acids 126-141 of VP3 [18].

Purified peptides were coupled to KLH using the Imject Maleimide Activated Immunogen Conjugation Kit (Pierce, Rockfort, IL) according to the manufacturers' instruction.

Immunization of mice and rabbits

Rabbit antibodies against 14VP1, 89VP1, PVP1A, PVP1B and PVP3A, respectively, were obtained by immunizing rabbits (Charles River, Kisslegg, Germany). Groups of five mice were also immunized subcutaneously with 5 μg of 89VP1 adsorbed to Alum in three-week intervals and bled from the tail veins [35]. Animals were maintained in the animal care unit of the Dept. of Pathophysiology, Medical University of Vienna, according to the local guidelines for animal care [35].

ELISA experiments

ELISA plates were coated with 5 μg/ml 89VP1, 14VP1 and recombinant fragments of 89VP1, respectively [36]. The mouse sera were diluted 1:500 and the rabbit sera $1:10^3$-$1:10^6$. Antigen-specific IgG_1 mouse antibodies were detected with 1:1000 diluted alkaline phosphatase-coupled mouse monoclonal anti-mouse IgG_1 antibodies (Pharmingen). The reactivity of the rabbit anti-VP1 and rabbit anti-peptide anti-sera with the VP1 proteins was tested on one ELISA plate to allow a comparison of the titers. Antigen-specific rabbit IgG antibodies were developed with a

1:2000 dilution of donkey anti-rabbit IgG peroxidase-coupled antibodies (Amersham Bioscience). Binding was quantified at 405 nm and 490 nm for rabbit antibodies and at 405 nm and 450 nm for mice antibodies in an ELISA reader (Dynatech, Denkendorf, Germany) [35, 37].

Reactivity of anti-VP1 antibodies in Western blots

Cell culture supernatants from HRV14 infected HeLa cells were centrifuged in a bench fuge (15 000 rpm, 10 min, 20°C) to remove cell debris. A solution (0.5 ml) of 40% v/v polyethylene glycol 6000, 2.4% w/v NaCl) was added to 2 ml of the virus-containing supernatant, incubated at 4°C over night and centrifuged at 2,300 x rpm for 45 minutes in a bench fuge at RT. The pellet was re-suspended in 100 µl PBS and solubilized by adding 50 µl SDS sample buffer. Ten µl of this HRV14 protein extract and 0.5 µg purified 14VP1 were separated in parallel by 12% SDS PAGE and blotted onto nitrocellulose membranes [34, 38]. Identically prepared blots were incubated with 1:500 dilutions of rabbit anti-14VP1 antibodies or the corresponding pre-immune Ig. Bound antibodies were detected with ^{125}I-labeled donkey anti-rabbit IgG and visualized by autoradiography [35, 37].

For immunogold electron microscopy, 4.2 µl aliquots of the re-suspended viral precipitate were pipetted onto carbon-coated, plasma-cleaned copper grids and air-dried. After 5 minutes, remaining liquid was removed with a piece of filter paper. The grids were then incubated face down (moist chamber at room temperature) in the following buffers: First, PBS containing 1 % (w/v) BSA at pH 7.4 and then 50 mM Tris buffer containing 1% (w/v) BSA at pH 8.2. Followed by: (a) 5 % (w/v) BSA, 5 min; (b) protein G-purified anti-VP1 Ig or pre-immune Ig adjusted to A_{280} = 0.6, 45 min; (c) 6 x PBS buffer, 5 seconds each; (d) 6 x 50 mM Tris buffer, 5 seconds each; (e) goat anti-rabbit Ig coupled to colloidal gold particles with a diameter of 10 nm (Plano, Wetzlar, Germany), diluted 1:20 in 50 mM Tris buffer, 30 min; (f) 6 x 50 mM Tris buffer, 5

seconds each; (g) 6 x distilled water, 5 seconds each. The immunogold labeling was followed by negative staining with a saturated solution of uranyl acetate. After 1 minute, surplus negative stain was removed with a wet filter paper. The grids were then air-dried and viewed in a Philips EM 410 transmission electron microscope (FEI, Eindhoven, The Netherlands) equipped with a high resolution CCD camera (Toshiba Teli Corporation, Tokyo, Japan). Micrographs were taken at a magnification of 165,000 x or 240,000 x.

HRV neutralization test

Rhinovirus stocks and the HRV-sensitive "Ohio" strain of HeLa cells [39] were kindly provided from the Institute of Virology, Medical University of Vienna. HeLa cells were seeded in 24 well plates and grown to approximately 90% confluence as described [18]. In a first set of experiments, 300 µl aliquots of HRV14 (100 $TCID_{50}$) in medium were incubated for 2 h at 37°C with 300 µl of rabbit anti-sera (anti-14VP1, anti-PVP1A, anti-PVP1B or PVP3A) or the corresponding pre-immune sera (undiluted or diluted 1:2-1.32) and added to the cells. MEM-Eagle medium (Invitrogen, Carlsbad, California) containing 1% FCS and 40 mM $MgCl_2$ was used as a diluent in the experiments. Plates were incubated at 34°C in a humidified 5% CO_2 atmosphere and viable cells were stained with crystal violet after three days [18]. Cross-neutralization tests were carried out in 96 well plates; HeLa cells were seeded in minimal essential medium (MEM) containing 2% fetal calf serum, 30 mM $MgCl_2$, and 1 mM glutamine (infection medium) and grown over night at 37°C to about 70% confluency. HRVs (100 $TCID_{50}$ in 100 µl infection medium) were mixed with 100 µl of the respective undiluted antiserum and serial twofold dilutions thereof in the same medium. After incubation for 3 h at 37°C, the cells were overlaid with these solutions and incubation was continued at 34°C for 3 days. The medium was removed and cells were stained with crystal violet (0.1% in water) for 10 min. After washing

with water, the plate was dried, stained cells were dissolved in 30 µl 1% SDS under shaking for 1 hour and cell protection was quantified as A_{560} in a plate reader. The identity of all HRVs used was repeatedly confirmed by neutralization with the respective type-specific quinea pig antisera from ATCC (Manassas, VA).

