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Human monoclonal antibodies that neutralize vaccine and wild-type poliovirus strains

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ABSTRACT

An essential requirement for eradication of poliomyelitis is the elimination of circulating vaccine derived polioviruses (cVDPV) and polioviruses excreted by chronically infected individuals with immunodeficiencies (iVDPV). As part of a post-eradication risk management strategy, a human monoclonal antibody (mAb) therapeutic could play a role in halting excretion in asymptomatic carriers and could be used, in combination with vaccines and antiviral drugs, to protect polio-exposed individuals. Cross-neutralizing mAbs may be particularly useful, as they would reduce the number of mAbs needed to create a comprehensive PV therapeutic. We cloned a panel of IgG mAbs from OPV-vaccinated, IPV-boosted healthy subjects. Many of the mAbs had potent neutralizing activities against PV wild-type (WT) and Sabin strains, and two of the mAbs, 12F8 and 1E4, were significantly cross-reactive against types 1 and 2 and types 1 and 3, respectively. Mapping the binding epitopes using strains resistant to neutralization (escape mutants) suggested that cross-specific PV binding epitopes may primarily reside within the canyon region, which interacts with the cellular receptor molecule CD155 and the cross-neutralizing chimpanzee/human mAb, A12. Despite their close proximity, the epitopes for the 12F8 and 1E4 mAbs on Sabin 1 were not functionally identical to the A12 epitope. When tested together, 12F8 and 1E4 neutralized a diverse panel of clinically relevant PV strains and did not exhibit interference. Virus mutants resistant to the anti-poliovirus drug V-073 were also neutralized by the mAbs. The 12F8 and 1E4 mAbs may suitable for use as anti-PV therapeutics.

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1. Introduction

The worldwide polio eradication campaign may soon end the circulation of wild type polioviruses (PV). The primary weapon in this fight has been vaccination with the attenuated live oral polio vaccine (OPV), performed in mass campaigns conducted on a national and sub-national level, coupled with surveillance for acute flaccid paralysis (AFP) (De Jesus, 2007). OPV is generally safe, but an estimated 200–250 cases of vaccine-associated paralytic poliomyelitis (VAPP) occur annually as a result of OPV vaccination (WHO, 2002). Furthermore, due to the highly mutable nature of the PV genome and the processes of natural selection, OPV virus strains can mutate to become virulent circulating vaccine-derived polio viruses (cVDPVs), which can spread in populations with insufficient levels of immunity (Kew et al., 2005). Multiple outbreaks of cVDPV-associated poliomyelitis caused by viruses of all three serotypes have been observed (Burns et al., 2013; Estivariz et al., 2008; Kew et al., 2002; Liang et al., 2006; Rakoto-Andrianarivelo et al., 2008; Yan et al., 2010). Halting the emergence and circulation of cVDPVs is therefore an absolute requirement for a successful polio eradication strategy. This will require universal replacement of OPV by IPV (Dowdle et al., 2003).

Abbreviations: AFP, acute flaccid paralysis; CPE, cytopathic effect; cVDPV, circulating vaccine derived polio virus; iVDPV, immunodeficiency-associated vaccine-derived poliovirus; aVDPV, ambiguous vaccine derived poliovirus; HRP, horseradish peroxidase; IPV, inactivated polio vaccine; IVIG, intravenous immunoglobulin; mAb, monoclonal antibody; OPV, oral polio vaccine; PV, polio virus; TCID, tissue culture infectious doses; VAPP, vaccine-associated paralytic poliovirus; VDPV, vaccine-derived polio virus; WT, wild type.

