Mechanism of Action and Capsid-Stabilizing Properties of VHHs with an *In Vitro* Antipolioviral Activity

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ABSTRACT

Previously, we reported on the *in vitro* antiviral activity of single-domain antibody fragments (VHHs) directed against poliovirus type 1. Five VHHs were found to neutralize poliovirus type 1 in an *in vitro* setting and showed 50% effective concentrations (EC₅₀s) in the nanomolar range. In the present study, we further investigated the mechanism of action of these VHHs. All five VHHs interfere at multiple levels of the viral replication cycle, as they interfere both with attachment of the virus to cells and with viral uncoating. The latter effect is consistent with their ability to stabilize the poliovirus capsid, as observed in a Thermo-Fluor thermal shift assay, in which the virus is gradually heated and the temperature causing 50% of the RNA to be released from the capsid is determined, either in the presence or in the absence of the VHHs. The VHH-capsid interactions were also seen to induce aggregation of the virus-VHH complexes. However, this observation cannot yet be linked to their mechanism of action. Cryo-electron microscopy (cryo-EM) reconstructions of two VHHs in complex with poliovirus type 1 show no conformational changes of the capsid to explain this aggregation. On the other hand, these reconstructions do show that the binding sites of VHHs PVSP6A and PVSP29F overlap the binding site for the poliovirus receptor (CD155/PVR) and span interfaces that are altered during receptor-induced conformational changes associated with cell entry. This may explain the interference at the level of cell attachment of the virus as well as their effect on uncoating.

IMPORTANCE

The study describes the mechanism of neutralization and the capsid-stabilizing activity of five single-domain antibody fragments (VHHs) that have an *in vitro* neutralizing activity against poliovirus type 1. The results show that the VHHs interfere at multiple levels of the viral replication cycle (cell attachment and viral uncoating). These mechanisms are possibly shared by some conventional antibodies and may therefore provide some insight into the natural immune responses. Since the binding sites of two VHHs studied by cryo-EM are very similar to that of the receptor, the VHHs can be used as probes to study the authentic virus-cell interaction. The structures and conclusions in this study are original and raise interesting findings regarding virus-receptor interactions and the order of key events early in infection.

Poliovirus, a member of the *Picornaviridae*, is known to be the causative agent of poliomyelitis. In 1988, the World Health Assembly launched the Global Polio Eradication Initiative (GPEI) to combat poliovirus using large-scale vaccination campaigns. The original goal to eradicate the virus by the year 2000 had to be adjusted since the last phase proved to be much more difficult than anticipated. Vaccination using the oral polio vaccine (OPV) was very successful but seemed to be hindering the final eradication at the same time (1). To complete the eradication effort and to have a tool to handle possible future outbreaks, it would be important to have an antipolioviral compound that could be used in combination with vaccination (2). Up to date, no antipolioviral compound was found to be both effective and clinically usable.

Single-domain antibodies (VHHs or Nanobodies) show some very interesting characteristics and are very promising candidates to be developed as antipolioviral drugs. VHHs are single-domain antibody fragments (3) derived from heavy chain antibodies, a class of antibodies found in camelids (4). They are small (~15-kDa) and stable proteins that have a tendency to bind in clefts and to recognize epitopes that are more hidden from classical antibodies (5). We previously reported on five of these VHHs that exert an *in vitro* neutralizing activity against poliovirus type 1 (6). They show 50% effective concentrations (EC₅₀s) in the nanomolar

range and, up to a tested concentration of 35 μ M, have no detectable cytotoxic effect in HeLa cells.

Poliovirus has a proteinaceous capsid that is assembled from 60 copies of four different viral proteins (VP1, VP2, VP3, and VP4), which are organized into an icosahedral capsid. The capsid is responsible for recognition of the host cells through binding to the poliovirus receptor (PVR/CD155). At physiological temperature, receptor binding induces conformational changes, producing an expanded form of the virion, the 135S or A particle. The conformational changes result in the externalization of the myristoylated protein VP4 (7, 8) and N-terminal extension of VP1 (9), both of which are located on the inner surface of the protein shell

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in native virions (10). The externalized polypeptides interact with the cell membrane (9, 11, 12). Following endocytosis of the 135S particle, the viral RNA genome is translocated across the endosomal membrane into the cytoplasm of the host cell, leaving behind an 80S empty capsid (reviewed by Levy et al. [13]). Recently, Strauss et al. (14) described the formation of two long “umbilical” density features that connect the virus to the cellular membrane. These umbilici presumably anchor the virus to the cell after the formation of the 135S particle (which is known to have little affinity for CD155) and may provide a conduit for protected RNA transfer. It has been assumed that VHH binding to the viral capsid may interfere with one of these early events of the infection cycle (6), but this had never been confirmed.

As reviewed by Reading and Dimmock (15), there are a number of different mechanisms for neutralization of viruses by conventional antibodies. Aggregation of virus particles through the binding of antibodies can cause a reduction in the amount of infectious virions available to interact with their receptor. Binding of the antibodies to the virion may also (i) inhibit the binding of the virus to cells via steric hindrance, (ii) stabilize the capsid in such a way that the RNA can no longer be released into the cell, (iii) cause a conformational change of the capsid, so that it is no longer capable of receptor binding, or (iv) disrupt the viral particle, triggering RNA release prematurely. The latter mechanism is an “intrinsic” neutralization that was found to occur only for some antibodies in a low-ionic-strength environment (16) or at 39°C (fever temperature) (17). Such antibodies were observed to irreversibly transform the native virus (N-antigenic 160S particles) into empty (RNA-free) capsids (H-antigenic 80S particles). After having converted the virion, the antibody was released from the capsid in a conformationally modified state (18). An important aspect of this “hit-and-run” neutralization is destabilization of the capsid, as was shown by Delaet and Boeye (19).