RESULTS

Expression and purification of recombinant VP1 proteins from HRV89 and HRV14

Recombinant VP1 of HRV89 (89VP1; Fig.1A) and HRV14 (14VP1; Fig.1B) were expressed in *E. coli* with a His_6-tag at their C-termini and purified from solubilized inclusion bodies by single step Nickel affinity chromatography. SDS-PAGE followed by Coomassie blue staining revealed the purified protein bands at approximately 34 kDa. Both, the recombinant proteins 89VP1 and 14VP1 reacted specifically with the anti-His-tag antibody due to their C-terminal hexa-histidine tag (right lanes; Fig. 1).

89VP1 and 14VP1 induce a VP1-specific immune response in animals

Immunization of rabbits with recombinant 89VP1 and 14VP1 induced VP1-specific IgG responses (Fig. 2A). The immune response to 89VP1 was stronger than that to 14VP1 with a strong signal detected up to a serum dilution of 10^{-5} after the second and up to a dilution of 10^{-6} after the third immunization The 14VP1-specific IgG response was detectable up to a serum dilution of 10^{-3} after the second and up to a 10^{-4} dilution after the third immunization (Fig. 2A). VP1-specific antibody responses were obtained also in mice immunized with Alum-adsorbed VP1 proteins. IgG_1 antibodies specific for 89VP1 were detected already after the first immunization and continued to increase after the second and third immunization (Fig. 2B).

Reactivity of antibodies raised against recombinant VP1 proteins toward virus-derived VP1 and entire virions

The reactivity of antibodies induced by immunization with recombinant VP1 proteins with natural, virus-derived VP1 and whole virus was studied by immunoblotting and electron microscopy, respectively. As a representative example, binding of rabbit anti-14VP1 antibodies and lack of binding of pre-immune Ig to nitrocellulose-blotted HRV14 proteins and 14VP1 is shown Fig. 3A. Antibodies raised against recombinant 14VP1, but not the pre-immune Ig, reacted with natural and recombinant 14VP1 (band at approximately 34 kDa) (Fig. 3A). Specific binding of anti-89VP1 antibodies to HRV89 was visualized using the immunogold electron microscopy method. When immobilized virions were exposed to anti-89VP1 antibodies and gold-conjugated secondary antibodies approximately 10% of the virus particles appeared coated with one up to five colloidal gold particles (Fig. 3B). No viral particles were stained in the control preparations with the pre-immune Ig; few gold particles were present but not associated with virus particles (Fig. 3B; right panel).

Immunization of rabbits with recombinant 14VP1 yields higher 14VP1- and 89VP1-specific antibody titers than immunization with KLH-coupled HRV14-derived peptides

Antisera were raised against KLH-coupled peptides which have been earlier described as possible vaccine candidates [18]. The anti-peptide antisera contained high titers of peptide-specific antibodies (PVP1A:10^{-3}; PVP1B:10^{-5}; PVP3A:10^{-5}; data not shown). However, in comparison with antisera raised against recombinant 14VP1, they reacted only weakly with the 14VP1 protein and showed weak cross-reactivity with 89VP1 (Fig. 4). Most remarkably, antiserum obtained upon immunization with recombinant 14VP1 showed a comparable reactivity with

14VP1 and 89VP1. This antiserum reacted with VP1 of both viral serotypes at least tenfold more strongly than the peptide antisera (Fig. 4).

14VP1-specific antibodies inhibit HRV infection of HeLa cells better than peptide-specific antibodies

Next, we were investigated whether rabbit IgG antibodies raised against the two recombinant proteins (14VP1 and 89VP1) inhibit HRV infection of HeLa cells. As seen in Fig. 5, HRV89 infectivity was strongly neutralized by both anti-14VP1 and anti-89VP1 rabbit IgG antibodies in a dose-dependent manner up to a 1:32 dilution. However, the antiserum against VP1 of HRV14 ony weakly protected against infection by HRV14. Antibodies from non-immunized rabbits failed to protect (compare with non-infected cells used as a control). We also compared the ability of antibodies raised against complete 14VP1 with antibodies raised against 14VP1-derived peptides for protection of the cells against viral infection. Serial dilutions (undiluted or diluted 1:2-1:32) of anti-14VP1, -PVP1A, -PVP1B and -PVP3A antisera, respectively, were pre-incubated with HRV14 and these mixtures were added to HeLa cells grown in 96-well plates. All three anti-peptide anti-sera were comparable with respect to their ability to inhibit infection. A clear reduction of CPE was seen at a dilution of 1:8 with anti-PVP1A and anti-PVP1B and at a dilution of 1:4 with anti-PVP3A. A similar degree of cell protection (i.e., only partial CPE) was obtained with the anti-14VP1 antiserum up to dilution of 1:32. This suggests that the latter antiserum was more potent in inhibiting viral infection (Table I).

Antibodies raised against recombinant VP1 proteins show cross-protection against distantly related HRV strains.

Figure 6A shows the evolutionary relationship of the rhinovirus types used for the cross-protection experiments. They were selected to belong to different species and different receptor groups: HRV37 and 89 are major group genus A, HRV3, 14 and 72 are major group genus B, HRV1A, 18 and 54 are K-types (i.e., major group HRVs possessing a lysine in the HI loop of VP1) [40] and HRV1A, HRV29 and 44 are minor group genus A. Both, the anti-89VP1 and anti-14VP1 antibodies inhibited infection of HeLa cells by half of these HRV serotypes in a concentration-dependent manner independent of their evolutionary relationship (Figure 6B). Interestingly, anti-14VP1 antibodies inhibited HRV89 infection more strongly than anti-89VP1 antibodies whereas anti-89VP1 antibodies only weakly inhibited infection by HRV14 (Figure 6B). Remarkably, both antisera neutralized HRV44 more strongly than all other strains and showed extensive cross-reactivity with weakly related HRVs (compare to Fig. 6A). We tentatively explain this latter fact by VP1 from the different HRV strains possessing highly conserved sequence stretches that are most probably recognized by the anti-sera. In this context, it is noteworthy, that the anti-VP1 anti-sera obtained by immunization with VP1 from HRV14 and HRV89 differentially reacted with recombinant fragments of 89VP1 spanning aa1-100, aa 101-200 and aa 201-293 (Fig. 7). It thus appears that this latter anti-serum contains IgGs reacting with many more epitopes than the anti-serum raised against 14VP1.