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An additional complication is that individuals with B-cell immune deficiencies may become chronic excretors of virus, termed immunodeficiency-associated vaccine-derived polioviruses (iVDPVs), and shed these viruses for years (Khetsuriani et al., 2003). Multiple cases of isolation from the environment of highly-evolved vaccine polioviruses of ambiguous origin (aVDPV) have also been documented (Al-Hello et al., 2013). These strains may be excreted by unidentified chronic shedders or represent a long-term cryptic circulation of ambiguous vaccine-derived polioviruses (aVDPV). While stopping emergence of cVDPVs can be achieved by cessation of OPV use (Dowdle et al., 2003), dealing with the threat posed by iVDPV and aVDPV is not straightforward. The prevalence of chronic excretors in the population is currently unknown, and their identification will require better understanding of the biology of chronic infection and massive surveillance efforts. But, more importantly, there is no known cure that would enable them to stop shedding the virus (MacLennan et al., 2004). It has been proposed that antiviral drugs effective against PV be developed and used to clear these patients from PV infection (Council, 2006). Development and clinical evaluation of small-molecule drugs effective against PVs are underway (Collett et al., 2008). Use of IgG and/or IgA mAbs could complement drug treatment.

The PV-specific antibody response is critical to PV immunity (Burnet and Macnamara, 1931; Flexner and Lewis, 1910). In PV-infected patients, even low titers of PV-specific IgG provided by IVIG may provide protection against neuroparalytic activity (Hammon et al., 1954). Studies have suggested that intestinal anti-poliovirus IgA titers correlate with decreased viral shedding (Valtanen et al., 2000). However, little is known about the potential activity of antibodies against chronic infection. Administration of breast milk (a source of secretory IgA) to a subject with common variable immunodeficiency and chronic VDPV infection significantly reduced excretion, although the effect was transient (MacLennan et al., 2004). Repeated dosing of intravenous immunoglobulin (IVIG) to patients with B-cell immunodeficiencies does not lead to viral clearance. However, even after IVIG administration, the level of polio-neutralizing antibodies in these patients remains relatively low. High doses of PV-specific mAbs (either IgG or IgA) may be more effective than IVIG in clearing virus from immune deficient subjects.

Another potential use of human mAbs is emergency prophylaxis for non-immune exposed subjects. While there is no doubt that such treatment will offer protection from developing paralytic disease (Hammon et al., 1954), it is less certain that it can prevent them from being infected and transmitting virus. Immunization with IPV prior to viral challenge leads to reductions in the prevalence of virus shedding, the duration of excretion, and its intensity, but these effects are smaller than those provided by immunization with OPV (Hird and Grassly, 2012). Availability of additional human mAbs would open a unique opportunity to study this question and to determine whether a high level of neutralizing antibodies could protect subjects, not only from the disease, but also from infection and viral replication. The human antibody response to PV has not been adequately characterized, but evidence suggests that it generates antibodies with important neutralizing functions. In one study, 4 human mAbs were cloned from tonsil B-cells, and each of these mAbs neutralized PV *in vitro* (Uhlig and Dernick, 1988). Two of the mAbs neutralized type 1 and 2 strains, and another neutralized type 1, 2, and 3 strains.

Recently, we generated chimeric chimpanzee/human anti-PV mAbs by combining Ig variable domains isolated from bone marrow of immunized chimpanzees with the constant domain of human IgG (Chen et al., 2011). These mAbs exhibited neutralization activity *in vitro* and protected transgenic mice against lethal challenge with wild type PV, even after post-exposure administration. Because of the close relatedness between human and chimpanzee IgG, these antibodies are not expected to induce an immune response in humans. Furthermore, two of the mAbs were cross-neutralizing, confirming the existence of conserved epitopes that may be targeted by mAbs to create PV therapeutics that have broad strain specificity (Chen et al., 2011, 2013). In this study, we used a highly efficient human hybridoma technology (Adekar et al., 2008) to clone a panel of mAbs from OPV-immunized human subjects boosted with IPV (Adekar et al., 2008). We identified additional cross-neutralizing mAbs and used PV escape mutant studies to begin to characterize their binding sites. Their properties suggest that they may be used to create cross-neutralizing mAb cocktails suitable for clinical application.