Since the VHHs are known to interact with the poliovirus capsid (20), they are likely to interfere with one of the early events of infection (cell attachment, entry, and uncoating). Other antipolioviral compounds, like 3(2H)-isoflavene and pirodavis, were demonstrated to exert their activity through binding to the capsid and, in the case of pirodavis, stabilizing the native virion (21, 22). Some antipicornaviral compounds were found to interfere with a later stage of replication and target nonstructural proteins. For a complete overview of these compounds, see the review by Thibaut et al. (23).

Here, we report our findings describing the mechanism of neutralization of the VHHs PVSP6A, PVSS8A, PVSP19B, PVSS21E, and PVSP29F on poliovirus type 1 as well as their stabilizing activity on the polioviral capsid.

MATERIALS AND METHODS

Virus and cells. Poliovirus type 1 strain Mahoney was used throughout this study. Both ³⁵S-radiolabeled poliovirus and nonlabeled virus were grown as previously described (24). HeLa monolayer cells were cultivated in minimum essential medium enriched with 5% bovine calf serum, 100 IU/ml penicillin G, and 100 µg/ml streptomycin sulfate.

Antipolioviral VHHs. The VHHs selected for these experiments originate from a dromedary that was immunized with poliovirus type 1 (strain Sabin). Six weeks postimmunization, blood was collected and peripheral blood lymphocytes were isolated. A VHH library was constructed from the total RNA of the lymphocytes and cloned into the phagemid vector pHEN4 (3). Specific VHHs against poliovirus type 1 were selected by phage display panning and recloned into the expression vector pHEN6(c)

(6). The recombinant VHHs are produced by a method similar to that described by Thys et al. (6), by growing *Escherichia coli* WK6 containing the VHH gene in Terrific Broth medium enriched with 0.1% glucose and 0.01% ampicillin in culture flasks for about 17 h. After induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h at 28°C, the cells were pelleted and the periplasmic fraction was extracted by osmotic shock. The VHHs were purified by immobilized metal affinity chromatography purification over a Bio-Scale Mini Profinity Ni-nitrilotriacetic acid (NTA) column (Bio-Rad) followed by a size exclusion chromatography purification on a HiLoad 16/600 Superdex 75-pg column (GE Healthcare). Fractions containing the single VHH were collected and concentrated on a Vivaspin 4 filter (Sartorius). The VHHs were dissolved in Tris buffer (137 mM NaCl, 25 mM Tris, pH 7.2) and stored at −80°C. Six different VHHs were used: PVSP6A, PVSS8A, PVSP19B, PVSS21E, PVSP29F, and Nb1. Nb1 is a VHH generated against the *lvpB* transcriptional regulator of *Sulfolobus solfataricus* and is known to have no interaction with poliovirus. The EC₅₀ (the concentration of VHH at which 50% of the virus is neutralized) for each VHH using this purification protocol was determined and found to be 0.3 nM, 26 nM, 11 nM, 84 nM, and 4.8 nM, respectively.

Pirodavis and arildone. Pirodavis (R77975) is ethyl-4-[3-[1-(6-methyl-3-pyridazinyl)-4-piperidinyl]ethoxy]benzoate and was synthesized by the Janssen Research Foundation. A stock solution of 10 mg/ml pirodavis in dimethyl sulfoxide was made, and it was further diluted in phosphate-buffered saline (PBS) buffer (145 mM NaCl, 50 mM Na₂HPO₄·12H₂O, pH 7.4) throughout the experiment.

Arildone (WIN 38020) is 4-[6-(2-chloro-4-methoxyphenoxy)hexyl]-3,5-heptanedione and was synthesized by Sterling-Winthrop, Inc. A stock solution of 100 µM arildone was prepared in dimethyl sulfoxide.

Time-of-addition test. A time-of-addition test was carried out to obtain an indication of the stage in the infection cycle in which the VHHs exert their activity. The test was performed as described by Garozzo et al. (25). Briefly, HeLa cells were cultivated in a 24-well plate and were infected with poliovirus Mahoney 1 (multiplicity of infection [MOI], 0.1) and incubated at 4°C for 2 h, to permit attachment. The inoculum was removed and replaced with fresh cell medium, and the cells were brought to 37°C, which normally triggers endocytosis and uncoating. Subsequently, VHH was added to obtain a final concentration of 0.07 µM (PVSP6A), 4.39 µM (PVSS8A), 6.80 µM (PVSP19B), 6.92 µM (PVSS21E), 0.16 µM (PVSP29F), or 7.00 µM (Nb1) at various time points: either during initial adsorption or at 0 h, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, or 6 h following adsorption of the virus. At 7 h postadsorption, the cells were frozen (at −20°C) and thawed three times. The viral titer in each well was determined in a plaque assay. Each time-of-addition test was repeated four times.

Heat stabilization of the viral capsid by VHHs. In a ThermoFluor thermal shift assay (26), the capsid-stabilizing properties of the VHHs were investigated. Poliovirus type 1 (final concentration, 97.4 µg/ml) was exposed to increasing temperatures in a Bio-Rad LightCycler3 in the presence or absence of either Nb1, PVSP6A, PVSS8A, PVSP19B, PVSS21E, or PVSP29F at a final concentration of 10 µM or the capsid-stabilizing compound pirodavis (27 µM). All of the samples were prepared in PBS buffer containing RNase inhibitor (400 U/ml), albumin (1 mg/ml), and SYBR green II RNA gel stain (Life Technologies). SYBR green II is a fluorescent dye that emits green light only when bound to nucleic acids. During heating of poliovirus particles, the capsid is destabilized and RNA is released. The samples were gradually heated from 30 to 73°C (steps of 0.5°C, one step per second). Because of the temperature sensitivity of the SYBR green II fluorescence, the temperature was changed to 30°C after each step to measure the fluorescent signal. For each control and compound, the temperature at which 50% of the maximal fluorescent signal was reached was determined, using Graph Pad Prism. The heat stabilization test was repeated four times.