DISCUSSION

A vaccine protecting against rhinovirus infections may be useful to prevent, or at least ameliorate, rhinovirus-induced exacerbations of asthma and COPD. We have selected the HRV-derived VP1 capsid protein as a potential vaccine antigen for several reasons. The work of Rossmann *et al.*, elucidating the crystal structure of HRV14 demonstrates that VP1 is critically involved in HRV binding to its receptor on human epithelial cells [17]. It was found that five

copies of VP1 form a depression, called canyon and that the ICAM-1 receptor binds into the central part of this canyon [17, 41]. Furthermore, studies of spontaneous mutations in the viral coat led to the identification of four neutralizing immunogenic (NIm) sites on the surface of HRV14. Additional investigations revealed that antibodies to two of the four antigenic sites which are located on the VP1 protein blocked cellular attachment [42-43]. McCray & Werner showed that purified antibodies raised against peptides derived from the HRV14 capsid proteins VP1 and VP3 not only inhibited infection in tissue culture, but also cross-protected against about 60% of 48 rhinovirus strains tested whereas almost no other serotypes were neutralized by a whole anti-HRV14 antiserum [18]. A more recent work by Katpally *et al.* demonstrates that antibodies against synthetic peptides corresponding to the N terminus of VP4 and VP1 from HRV14 are capable of neutralizing viral infectivity [44]. However, viral neutralization using anti-VP4 antiserum was assessed only for two other serotypes, HRV16 and HRV29, and no significant neutralization was found for the VP1 antiserum [44].

In the present study, we expressed the entire VP1 proteins from HRV89 and HRV14, which belong to the phylogenetically distant species HRV-A and HRV-B, respectively. Using the ClustalW program for alignment (http://www.ebi.ac.uk/clustalw) only a 41% amino acid identity is found between 89VP1 and 14VP1. Recombinant 14VP1 and 89VP1 were purified via a C-terminal His-tag by Nickel affinity chromatography in a single step procedure. Immunization of mice and rabbits with recombinant 14VP1 as well as 89VP1 proteins led to the development of VP1-specific antibody responses recognizing natural VP1 from the virus and even intact virus as demonstrated by immunogold electron microscopy.

The antibody responses obtained with the VP1 proteins were compared with those induced by HRV14 VP1- and VP3-derived peptides which had been earlier described as vaccine candidates [18] and with those obtained with a peptide (PVP1B) located at the C-terminus of the VP1

protein, being part of the ICAM-1 attachment site in HRV14 [45]. We found that the anti-HRV14 VP1 antisera reacted more strongly with VP1 than the anti-peptide antisera and exhibited a higher neutralization titer. The higher neutralization capacity and the remarkably broad cross-neutralizing effects of the antibodies raised against the entire proteins is most likely due to the recognition of several epitopes on VP1 leading to a higher avidity as compared to that of the peptide-specific antibodies. In summary, our results strongly suggest that vaccines based on complete VP1 proteins induce a polyclonal immune response against several segments of the protein. This increases the probability of cross-reaction. This is without doubt advantageous over the previously suggested peptide vaccines that necessarily must be less cross-protecting because of containing a limited repertoire of epitopes.

We have shown that antibodies raised against the recombinant VP1 proteins from both strains, HRV14 and HRV89, inhibited the infection of cultured HeLa cells by a variety of different rhinovirus strains belonging to the major and minor group. It is particularly notable that both anti-sera were more efficacious in neutralizing HRV44 than the cognate serotype corresponding to the immunizing antigen. This is corroborated by stronger binding of 89VP1 antibodies to VP1 of HRV44 as compared to VP1 of HRV2, 14 and 89 in Western blots (data not shown). Similar results were also obtained by McCray & Werner who found that anti-HRV14 peptide anti-sera neutralized other strains better than HRV14. Along the same lines, Katpally *et al.* also reported that anti-VP4 antibodies against VP4-derived peptides neutralize HRV16 much better than HRV14. These findings suggest either that a varying exposure of capsid proteins or protein regions in different strains may influence neutralization or that different epitopes are important for the neutralization of different HRVs [46]. Most likely, the cross-neutralization capacity of a rhinovirus vaccine might be increased by identifying and combining such epitopes, following the rationale of our work. The absence of a suitable animal model makes *in vivo* testing

of candidate vaccines for protection against rhinovirus infections difficult. In a pilot study, one person was vaccinated with recombinant 89VP1 and shown to produce VP1-specific IgG and IgA responses (data not shown).

Major advantages of a vaccine based on recombinant rhinovirus capsid proteins are that the vaccine antigens can be easily produced under controlled conditions by large scale recombinant expression in foreign hosts, such as *E. coli* at reasonable costs. A broadly cross-protective HRV vaccine may be especially useful for the vaccination of patients suffering from rhinovirus-induced respiratory diseases and may thus reduce asthma and COPD exacerbations.

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Figure legends

FIG. 1. Purification of recombinant VP1 proteins. (A) 89VP1 and (B) 14VP1 were stained with Coomassie blue after SDS-PAGE (left) and with an anti-His_6 antibody (right) after blotting on nitrocellulose. Positions of molecular weight markers run in parallel are indicated at the left.