2. Materials and methods

2.1. Human monoclonal antibodies

Human monoclonal antibodies were cloned following methods previously described (Adekar et al., 2008). Peripheral blood mononuclear cells from healthy donors 8 days following vaccination with IPV were stored frozen in 90% Hyclone Defined FBS (Invitrogen, Carlsbad, CA) and 10% DMSO (Sigma–Aldrich) under liquid nitrogen. Prior to cell fusion, CD27-positive cells were isolated with anti-CD27 magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions, and were cultured for 8 days on a monolayer of tCD40L cells (courtesy of Gordon Freeman, Dana Farber/Partners Cancer Care, Boston, MA) in IMDM supplemented with 10% FBS, IL4, IL10, transferrin, insulin, cyclosporine, and penicillin/streptomycin. The cultured cells were fused to the B5-6T heteromyeloma cell line and the nascent hybrid cells were selected with HAT (Sigma–Aldrich) in Advanced RPMI + 1% fetal calf serum. Hybridomas were stabilized by limiting dilution cloning, after which they were adapted to medium with 5% Ultra Low IgG fetal bovine serum medium (Life Technologies, Grand Island, NY), incubated for 5 days in a 500-ml roller bottle. Filtered supernatants were purified over protein G-Sepharose (Life Technologies). Antibody concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.2. Blocking ELISA

Antibodies with PV immunoreactivity in hybridoma supernatants were identified using a blocking ELISA, as previously described (Chen et al., 2011). Amounts of 0.5–1.0 D antigen units/ml of PV were captured on 96-well ELISA plates coated with purified rabbit polyclonal serotype-specific IgG (2 μg/ml). Then, some wells containing captured antigen were incubated with mAb or polyclonal serum (blocking reaction), while control wells were treated with normal rabbit serum. Wells with no antigen served as a background control. Anti-PV antibodies reacted with their respective antigenic sites and blocked them from subsequent reaction with biotin-conjugated polyclonal antipolio IgG (0.5 μg/ml). Next, ExtrAvidin-peroxidase conjugate (1:1,000 dilution; Sigma) was added, and the reaction of bound peroxidase with tetramethylbenzidine substrate (TMB, Sigma) resulted in the development of a color reaction. After the addition of a stop reagent (Sigma), the plate was scanned at 450 nm. The OD450 in the background wells that contained hyper-immune rabbit serum corresponded to 100% blocking activity, and the OD450 for antigen-containing wells incubated with normal rabbit serum corresponded to 0% blocking activity. The ratio of blocking activity of a specific MAb or serum with a particular antigen was calculated from the difference between the average ODs of blocked and non-blocked wells containing the same antigen.

2.3. Microneutralization test

PV-neutralizing mAb titers were determined in a microneutralization test (Chen et al., 2011; WHO, 2004). The mAbs were diluted to the indicated concentrations in maintenance medium (DMEM supplemented with 2% FBS and 1% antibiotic/antimycotic solution; Invitrogen) and sterilized by filtration through Spin-X columns (Corning, Corning, NY). The antibodies were tested at the concentrations indicated in the legends. Twofold serial dilutions of the mAbs (in triplicates) were incubated for 3 h at 36 °C with 100 50% tissue culture infectious doses ($TCID_{50}$) of the respective PV strain in an atmosphere of 5% CO_2. After the incubation, 1×10^4 HEp-2c cells were added to the wells. The plates were incubated for 10 days at 36 °C, 5% CO_2 and evaluated microscopically. Neutralizing mAb titers were calculated using the Kärber formula (WHO, 2004).