Sedimentation experiment. The effect of the VHH binding to poliovirus type 1 was also investigated in a sucrose gradient ultracentrifugation

experiment. Samples were prepared by adding VHH (in a final concentration of 10 μ M) to 76,000 dpm of ^{35} S-radiolabeled poliovirus (65.9 μ g virus/ml [estimated value], 15,000 dpm/ μ l, 0.39 nM virus in the final sample) in PBS buffer containing 0.01% (wt/vol) of bovine serum albumin. The samples were incubated for 1 h at 37°C and ultracentrifuged for 133 min at $175,000 \times g$ in a swing-out rotor on a 15 to 30% (wt/vol) sucrose density gradient in Tris buffer with a 70% Nycodenz cushion to collect aggregates. Fractions of 400 μ l were collected from the top of the gradient and were analyzed in a β -scintillation counter (PerkinElmer).

The formation of aggregates was also tested for control VHH Nb1 (final concentration, 10 μ M), with a different radiolabeled virus batch (39.5 μ g virus/ml, 22,000 dpm/ μ l). In this experiment, a control sample containing PVSS21E was included together with an untreated control. Aggregate formation was further verified by cryo-electron microscopy (see below).

Virus binding to cells. For the cell binding assays, 5 μ l of a ^{35} S-radiolabeled poliovirus solution at 39.5 μ g/ml (2,200 dpm/ μ l) was added to 100 μ l of minimum essential medium and incubated for 1 h at either 4°C or 37°C in the presence or absence (control) of a neutralizing VHH. This corresponds to a virus concentration of 0.22 nM and a 13.2 nM concentration of VHH binding sites (assuming 60 sites per virion). Concentrations of VHH were chosen between 0.001 and 1.0 μ M (corresponding to 4.5 to 4,500 VHHs per virion). After this first incubation step, the HeLa cells and the virus-VHH mixtures were separately given 20 min at 4°C or 37°C (depending on the subsequent incubation temperature) to ensure temperature stability. The virus-VHH mixtures were then placed on the cells, and the samples were incubated for 2 h at either 4°C or 37°C. The supernatant was removed, and cells were rinsed twice with PBS buffer to remove unbound virus. The cells were lysed and collected from the plate using a 0.1% Nonidet P-40 solution in PBS. Scintillation fluid was added, and the cell-associated radioactivity was determined in a β -scintillation counter (PerkinElmer). A control sample containing polyclonal serum against poliovirus was included in each binding assay to properly correct for nonspecific binding. Each binding assay was repeated four times.

Virus entry and uncoating. To determine the effect of the VHHs on the formation of intermediate particles necessary for entry and uncoating of poliovirus, a monolayer of HeLa cells was infected with 190,000 dpm of ^{35} S-radiolabeled poliovirus (96.1 μ g virus/ml, 19,000 dpm/ μ l) and incubated for 2 h at 4°C to allow binding of virions to the cells. VHH (0.1 $\times$ EC₅₀, 1 $\times$ EC₅₀, and 10 $\times$ EC₅₀ for PVSP6A, PVSS21E, and PVSP29F and 10 $\times$ EC₅₀ for PVSS8A and PVSP19B) or arildone (2.7 μ M, as a positive control) was added, and the cells were incubated for another hour at 4°C. After replacing the inoculum by cell culture medium containing VHH or arildone, the cells were incubated for 2 h at 37°C. The medium containing VHH or arildone was removed, and cells were washed with PBS before being subjected to freeze-thawing three times. The lysed uncoating samples were centrifuged for 5 min at $12,000 \times g$ and analyzed on a 15 to 30% (wt/vol) sucrose density gradient in Tris buffer for 133 min at $175,000 \times g$ in a swing-out rotor. Fractions of 400 μ l were collected from the top of the gradient and were measured in a β -scintillation counter (PerkinElmer).

Cryo-electron microscopy. Three microliters of virus-VHH complex was added to a glow-discharged Quantifoil electron microscopy grid (Quantifoil Micro Tools), the excess was removed by blotting with filter paper, and the remainder was rapidly frozen by plunging in liquid ethane. The grids were then transferred into liquid nitrogen for storage until imaging. Single-particle data sets for PVSP6A and PVSP29F were collected on a Titan Krios (FEI Company) instrument operating at 200 kV and equipped with an Ultrascan 4000 charge-coupled-device (CCD) camera (Gatan) using a calibrated pixel size of 0.841 Å. Automated data collection was carried out using EPU software (FEI Company), yielding 8,563 micrographs (of which 6,223 were usable) for PVSP6A and 3,575 micrographs (1,503 usable) for PVSP29F. Contrast transfer function (CTF) es-

timization was performed using CTFFIND3 (27), and micrographs with poor CTF estimation were discarded. Semiautomated particle selection was carried out using EMAN2 (28), resulting in 57,282 and 9,764 particles for PVSP6A and PVSP29F, respectively. Orientation determination and refinement were done using a graphics-processing-unit (GPU)-enabled version of FREALIGN (29, 30), using a map of poliovirus Fourier filtered to 35-Å resolution. The final resulting maps were sharpened using either EM-BFACTOR (31) or Sparx (32).

Reversibility of inactivation. The reversibility of the neutralization by two VHHs (PVSP6A and PVSP29F) was tested by plaque counting of diluted neutralized samples. We examined this under three different conditions. In detail, 4×10^4 PFU/ml of poliovirus was incubated in the presence of a neutralizing VHH (0.7 μ M PVSP6A or 1.6 μ M PVSP29F) either for 1 h at 37°C or 39°C in a PBS buffer with physiological ionic strength (137 mM NaCl, 2.7 mM KCl, and 9.5 mM Na₂HPO₄, pH 7.3) or for 1 h at 37°C in a buffer with low ionic strength (0.6 mM Na₂HPO₄, pH 7.3). The samples were serially diluted, and a plaque assay was performed. The plaques were counted, and the reversibility of the poliovirus neutralization was determined.

Protein structure accession numbers. The coordinates and cryo-electron microscopy (cryo-EM) reconstructions of the complexes of poliovirus type 1 and PVSP6A (PDB accession no. 3J69, EMDB accession no. 5886) and poliovirus type 1 and PVSP29F (PDB accession no. 3J6A, EMDB accession no. 5888) can be found in the Protein Data Bank and the Electron Microscopy Data Bank.