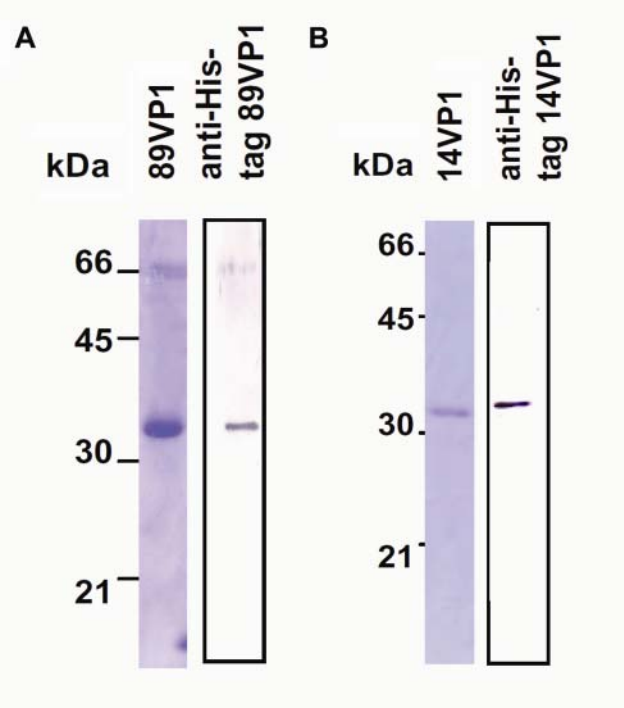


FIG. 2. VP1-specific immune responses of immunized rabbits and mice. (A) 89VP1- and 14VP1-specific IgG responses in rabbits. Rabbits were immunized with 89VP1 or 14VP1. Serum samples were taken on the day of the first immunization (pre-immune serum) and after the

second and third injection in 3-4 weeks intervals (top of the box: Immune serum 1; Immune serum 2). Dilutions of the sera (rabbitα89VP1; rabbitα14VP1) are presented on the x-axes (10^{-3}-10^{-6} indicated as log). IgG reactivities toward the immunogens (89VP1, 14VP1) were determined by ELISA and are depicted as bars. (B) A group of five mice was immunized with 89VP1. Serum samples were taken on the day of the first immunization (0) and in three weeks intervals (w3-w9) (x-axis). IgG_1 reactivities are displayed for the group as box plot where 50% of the values are within the boxes and non-outliers between the bars. Lines within the boxes indicate the median values. IgG_1 levels specific for 89VP1 were determined by ELISA as above.

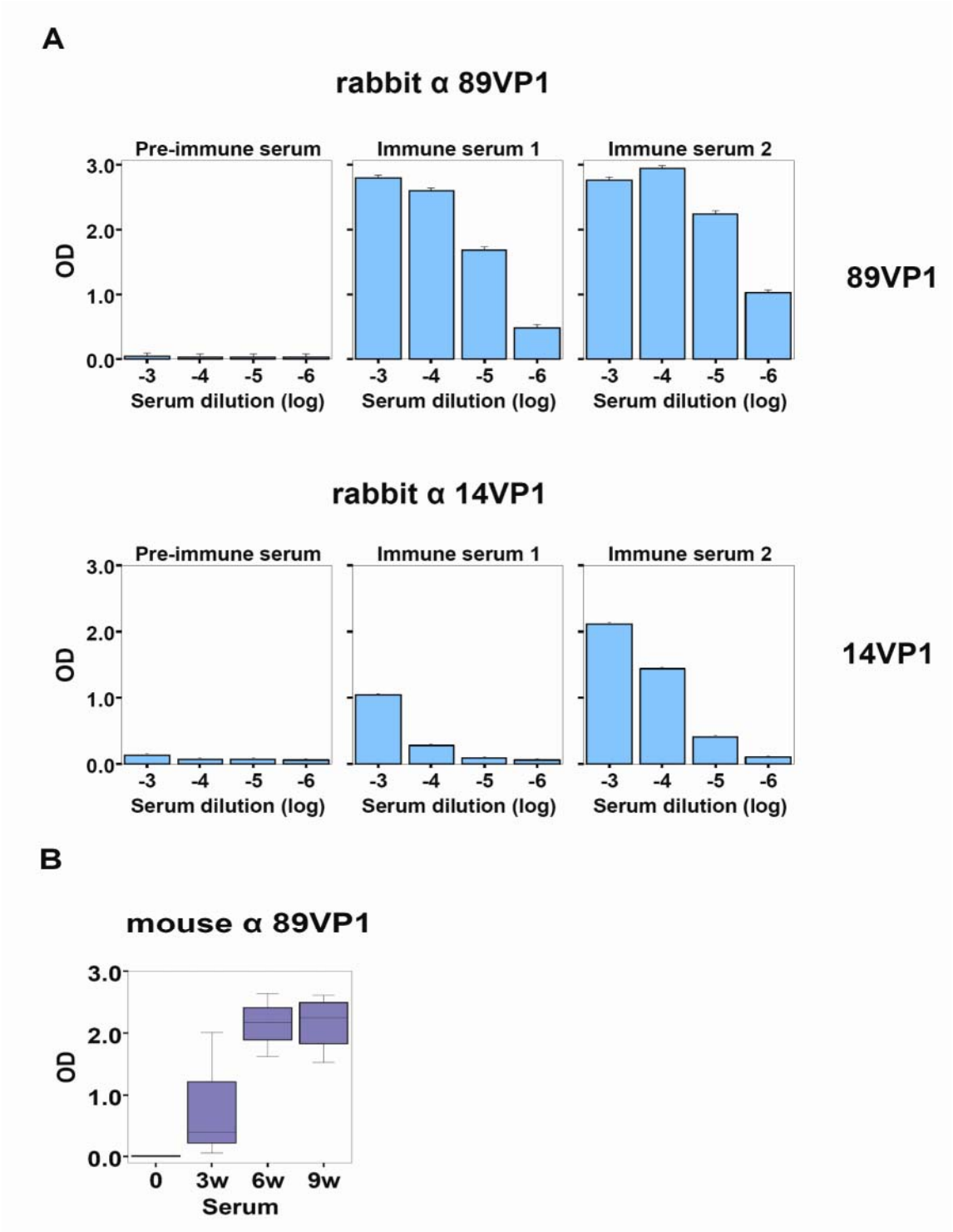


FIG. 3. Anti-VP1 antibody raised against recombinant VP1 react with rhinovirus-derived VP1 and whole virus. (A) Nitrocellulose-blotted HRV14 protein extract and recombinant 14VP1 were