2.4. Generation of escape mutants

Fivefold dilutions of the mAbs, starting at 200 μg/mL, were incubated with 10^8 $CCID_{50}$ of virus for 1 h at room temperature, followed by 2 h at 36 °C. Virus samples were inoculated onto Hep-2C cell monolayers and incubated at 36° C until the cytopathic effect (CPE) developed. Supernatants after three freeze–thaw cycles were clarified by centrifugation. For cloning by plaque purification, serial dilutions of mAb-resistant viruses (10^1–10^4 $CCID_{50}$/mL) were inoculated onto Hep-2C monolayers in six-well plates, incubated for 1 h at room temperature, then replaced with 4 mL MEM/0.5% agarose overlay with 6% FBS (all reagents from Invitrogen). Plaques were picked after 48 h of incubation at 36 °C, 5% CO_2, transferred into 12-well plates with confluent monolayers, and incubated until CPE developed. Supernatants after three freeze–thaw cycles were clarified by centrifugation and nucleotide sequences were determined by deep sequencing. Briefly, random cDNA libraries were prepared and subjected to Illumina sequencing using the MySeq instrument. Individual 150-bp-long sequence reads were aligned against a representative set of ~300 full-length enteroviruses, and assembled into quantitative SNP profiles indicating positions of mutations or sequence heterogeneity. Virus grown in the same experiment without antibodies was used as a control.

3. Results

3.1. Isolation and PV binding activity of human mAbs that neutralize PV

Peripheral blood mononuclear cells were obtained from adults 8 days following IPV vaccination. Each had received OPV in childhood. Three cell fusions were performed, resulting in the 10 IgG mAbs listed in Table 1. All mAbs were IgG1; six had lambda light chains (6D11, 6D2, 7A1, 7E2, 8A12, and 1E4), and four had kappa light chains (8F9, 2H5, 12F8, 2F3).

The binding of each mAb in hybridoma supernatants was assessed against the three Sabin vaccine strains and the three wild type PV strains used in the manufacture of IPV: Mahoney (type 1), MEF-1 (type 2), and Saukett (type 3). We used a competition (blocking) ELISA, in which we measured the ability of IgGs to compete for binding of a PV antiserum to whole PV particles. The readout of the reaction was the ratio of the reduction in binding caused by the mAbs compared to that seen with the positive control hyperimmune rabbit serum (Table 1). Many of the mAbs exhibited measurable binding, and three of the mAbs showed multi-strain reactivity. 1E4 bound to type 1 and 3 strains, whereas both 2H5 and 12F8 bound to type 1 and 2 strains. There was also a strong concordance between binding of vaccine and wild-type strains, such that mAb binding to particular Sabin strains correlated with binding to the homologous wild-type strains. One mAb (2F3) exhibited a weak activity against wild-type PV strains of type 2 and 3, but the hybridoma lost mAb expression and was excluded from further study.

3.2. Neutralization of vaccine and wild-type poliovirus strains

The *in vitro* PV neutralizing activity by purified mAbs was measured using the micro-neutralization test. In Table 2, neutralizing activity of the noted PV strains is recorded as the reciprocal of the highest dilution of 1 mg/ml mAb solution protecting 50% of the cell cultures against 100 $TCID_{50}$ of virus. Each of the mAbs showed some neutralizing activity, although the range of titers was substantial, from 100 (12F8 against Saukett) to 1,459,600 for 7E2 against Saukett). Three of the mAbs were active against more than one strain type. The most potent, 12F8, had titers for Sabin 1: 57,500; Mahoney: 1,158,500; Sabin 2: 57,500; and MEF1: 229,900. These were comparable to what we previously measured for the A12 mAb, Sabin 1: 102,400; Mahoney: 121,800; Sabin 2: 121,800; and MEF1: 172,200 (Chen et al., 2011). For each of the new mAbs tested, there appeared to be a bias with equivalent or higher neutralization titers against WT compared to the homologous Sabin strains, the only exceptions being 1E4 (Sabin 3 57,500 vs. Saukett 17,800) and 6D2 (Sabin 3 14,000 vs. Saukett 7,200).

3.3. Neutralization of escape mutant PV strains

The potent neutralizing activities of the 8A12, 1E4, 12F8, and 8F9 mAbs, and the cross-serotype neutralization of 1E4 and 12F8, suggested that they may be useful as antiviral therapeutics, potentially in combination with the two cross-neutralizing

Table 1
Human mAbs tested in a competition binding ELISA with attenuated and wild type polioviruses.