RESULTS

Time-of-addition test. To define the time frame of the viral replication within which the VHHs interfere with the infection, the effects of VHH addition at various stages of the infection of HeLa cells with poliovirus type 1 were studied. The results from these time-of-addition tests show that all of the tested VHHs interfere with an early phase of the viral replication (Fig. 1). At this concentration, they gave a 2 log₁₀ reduction in infectious particles (4 log₁₀ for PVSP6A) when they were present during the adsorption phase, and the reduction of titer dropped significantly at later times of addition. Nb1, a VHH against the *lprB* transcriptional regulator of *Sulfolobus solfataricus*, shows no interaction with poliovirus and was used as a control.

Heat stabilization of the viral capsid by VHHs. We were interested in finding out whether the VHHs exert a stabilizing activity against heat on the poliovirus capsid. When a 160S particle is heated, the capsid is destabilized and RNA is externalized to form an empty capsid or 80S particle. The thermostability of each complex was assessed using a ThermoFluor assay, in which the complexes are gradually heated in the presence of SYBR green II to detect the release of viral RNA upon heat inactivation. The fluorescent signals are continuously measured and result in a sigmoidal curve when plotted as a function of the temperature. From these curves, the temperature at which 50% of the RNA was released from the capsid (50% effective temperature [ET₅₀]) could be calculated. These ET₅₀ values are given in Table 1 and show that all five neutralizing VHHs were able to significantly stabilize the polioviral capsid, as their ET₅₀ values are significantly different ($P < 0.01$) from that from the control sample for each VHH. Pirodavir was tested as a positive control. Using a one-way analysis of variance (ANOVA) test followed by a Bonferroni *post hoc* test, it is seen that the stabilizing activity varies statistically for the five VHHs. PVSP6A and PVSS8A exert stabilizing activities that are smaller than those of PVSP19B, PVSS21E, and PVSP29F. Furthermore, the last three VHHs appear to have a

FIG 1 Time of addition of VHHs. VHH was added to a monolayer of HeLa cells at different times during the infection cycle of poliovirus type 1: during a 2-h adsorption step at 4°C or at 0, 15, 30, 45, 60, 120, 240, or 360 min after placing the infected cells at 37°C. At 7 h postadsorption, the cells were lysed and the viral titer was defined in a plaque assay. The log₁₀ reduction in viral titer is represented as a function of the time of addition of the VHH. Each test was repeated four times, and the data followed a normal distribution as defined in a Shapiro-Wilk test ($\alpha = 0.05$) and are thus represented as the mean values $\pm$ standard errors of the means (SEM). The VHHs seem to be effective when added during or right after the adsorption phase. Nb1, a VHH with no antipoliovirus activity, was measured as a control.

TABLE 1 Heat stabilizing activity of the VHHs on the polioviral capsid^a

VHH or compound	ET ₅₀ (°C) ^b	P value for comparison with:		
		Control	PVSP6A and PVSS8A	Pirodavar
Control	50.19 $\pm$ 0.30			
Nb1	50.40 $\pm$ 0.12			
Pirodavar	54.11 $\pm$ 0.03	<0.005		
PVSP6A	51.89 $\pm$ 0.31	<0.01		
PVSS8A	52.61 $\pm$ 0.31	<0.005		
PVSP19B	55.81 $\pm$ 0.24	<0.005	<0.005	<0.05
PVSS21E	56.99 $\pm$ 0.37	<0.005	<0.005	<0.005
PVSP29F	57.04 $\pm$ 0.35	<0.005	<0.005	<0.005

^a Poliovirus was gradually heated from 30 to 73°C in the presence or absence of the *in vitro* neutralizing VHHs. The release of RNA from the capsid was detected with SYBR green II, and the temperature at which 50% of the RNA was released from the capsid was defined. The capsid-stabilizing compound pirodavar and Nb1, a VHH without neutralizing activity against poliovirus, were tested as controls. All the VHHs (apart from Nb1) have a statistically significant stabilizing effect on the capsid.

^b Temperature at which 50% of the RNA was released from the polioviral capsid. Each ET₅₀ value represents the mean value $\pm$ standard error of the mean (SEM) of four separate experiments. Normal distribution was verified in a Shapiro-Wilk test ($\alpha = 0.05$). The significance of the differences between the ET₅₀ values was calculated in a one-way ANOVA followed by a Bonferroni *post hoc* test.

greater stabilizing effect than pirodavar, since they have a significantly ($P < 0.05$) higher ET₅₀ value, even when present at a lower concentration than pirodavar (10 μ M versus 27 μ M, respectively).

Formation of aggregates. In sedimentation experiments (Fig. 2A), it is seen that the poliovirus peak is shifted from around fraction 20 in the control sample to fraction 27 in the samples containing virus treated with 10 μ M (a $\sim$ 25,600-fold molar excess) of all different neutralizing VHHs for 1 h. Addition of control VHH Nb1 did not result in the formation of aggregates. This aggregation phenomenon was also observed on EM images (Fig. 2B).

Virus binding to cells. The binding assay is used to investigate whether the VHHs prevent the infection of cells by hindering the virus from attaching to the cell receptor. The first binding assay measured the cell association of radioactive virus in samples containing VHH. The effect of varying the VHH concentration was evaluated on VHH-virus samples that were preincubated at 37°C and then incubated with cells at 4°C. Assuming 60 binding sites per virus (as indicated by the cryo-EM reconstructions of the VHH-poliovirus complexes, see below), the initial concentration

FIG 2 Formation of aggregates. (A) Sedimentation profiles of untreated poliovirus type 1 (peak at fraction 20) and virus that was treated for 1 h at 37°C with 10 μ M VHHs PVSP6A, PVSS8A, PVSP19B, PVSS21E, and PVSP29F. The samples were analyzed on a 15 to 30% (wt/vol) sucrose density gradient with a 70% Nycodenz cushion to collect aggregates. The viral particles clearly shift toward the fractions with faster sedimentation (right) after treatment with VHH, indicating the formation of aggregates. Treatment with VHH Nb1 does not result in aggregation of the virus-VHH complexes, as shown by the sedimentation profiles in the inset. (B) Aggregates as seen by EM. Bar, 100 nm.

of binding sites on the virus surface was ~ 13 nM. The final concentration of VHH was 1 nM, 10 nM, or 100 nM (also 1 μ M for PVSS21E) (Fig. 3).