incubated with anti-14VP1 antibodies and the corresponding pre-immune serum (pre-IS). Molecular weights in kDa are indicated at the left. (B) Electron micrographs of labelled virus preparations after negative staining. Immobilized HRV89 was incubated with anti-89VP1 IgG antibodies and the binding sites were visualized by a secondary IgG antibody probe coupled to colloidal gold particles with a diameter of 10 nm. The left micrograph gives a detail from a virus particle (VP) connected with four gold particles (GP). The right micrograph shows the control preparation using the pre-immune Ig. Bars: left micrograph, 50 nm; right micrograph, 100 nm.

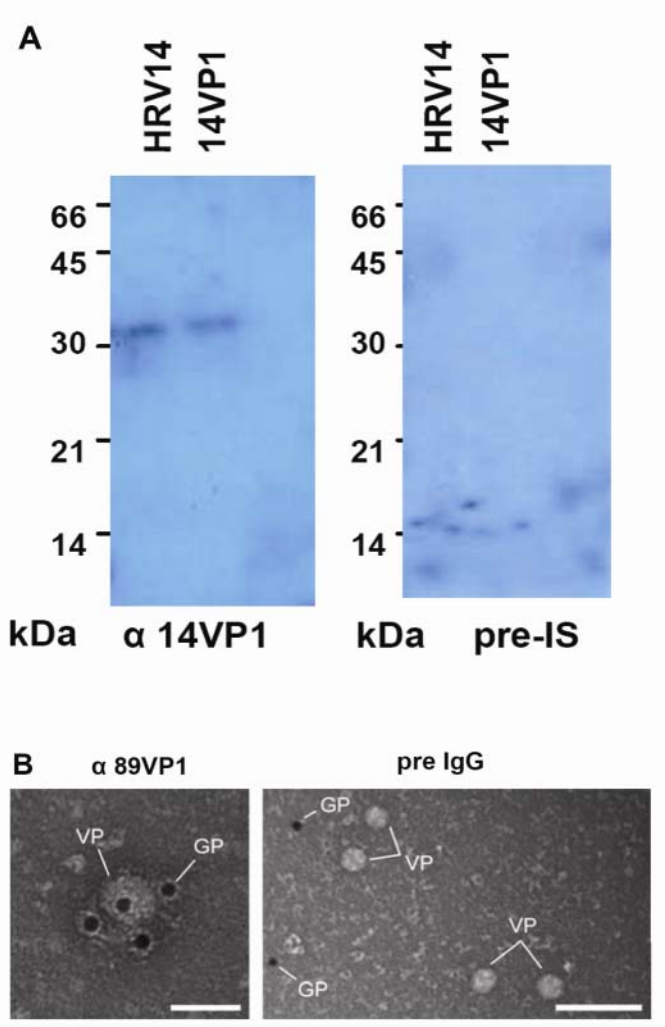


FIG. 4. Reactivity of rabbit antisera raised against recombinant 14VP1 protein or 14VP1-derived peptides. Rabbits were immunized with recombinant 14VP1, PVP1A, PVP1B or PVP3A (top of

the box) and sera exposed to 14VP1 (top) or 89VP1 (bottom). The dilutions of the sera are displayed on the x-axe (10^{-3}-10^{-6} indicated as log). IgG levels specific for 14VP1 and 89VP1 correspond to the optical density values (bars: y-axe).