Antibody	Isotype	Type 1		Type 2		Type 3	
		Sabin 1	Mahoney	Sabin 2	MEF-1	Sabin 3	Saukett
6D11	IgG-λ		0.02 ± 0.00		0.01 ± 0.00	0.37 ± 0.02	0.51 ± 0.02
6D2	IgG-λ		0.04 ± 0.00		0.01 ± 0.00	0.41 ± 0.02	0.26 ± 0.01
7A1	IgG-λ				0.01 ± 0.00	0.47 ± 0.03	0.57 ± 0.02
7E2	IgG-λ				0.04 ± 0.00	0.40 ± 0.02	0.56 ± 0.02
8A12	IgG-λ	0.46 ± 0.06	0.42 ± 0.04		0.01 ± 0.00	0.04 ± 0.00	
1E4	IgG-λ	0.44 ± 0.05	0.45 ± 0.04	0.03 ± 0.00	0.09 ± 0.00	0.26 ± 0.01	0.07 ± 0.00
8F9	IgG-κ	0.02 ± 0.00	0.01 ± 0.00	0.90 ± 0.02	0.77 ± 0.02	0.01 ± 0.00	
2F5	IgG-κ	0.15 ± 0.02	0.25 ± 0.02	0.09 ± 0.00	0.12 ± 0.00	0.01 ± 0.00	
12F8	IgG-κ	0.57 ± 0.07	0.43 ± 0.04	0.79 ± 0.02	0.70 ± 0.02	0.00 ± 0.00	0.04 ± 0.00
2F3	IgG-κ	0.02 ± 0.00	0.20 ± 0.02		0.07 ± 0.00	0.01 ± 0.00	0.13 ± 0.00
HIRS		1.00 ± 0.12	1.00 ± 0.09	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.06	1.00 ± 0.03

Note: Data are reported as the ratios of blocking observed with the human mAbs relative to hyper-immune rabbit serum (HIRS).
Empty cells refer to zero values.

Table 2
Neutralization activity of monoclonal antibodies.

Strain	Type 1		Type 2		Type 3	
mAb	Sabin 1	Mahoney	Sabin 2	MEF1	Sabin 3	Saukett
6D11					364,900	1,158,500
6D2					14,400	7200
7A1					91,200	444,800
7E2					459,800	1,459,600
8A12	182,500	364,900				
1E4	45,600	91,200		200	57,500	17,800
8F9			364,900	1,158,500		
2H5	300	900	200	200		
12F8	57,500	1,158,500	57,500	229,900	200	100

The mAbs were tested in the poliovirus microneutralization assay of serial dilutions, and the results presented in neutralization titers recalculated per 1 mg of protein. The highest antibody concentration tested in the assay was 10 μg/ml, therefore antibody – virus combinations represented by empty cells may have neutralization activity of less than 100.

chimpanzee-human antibodies that we previously isolated (A12 and F12) (Chen et al., 2011, 2013). We assessed the ability of these new mAbs to neutralize a panel of escape mutant strains generated against A12, F12, and other mAbs (Table 3). Microneutralization tests were performed with 100 $TCID_{50}$ of the escape mutant PV strains used as challenge viruses, reported as the PV neutralizing titers (reciprocal of the highest dilution that protects 50% of cell cultures from viral challenge) (Table 4).