For all five VHHs, we detected a concentration-dependent interference with the attachment of poliovirus type 1 to HeLa cells. A significant decrease ($P < 0.005$) in cell attachment was observed starting from a concentration of 0.01 μ M (i.e., 45 VHHs per virion; see “Virus binding to cells” in Materials and Methods) for PVSP6A, PVSS8A, PVSP19B, and PVSP29F and of 0.1 μ M (i.e., 450 VHHs per virion) for PVSS21E. As a control, the same test was performed with Nb1. Even at 0.1 μ M, Nb1 did not interfere with viral attachment to the cells.

Subsequently, in view of the capsid-stabilizing properties of the VHHs (see Discussion), the single highest concentration of VHH in each cell-binding assay was repeated at a different temperature. In one variation, the temperature of the 2-h incubation of cells was raised to 37°C (Fig. 3, columns with horizontal stripes). In the other variation, the temperature of the 1-h preincubation step was lowered to 4°C (Fig. 3, columns with vertical bars).

Even with changes in either the preincubation or the incubation temperature, all five VHHs continued to be able to significantly ($P < 0.005$) decrease the attachment of virus to its receptor, relative either to the nonspecific VHH control, Nb1, or to the VHH-free sample. Additionally, there was no instance in which lowering the temperature of the preincubation step made a significant difference in the result (Fig. 3, vertical bars). However, two distinctly different results were obtained when the temperature of the incubation on cells was raised to 37°C. For two of the VHHs (PVSP6A and PVSS21E), the effectiveness of the VHH in inhibiting cell attachment was not significantly affected. For the other three of the VHHs (PVSS8A, PVSP19B, and PVSP29F), the effect of raising the temperature of the incubation on cells was to significantly diminish the effectiveness of the VHH in preventing attachment. Possible explanations for this are considered in Discussion below.

We attempted internalization experiments that measured the amount of virus associated with the cell after external receptor-bound virus was washed away, as described by Garozzo et al. (25),

but were unable to detect any difference from the control experiments, so these data have not been included. We suspect that the EDTA wash is not effective at removing receptor-bound virus from the cell surface.

Effects on the formation of intermediate particles during viral entry and uncoating. After attachment of viral particles to the cell-associated CD155 receptors at physiological temperature, the capsid is triggered to undergo a series of conformational changes, resulting first in an expanded 135S particle, followed by an empty capsid. The results indicate that the VHHs do show an activity on the formation of these eclipse particles. The cell-induced conversion of 160S to 80S particles during infection can be seen on a sucrose density gradient, where bands containing the two types of particles are separated. For each sample, the areas under the curve of the 80S and the 160S peak were calculated and the 160S/80S ratio was defined relative to the 160S/80S ratio of the respective control sample. As represented in Fig. 4, this relative 160S/80S ratio shows an increasing trend with increasing concentrations (logarithmic scale) of VHH. For VHHs PVSS8A and PVSP19B, only one concentration was measured, but for those single concentrations the ratios are 2.6 and 1.7, respectively, showing a decrease in 80S formation. This decrease in the formation of 80S particles can be the consequence of the (partial) inhibition of the formation of 135S particles or of interference at the level of the 135S-to-80S transition.

Cryo-EM reconstructions. Cryo-EM reconstructions were obtained for the complexes of poliovirus type 1 with the VHHs PVSP6A and PVSP29F. Fourier shell correlations of both complexes (Fig. 5C) indicate resolutions of 4.8 Å and 6.5 Å for the poliovirus-PVSP6A and the poliovirus-PVSP29F complexes, respectively. The outer isocontour surface of the poliovirus-VHH complexes (Fig. 5A and B) show that the VHHs are well ordered and that the VHH binding sites are positioned far enough apart that neighboring VHHs do not contact another, allowing 60 of them to bind at saturation. Each of the VHHs is bound to the virus surface at the center of the 5-3-3 icosahedral triangle (Fig. 5B and D). Footprints of PVSP6A and PVSP29F on the virus surface were calculated from preliminary fitted atomic models, using 5- and 9-Å distance criteria (Fig. 5D). Numerous cryo-EM studies of the poliovirus complex with its receptor (14, 33–35), which is stable at low temperature, have established that the N-terminal domain of the poliovirus receptor (CD155) binds deeply within a depression on the poliovirus surface called the “canyon” and that it makes extensive contacts with three distinct patches on the poliovirus surface (Fig. 5D). Like CD155, the VHH domains both bind within the “canyon.” PVSP6A is oriented to lie flatter along the virus surface and makes extensive contacts with the same three surface patches that CD155 does, contacting many of the same aminoacyl residues. PVSP29F has a more radial orientation, making extensive contacts with two of the patches and more-tenuous contacts with the third patch.