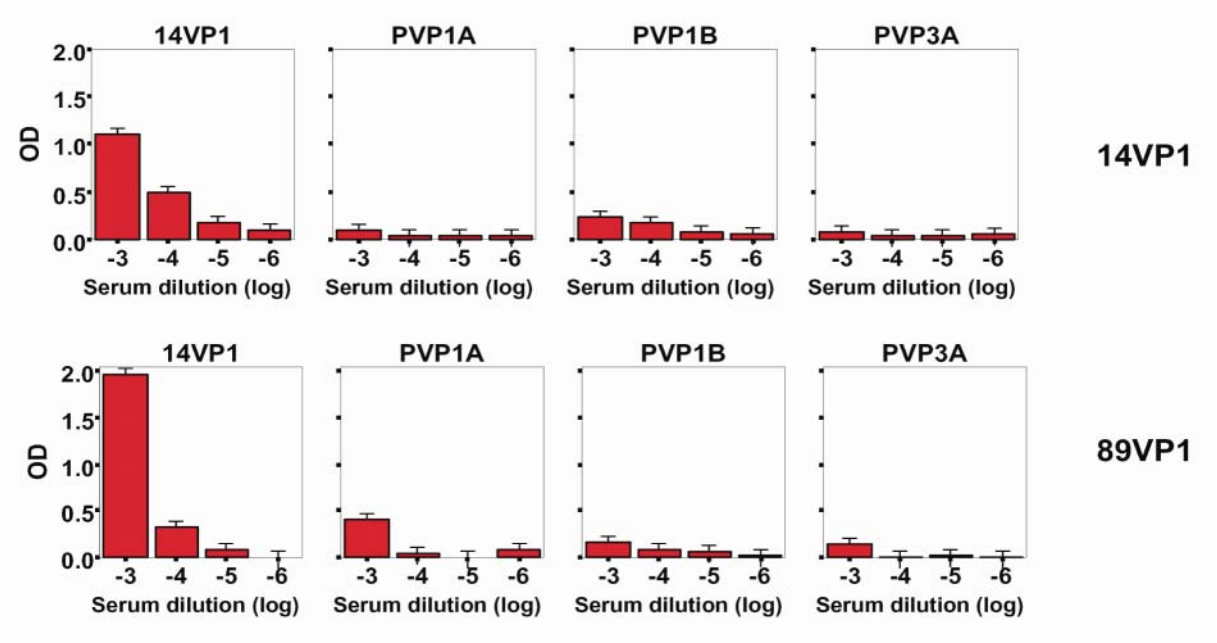


FIG. 5. Neutralization of HRV14 and HRV89 by anti-14VP1 and anti-89VP1 antibodies. HRV14 and HRV89 at 100 $TCID_{50}$ were preincubated with serial two-fold dilutions of the anti-sera as indicated (1:4, 1:8, 1:16, 1:32) for 2 h at 37°C and the mixture was added to subconfluent HeLa cells in 24 well plates. After 3 days at 34°C remaining cells were stained with crystal violet, washed, the stain was dissolved, and A_{560} was determined. Normal rabbit serum (nrs), type-specific quinea pig anti-sera from ATCC (gp) and non-infected cells were used as controls.

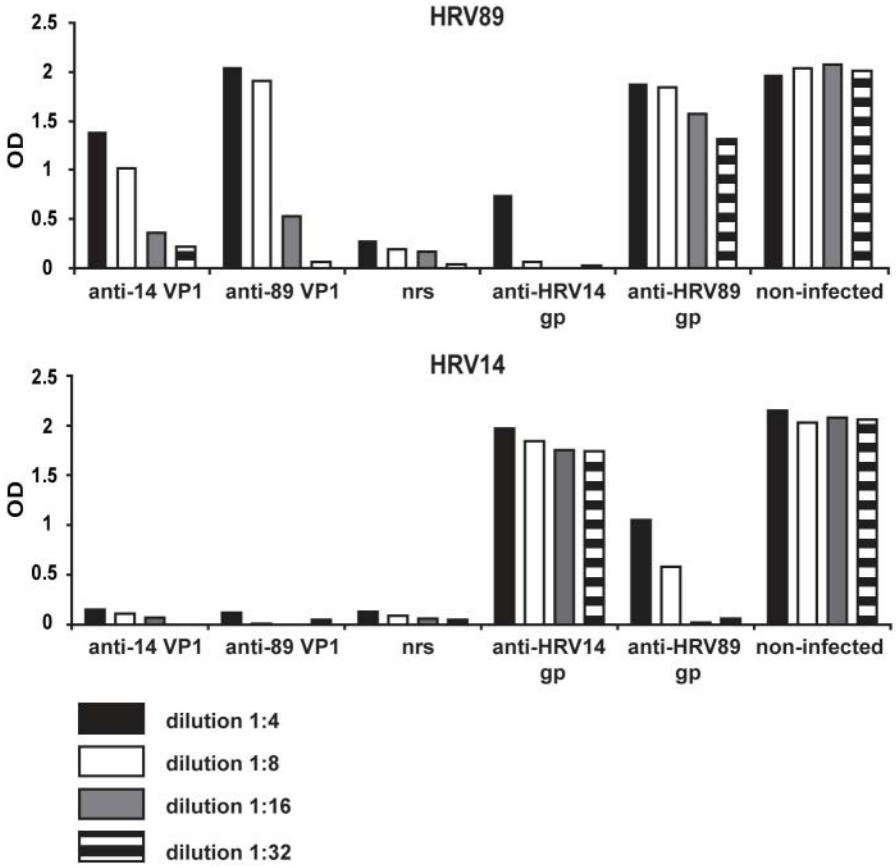
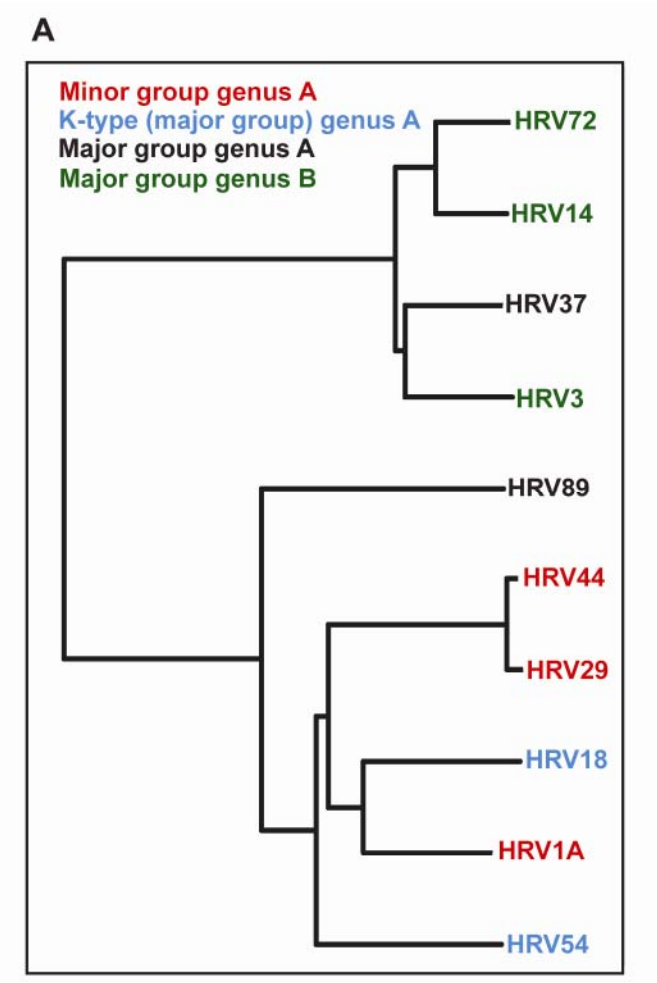
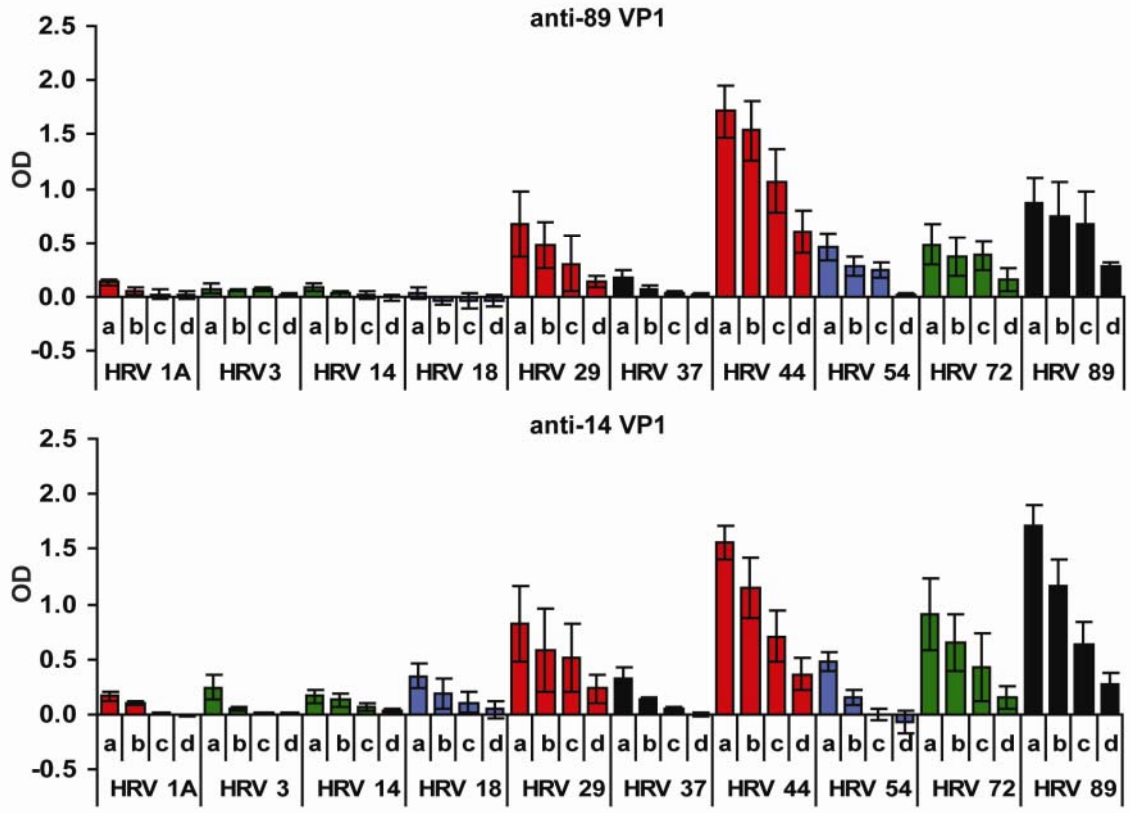