In poliovirus, all three patches include polypeptide segments that are known to rearrange upon capsid expansion during receptor-induced uncoating at 37°C (36). It is believed that receptor-induced release of a few of these capsid-stabilizing polypeptide segments is sufficient to permit icosahedral symmetric expansion of the entire capsid (14). We conjecture that both PVSP6A and PVSP29F act to lock these same movable segments in place, thereby preventing concerted icosahedral symmetric changes from occurring everywhere else in the capsid. However, further

FIG 3 Concentration-dependent interference with cell attachment of poliovirus by VHHs. The inhibitory activity on the binding of poliovirus to its receptor is shown for each of the five VHHs. Nb1 is a VHH that has no affinity for the virus and is used as a control. The amount of radiolabeled virus attached to untreated control cells was set to be 100%, and the samples were all corrected for nonspecific binding. The results of the binding assay with a 1-h preincubation at 37°C and a 2-h incubation at 4°C are represented in gray without pattern. With increasing concentration of VHH, the virus is increasingly blocked from binding the HeLa cells. Due to the capsid-stabilizing features of the VHHs, the binding assay was repeated under a 2-h incubation at 37°C (horizontal stripes), and to find out if the VHHs recognize structures exposed during breathing, it was repeated once more with both the preincubation and incubation steps at 4°C (vertical stripes). All data sets followed a normal distribution (Shapiro-Wilk test, $\alpha = 0.05$) and are therefore represented as the mean values $\pm$ SEM. The differences between the control and the samples as well as the samples at different temperatures were calculated using one-way ANOVA followed by a Dunnet's *post hoc* test (ns, nonsignificant; ***, $P < 0.005$).

FIG 4 Effect of VHHs on poliovirus uncoating. During uncoating, the 160S particle is transformed into an 80S particle by the release of its RNA into the cytoplasm. Each sample was analyzed on a 15 to 30% (wt/vol) sucrose density gradient, and the 160S/80S (area under the peak) ratio was calculated relative to the 160S/80S ratio of the respective control sample. The 160S/80S ratio increases with increasing concentrations of VHH (logarithmic scale), indicating an inhibitory activity of the VHHs on the formation of 80S particles. For VHHs PVSS8A and PVSP19B, only one concentration was measured.

experiments and a more detailed characterization of the virus-nanobody interaction will be required to test this hypothesis.

Reversibility of inactivation. To elucidate the mechanism(s) of neutralization, two of the VHHs, PVSP6A (with the lowest ET_{50}) and PVSP29F (with the highest ET_{50}), were tested for the reversibility of neutralization by dilution of the complex under various conditions. For each condition tested, the plaque titration revealed that infectivity was at least partly restored after dilution of the sample (Fig. 6). When the samples were further diluted, a decrease is observed parallel to a decrease in the control samples as a result of the dilution. Very similar results were obtained under the two other conditions (at 37°C in a buffer with low ionic strength or at 39°C) and are therefore not shown. These results demonstrate that neither of the tested VHHs causes an irreversible neutralization of the virus (as would be required for a hit-and-run mechanism).

FIG 5 Cryo-electron microscopy reconstructions of VHH-poliovirus complexes. (A and B) The isosurface rendering of poliovirus-PVSP6A (A) and poliovirus-PVSP29F (B) complexes. The outer isocontour surface of each complex is shaded by radius and depth. Poliovirus is in blue, and PVSP6A and PVSP29F are in purple and green, respectively. They both bind in the center of the 5-3-3 triangle (yellow). (C) Fourier shell correlation curves for poliovirus-PVSP6A (purple) and poliovirus-PVSP29F (green) complexes, processed using FREALIGN (29). (D) Intermolecular contact areas (yellow, 9 Å from the surface; red, 5 Å or closer) for poliovirus-PVSP6A, poliovirus-PVSP29F, and poliovirus-CD155. In all three complexes with virus, a similar portion of the virus surface is covered, and many of the same polypeptide segments are involved in binding.

FIG 6 Reversibility by dilution of poliovirus neutralization by VHHs. The reversibility of the neutralization by two VHHs (PVSP6A and PVSP29F) was tested by a plaque titration of diluted neutralized samples. The reversibility was tested under three different conditions: at 37°C or 39°C in a buffer with physiological ionic strength or at 37°C in a buffer with low ionic strength. The results from the plaque titration at 37°C in a buffer with physiological ionic strength are represented. It is seen that the amount of infectious particles (like the amount of plaques) increases after dilution of a sample containing VHH and virus, indicating a reversible neutralization by the VHHs. After this increase, the amount of plaques decreases, parallel to the amount of plaques in the untreated control samples.

DISCUSSION

Timing of neutralization. In the present study, five VHHs with an *in vitro* neutralizing activity against poliovirus type 1 were further investigated to determine the consequences of poliovirus-VHH binding, as well as the mechanism of neutralization of viral infectivity. All five of the VHHs are specific for the native (N-antigenic) form of the capsid, and none show any specificity for the expanded (H-antigenic) form (i.e., 80S particles) (20). The first step was to look at the time frame of action of the VHHs. As expected from their poliovirus capsid-binding properties, the VHHs are most effective when added at or near the time of addition of the virus to the cells (Fig. 1).

Virus binding to cells. We then measured the effect of the VHHs on the binding of poliovirus type 1 to HeLa cells (Fig. 3). The binding of specific VHHs to poliovirus, either at 4°C or 37°C, inhibits the subsequent attachment of poliovirus to cells. The results of the binding assays with the control VHH, Nb1, confirm that the effect observed for the other VHHs is due to their poliovirus-neutralizing (and binding) properties, rather than merely a result of the presence of the VHH as such. For the most effective of the VHHs, quantities of 45 VHHs per virion were sufficient to show a significant ($P < 0.005$) effect on cell attachment of the virus. This VHH-to-virus ratio was calculated based on the con-

centrations of the VHH and the virus determined using the absorbance at 280 nm and 260 nm, respectively, measured on a Nano-Drop spectrophotometer ND-1000. Presuming both statistical and alternating (hop-on, hop-off) binding of the VHHs to the poliovirus capsid, this would imply that not all binding sites need to be occupied to prevent receptor binding and a subsequent replication cycle. At 5°C, PVR/CD155 is known to bind to the capsid, though the majority of the interactions were found to be of a weak affinity mode (37). To allow this binding at 4°C/5°C, minor conformational changes are required (38). Presumably, these small changes would precede the major polypeptide rearrangements that accompany expansion of the virus to the 135S state. Thermodynamic studies (37) are also consistent with this picture, because in several picornaviruses, including poliovirus, the initial weak mode of receptor binding (which is stable at 4°C) is followed by a second, stronger binding mode (which is prevalent at 25°C), with both modes preceding the externalization of internal polypeptides that accompanies the expansion of the capsid to the 135S state (which occurs at 37°C). Possibly, binding of several VHHs to the capsid blocks such a conformational change necessary for receptor binding with icosahedral symmetry. A similar effect, preventing the binding of receptors to poliovirus, has previously been reported for capsid-stabilizing compounds that bind in the hydrophobic core of the VP1 beta barrel (38). As an exception, PVSS21E seems to hinder the cell attachment of poliovirus through a steric clash, as this VHH needs many more VHH copies per virion (between 450 and 4,500) to interfere with this attachment effectively.

A second, and in our view, more likely mechanism for inhibition of attachment comes from the prevention of cooperative activity in receptor binding. For most of the VHHs tested, the attachment of poliovirus to cells at 4°C, strong enough to withstand washing, was significantly inhibited at a concentration of ~45 VHHs per virion but not at ~4 per virion. Moreover, the VHH binding was shown to be reversible upon subsequent dilution. This furnishes an important clue to the mechanism of inhibition of binding, as this concentration is not sufficient to cover the viral surface and prevent the univalent attachment of PVR.

The relevant explanation may be that, in the absence of VHHs, viral attachment to cells normally involves the binding of several receptor molecules simultaneously. Bubeck et al. (39) and Bostina et al. (40) showed that most of the virions that bind to membranes bind pentavalently, with a viral 5-fold axis facing the membrane. Presumably, only a single receptor molecule is required for initial attachment, but the strength of attachment increases exponentially as additional receptors bind to it. Thus, the probability of all of the receptors releasing simultaneously is exceedingly small; and the virion remains localized at the cell surface and is able to rebinding individual receptors as long as any of the other receptors remain attached.

We propose, therefore, that VHH binding may weaken cell attachment by reducing the average number of PVR molecules that can bind simultaneously, which would have a dramatic effect on the association constant. Notably, the random binding of ~45 VHH monomers would prevent pentameric PVR binding from happening in more than 99.9% of virions. Since the binding of the VHHs is generally tighter than the receptor (dissociation constant [K_d] for VHHs, 6 to 84 nM [6]; K_d for PVR, 110 nM and 670 nM for strong and weak binding modes, respectively [37]), the displacement of the VHHs by the receptor is not likely to occur. It may also be relevant that multimeric binding of PVR to virions

causes a localized distortion of the membrane (39, 40) that might play a role in either the endocytosis or RNA transfer steps of infection subsequently; and proper umbilicus formation (14) may depend on the geometry of the initial virus-receptor complex. Thus, by disrupting the pentameric binding of PVR, VHHs could potentially affect infection at multiple stages.

Among the five specific VHHs that were tested, three of the VHHs (PVSS8A, PVSP19B, and PVSP29F) were significantly less effective at blocking attachment at 37°C but still remained more effective than the nonspecific control. (In particular, all three were still able to reduce the cell attachment by approximately 50% at a concentration of 0.1 μ M, being ~450 VHHs per PV.) Two of the VHHs (PVSP6A and PVSS21E) were just as effective at 37°C as at 4°C. The simplest explanation is that the native virus normally exhibits a balance between structural stability and instability. The balance can be shifted toward instability by (destabilizing) mutations or heat, and it can be shifted toward stability by other (stabilizing) mutations, by capsid-binding antiviral compounds, or by VHHs (to an extent that varies among the VHHs tested and is concentration dependent). Consistent with this, the simplest possibility is that the VHH block to receptor binding operates by preventing the initial binding event. We surmise that for those VHHs that need more copies per virion at 37°C, the elevated temperature delivers sufficient energy for the receptor to overcome an activation barrier and compete successfully for virion binding. These three VHHs can, however, still inhibit the receptor attachment, probably by steric hindrance, thanks to full occupancy of the receptor's binding sites on the poliovirus capsid. The differences observed between the five VHHs can be explained by their affinity for the poliovirus capsid. VHH PVSP6A has a K_d of 5.6 nM, while PVSS21E has a K_d of 83.5 nM. The K_d values of PVSS8A, PVSP19B, and PVSP29F are between these two extremes and are 31.4 nM, 7.3 nM, and 8.5 nM, respectively (20).

Formation of intermediate virus particles. To assess the effect of the VHHs on viral entry and uncoating during infection, the relative amount of 80S empty capsids and RNA-containing virions was analyzed on a sucrose density gradient (Fig. 4). Notably, the VHH that is the most effective at preventing infection (i.e., PVSP6A, having the lowest EC_{50}) is also the most effective at preventing 80S particles from forming. One possibility is that the block between the 160S and 80S forms occurs at the stage of 135S particle formation from 160S. That kind of block could prevent virus from becoming internalized into cells, as it was previously (41) suggested that poliovirus needs to be converted to its 135S conformation in order to be internalized. As a second possibility, the RNA release that changes the 135S particles into 80S empty capsids might be inhibited. This corresponds to the possibility that the virus gets internalized but the uncoating is blocked. Due to the fragility of 135S particles to freeze-thawing, the failure to see 135S particles in the sedimentation profiles does not rule out either alternative.

Temperature stability. The ability of VHHs to prevent conformational changes in the virion is also suggested by the stabilization of virions, by all five of the VHHs, against heat-induced degradation above 50°C (Table 1). In each case, the viral RNA genome is released at significantly higher temperatures than in a control sample. For each VHH, the temperature at which 50% of the RNA was released (ET_{50}) could be calculated. Ranking the VHHs by their ET_{50} values (PVSP6A < PVSS8A < PVSP19B < PVSS21E < PVSP29F) does parallel

the ranking by EC_{50} (PVSP6A < PVSP29F < PVSS8A < PVSP19B < PVSS21E), except for the position of PVSP29F in the series. The EC_{50} is the concentration of VHH at which 50% of the virus is neutralized as defined in a multicycle cytopathic reduction assay (6). Note, however, that the most effective stabilizers of the capsid at high temperature (those with the highest ET_{50} values) were the least effective at neutralization of infection (i.e., requiring the highest VHH concentration for neutralization). An analogous lack of a quantitative correlation between the stabilizing and neutralizing properties of an antiviral drug was previously described for the compound R61837 against rhinoviruses (42). Several factors could account for the lack of correlation, including the ability of the VHH to inhibit cell attachment of the virus, the ability to shift from low-affinity to high-affinity binding, and the ability to undergo the 135S transition.