FIG. 6. (A) Phylogenetic tree of the VP1 sequences of the HRVs investigated. VP1 sequences were retrieved from the data bank and their similarity was analyzed with ClustalW. (B) Inhibition of HRV infections by the respective VP1-specific antibodies. HRVs at 100 $TCID_{50}$ were pre-incubated with twofold serial dilutions of the respective antisera at 1:2 (a) to 1:16 (d) for 3 hours at 37^{o}C and the mixtures were applied to sub-confluent HeLa cells in 96 well plates. After incubation for 3 days at 34^{o}C, cells were stained with crystal violet, washed, the stain was dissolved, and A_{560} was read. Mean ± standard error of four independent experiments is shown. (C) Alignment of amino acid sequences of VP1 from the HRVs studied in this report. Dots represent identity.


A
Minor group genus A
K-type (major group) genus A
Major group genus A
Major group genus B
HRV72
HRV14
HRV37
HRV3
HRV89
HRV44
HRV29
HRV18
HRV1A
HRV54

B



C

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HRV_89  NPVENYIDSVLNEVLVVPNIQPSTSVSSHAAPALDAAETGHTSSVQPEDMIETRYVITDQTRDETSIESFLGRSGCIAMIEFNTSSD-KTEHDKIGKGFK   99
HRV_37  GLGDELEEV..EKTKQTLASIS.GPKH.QSV.T.T.N...A.MPTN.S.N....TTYMHFNGS..D.......AA..H.T.IENKNSTG.VNH.SD.L.N  100
HRV_18  ........E...........NE.HA...NS.............N............Q.S.....M...C........H.SKLVVHYE-DYNAETR--N.V   97
HRV_54  ....R...E...........RE.HPA..NS.............G.....T......Q.S.....M........A...HESTITIQN--DV.Y.DH--H..   97
HRV_1A  ........E...........KE.HHT..NS..L..........N.....A........S.....M............H.SRIKVDYT-DYNGQD.--N.T   97
HRV_29  ........E...........RE.HPS..NS..I..........N.....T......Q.SH....M............H.STIKAN-----.AHDA--K.D   93
HRV_44  ........E...........RE.HPSI.NS..I..........N.....T......Q.S.....M............H.STIK.N-----.AHNT--K.D   93
HRV_3   GLSDELEEV..EKTKQTLASVS.GPKH.QSV...T.N...A.LPTR.S.N....TTYMHFNGS..D.......AA..H.T.IKNKNAAGLDNH.KEGL.N  100
HRV_14  GLGDELEEV..EKTKQTVASIS.GPKH.QKV.I.T.N...A.MP.L.S.S....TTYMHFNGS..D..C....AA..H.T.IQNKDATGIDNH.EA.L.N  100
HRV_72  GLNDELEEV..EKTKQTLASIS.GPKY.QSV.T.T.N...A.MPTL.S.N....TTYMHFNGS..D..C....AA..H.T.IENKNPNGISNH.AE.L.N  100