Footprint. When looking at the footprints of PVSP6A and PVSP29F on the poliovirus surface (Fig. 5), they appear remarkably similar to the footprint of the natural poliovirus receptor. Thus, both the receptor and the VHHs bind to the same site in the depression ("canyon") at the center of the 5-3-3 triangle and make contacts with three discrete patches on the walls of the canyon. If a high copy number were required for neutralization, as in PVSS21E, this similarity could explain the interference of the VHHs at the level of cell attachment, as they are sterically blocking the interaction site of the receptor at the capsid. However, the ability of PVSP6A to block attachment with 45 VHHs per virion implies that steric blockage is not the only mechanism for the most effective of the VHHs. The possibility of preventing localized multivalent receptor attachment has been discussed above.

As another possibility, the key for this VHH (PVSP6A) may well lie in the ability of the virion to undergo concerted conformational transitions, where structural changes in some of the icosahedrally related copies are propagated to most or all of the other copies nearly simultaneously. When the virus first assembles, flexible polypeptide segments (from loops and terminal extensions) stabilize the virus by wrapping across the inner and outer surfaces of neighboring beta barrels. The localized binding of a relatively small number of receptor molecules catalyzes the detachment or rearrangement of a number of these polypeptides, an activation energy barrier is overcome, and a similar detachment or rearrangement is seen throughout the virus. In a similar way, we propose that the binding of PVSP6A stabilizes a small number of the movable polypeptide segments, locking them in place, and might be sufficient to prevent icosahedrally symmetric expansion. The similarity of the VHH and CD155 footprints on the virus surface suggests that many of the same polypeptide segments are bound in both cases. However, it is easy to imagine that the receptor might act to destabilize those polypeptide segments, leading to expansion and uncoating, while the VHHs might act instead to stabilize them.

A recent Fab-poliovirus structure (43) shows that this bulkier antibody binds higher in the canyon and is sterically hindered from reaching critical receptor binding sites at the base of the canyon. The smaller VHHs are able to enter the canyon and bind at its base, but unlike the Fab fragment, which neutralizes two serotypes of poliovirus, PVSP6A and PVSP29F neutralize only poliovirus type 1 (6).

Owing to the less-than-atomic resolution of the cryo-EM reconstructions, it is not yet certain what structures are bound by

VHHs, what structural changes occur upon receptor binding, and from a structural standpoint, exactly how the formation of 135S particles is triggered. Nevertheless, it is clear from the expanded structures of poliovirus (36), coxsackievirus A16 (CVA16) (44), enterovirus 71 (EV71) (45), and human rhinovirus 2 (HRV2) (46) that the GH loops of VP3 rearrange to protrude perpendicularly away from the virus surface, thereby opening a hole at the quasi-3-fold point to accommodate and anchor the externalized N terminus of VP1. Additionally, in poliovirus, the GH loop of VP1 and the C-terminal extensions of VP1 and VP2 become extensively disordered (36). These are among the most significant rearrangements on the outer surface of the capsid that accompany shifts of the beta barrels that act to separate neighboring protomers and expand the virus.

PVSP6A is one of the VHHs that blocked virus binding to the cell equally well at 4°C and at 37°C, whereas PVSP29F permitted more cell binding to occur at 37°C. This difference may be reflected in the footprints of these two VHHs, as PVSP6A overlaps more extensively with the receptor's footprint. Since the structures on the virus surface that are contacted by the two VHHs are not identical, it is certainly possible that either the areas of difference or the specific interatomic interactions are responsible for the differences.

Concerning the interference of the VHHs at the level of the formation of eclipse particles, the cryo-EM reconstructions of poliovirus-PVSP6A and poliovirus-PVSP29F complexes indicate that the VHHs bind to the polypeptide segments of poliovirus that rearrange during assembly to stabilize mature virus. Correspondingly, these same segments typically shift, rearrange, or disorder to allow the metastable virus to expand during the formation of 135S particles. The cryo-EM data are therefore indicating that the transition of 160S to 135S particles is likely to be blocked as long as VHH molecules remain bound.

Aggregation. Another interesting but rather puzzling observation is the formation of virus aggregates when treated with VHH. Classical antibodies (47, 48) can undergo a form of aggregation whereby the two antigen-recognizing arms of the antibody can bind to two different viral particles and, in doing so, create a network of poliovirus-antibody units. This cannot be the case for VHHs, so we considered the possibility that the VHH traps the virus in an (intermediate) breathing state (49), which may display more hydrophobic residues and thus have a tendency to aggregate. The cryo-EM reconstructions showed that the VHHs did not induce a conformational change in the capsid but bound to the native form of the virus. We were unable to decipher the cause of the aggregation, and further investigation is required to determine whether this plays any significant role in poliovirus neutralization.

Conclusion. In conclusion, the results presented in this paper show that all five single-domain antibody fragments tested (PVSP6A, PVSS8A, PVSP19B, PVSS21E, and PVSP29F) exert their *in vitro* neutralizing activity on poliovirus type 1 at multiple levels of the replication cycle. First, they prevent infection of the cell through inhibition of the cell attachment of the virus. The prevention of localized multivalent attachment or the blockage of a conformational change needed for receptor binding may well be factors. Second, the VHHs have a stabilizing effect on the capsid that may cause the VHHs to interfere at some level of the viral entry and uncoating process, likely by blocking the expansion of 160S particles to 135S. These mechanisms may well be shared by some conventional antibodies and may therefore provide some

insight into the natural immune responses. Finally, since the binding sites of two VHHs studied by cryo-EM are very similar to that of the receptor, the VHHs can be used as probes to study the authentic virus-cell interaction.

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