HRV_89  TWKVSLQEMAQIRRKYELFTYTRFDSEITIVTAAAA--QGNDSGHIVLQFMYVPPGAPVPEKRDDYTWQSGTNASVFWQEGQPYPRFTIPFMSIASAYYMFY  199
HRV_37  D...N.SS.V.....L.....V.....Y...AT.SQPSKS.YASN....A........N.KEW.......A..P....KV.DTA-.......G.....NC..  201
HRV_18  K.Q.N..................V.........PSV..--K...I.................KT....A.............H..T.....................  197
HRV_54  K.D..............F...V.........PCI.G--K.V.I.................K...........S..P.....H..A.....................  196
HRV_1A  K.....................V.........PCI.G--R...I.................S............M.....H.....................  197
HRV_29  K.N.N.................V.........PCI.G--R...I.................ND..H.A............H.....................  193
HRV_44  K.N.N.................V.........PCI.G--R...I.................DD.IH.A....N.......H.....................  193
HRV_3   D...N.SS.V.....L.....V.....Y...AT.SQPEASSY.SN.T..A........N.KEW.......A..P....KV..TS-.......G.....NC..  201
HRV_14  D...N.SS.V.....L.....V.....Y...AT.SQPDSA.Y.SN....A........N.KEW.......A..P....KV.DTS-.......G.....NC..  201
HRV_72  D.....SS.V.....L.....V.....Y...AT.SQPDTA.Y.SN....A........N.VEW.......A..P....KV.DTS-.......G.....NC..  201

HRV_89  DGYDGDSAASKYGSVVTNDMGTICVRIVTSNQKHDLNIVCRIYHKAKHIKAWCPRPPRAVAYQHTHSTNYIPSNGEATTQIKTRP-DVFTVTNV   292
HRV_37  ...SH.DEN.P..IT.L.H....AF...NEHDA.TTL.KI.........E..I..A....P..AIGK...-.KM--I.PV..K.D-..T.Y---   288
HRV_18  ......QTS......A........S....DKH.NE.E.TT.....................E.T...V...K.KE.REK.A.VP.A-R.TMA---   287
HRV_54  ......APG.....S...H.....S....DK...P.E.TT.....................A....P.T..R......RE.DP.IF..H.T-..V.A---   286
HRV_1A  ......NTS..............S....EK...S.V.TTH.......T............P.T...V.....ET.DV..A.VR.N-T.T.A---   287
HRV_29  .....GDHTAT...T.V.R..........GK.A...Q.TTS.................V.P.KYVGL...TLKE--ED.V.E...-S.M.A---   281
HRV_44  .....GDHTAT...T.V.R..........GK.A...Q.TTS.................V.P.KYVGL...TLKE--TD.V.EP.H-S.M.A---   281
HRV_3   ...SH.DPD.P..IT.L.H....AF...NEHDV.TTI.KI.........E..I..A....P.VSIGR...-.R.--SK.I..K.T-..K.Y---   288
HRV_14  ...SH.D.E.Q..IT.L.H....AF...NEHDE.KTL.KI.........E..I..A....P.TSIGR...-.K.--TEPV..K.KG..K.Y---   289
HRV_72  ...SH.D.E.Q..IS.L.H....AF...NEHDT.RTL.KI.........E..V..A....P.TSIGR...-.K.--PKPV..K.EG..K.Y---   289
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FIG. 7. Reactivity of rabbit anti-89VP1 and anti-14VP1 antibodies with 14VP1, 89VP1 and three recombinant 89VP1 fragments. Rabbit sera were diluted 1:5000 and A_{560} corresponding to bound IgG antibodies is shown on the y-axis.

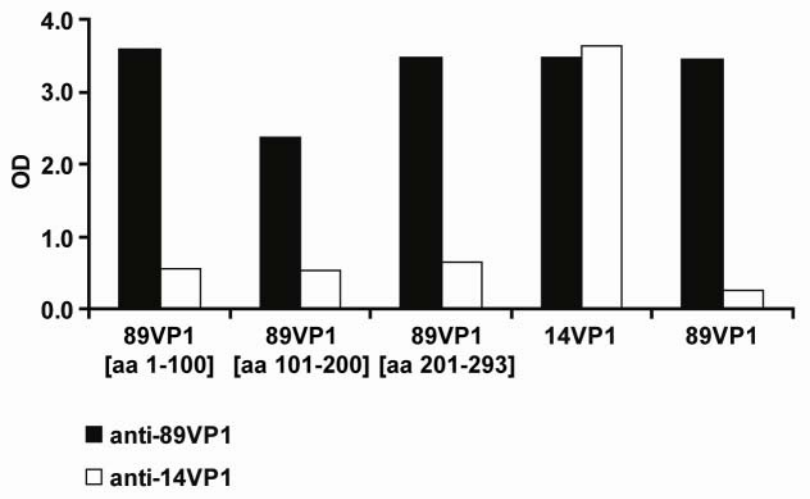

TABLE 1. Neutralization of viral infectivity by antibodies raised against 14VP1 and HRV14-derived peptides. Serial two-fold dilutions of anti-14VP1, anti-PVP1A, anti-PVP1B or anti-PVP3A antibodies (undiluted or diluted 1:2-1:32) were pre-incubated with 100 $TCID_{50}$ HRV14 and added to HeLa cells in 96-well plates. The respective cytopathic effects (CPE) observed is indicated: +++: complete neutralization; ++: minimal CPE; +: partial CPE; +/-: almost complete CPE; - complete CPE.

HRV14

	undiluted	1:2	1:4	1:8	1:16	1:32	1:64
α 14VP1	+++	+++	++	++	+	+	-
α PVP1A	+++	+++	+	+	+/-	+/-	-
α PVP1B	+++	+++	+	+	+/-	+/-	-
α PVP3A	+++	+++	+	+/-	+/-	+/-	-

TABLE 1. Inhibition of HRV14 infection with antisera raised against 14VP1 (highlighted in grey) and HRV14 derived peptides.