The 8A12 mAb, which was specific only against serotype 1, neutralized equally well both Sabin 1 virus and escape mutants resistant to neutralization by the A12 and H2 chimpanzee/human mAbs, as well as the murine mAb, 1o. This suggests that 8A12 does not bind antigenic site 1 (H2 mAb), antigenic site 3 (1o) or the new antigenic site located in the canyon that is used by cross-neutralizing mAb A12 (Chen et al., 2013). The two bi-specific human mAbs, 1E4 and 12F8, had reduced neutralization of two of the Sabin 1 A12 escape mutants, ES15-1/5 and ES15-1/6, but efficiently neutralized the H2 and 1o mutants. The 12F8 fully neutralized all of the Sabin 2 mutants, including the A12 E15-6/3 mutant, as well as those generated against the human/chimpanzee mAbs F12, A6, and B2. This is in contrast to the Sabin 2-specific human mAb 8F9, which had minimal activity against two of the A6 escape mutants, but was equipotent against the A12, F12, and B2 mutants. 8F9 and A6 likely share the same epitope, which is distinct from the cross-reactive epitope bound by 12F8 and may include arginine at position 109 of VP1. Taken together, these observations suggest that the cross-specific epitopes recognized by 12F8 and 1E4 on Sabin 1 only partially overlap the A12 epitope. Furthermore, the epitopes bound by A12 and 12F8 on Sabin 2 appear to be functionally distinct.

We next quantified the neutralization titers of the mAbs against the A12 escape mutants (Table 5). The results are shown as the reciprocals of the highest mAb dilutions that protect 50% of the cell cultures against challenge with 100 $TCID_{50}$ of A12 escape mutant PV strains or corresponding Sabin strains, starting from a 5 μg/ml concentration (Chen et al., 2011, 2013). The 12F8 retained activity against the A12 mutants of type 1 PV ES15-1/5 and ES15-1/6 and neutralized escape mutants of type 2 PV ES15-6/3 even better than Sabin 2. Both mono-specific mAbs, 8A12 and 8F9, were fully active against their respective Sabin and mutant strains (type 1 and type 2, respectively).

Table 3
Escape mutant strains used in the present study (Chen et al., 2011).

Antibody used for escape generation	mAb source*	Parental strain	Escape strain name	Mutation(s)
A12	H/C	Sabin 1	ES15-1/5	VP1:V166E
A12	H/C	Sabin 1	ES15-1/6	VP1:V166E, I90M
A12	H/C	Sabin 2	ES15-6/3	VP1:G225D
H2	H/C	Sabin 1	ES13-3	VP1:K144R
H2	H/C	Sabin 1	ES13-5	VP1:K144R, D288N
H2	H/C	Sabin 1	ES13-10	VP1:V107A, D288G
1o	M	Sabin 1	EX4-1	VP1:D298N VP3:S58C
1o	M	Sabin 1	EX4-2	VP1:D298G, VP3:D181Y
1o	M	Sabin 1	EX4-5	VP1:D298G VP1:L234V
F12	H/C	Sabin 2	ES17-4/3	VP1:K109E
F12	H/C	Sabin 2	ES17-4/10	VP1:K109E VP1:H65R
A6	H/C	Sabin 2	ES15-9/1	VP1:R100C VP1:H65R
A6	H/C	Sabin 2	ES15-10/3	VP1:R100L
B2	H/C	Sabin 2	ES15-13/2	VP1:A101D VP2:N165S
B2	H/C	Sabin 2	ES15/13/5	VP1:A101D VP4:I62T

3.4. Escape mutants of human anti-PV mAbs

Because cross-neutralizing mAbs will be most useful as PV therapeutics, we generated escape mutant PV strains for the 1E4 and 12F8 mAbs (Table 6). For comparison, we also analyzed the most potent mono-specific mAbs, 8F9 (type 2) and 7E2 (type 3). Resistant virus populations were cloned and sequenced. The locations of amino acids in which mutations were observed are shown in Fig. 1. The residues that, when mutated, affected binding of the mono-specific 7E2 (VP1:98R) and 8F9 (VP1:100R and VP1:223S) mAbs are located in the B-C loop that constitutes antigenic site 1. In contrast, amino acids involved in binding of cross-neutralizing antibodies 1E4 (VP1:105M) and 12F8 (VP1:233S and VP3:234K) reside deeper within the canyon domain, similar to the epitope of the A12 mAb (Chen et al., 2013).

3.5. Neutralization of a panel of type 1 poliovirus strains

An essential requirement for effective antiviral therapy is broad activity against a variety of clinically relevant strains, and cross-neutralizing mAbs may be ideal for this purpose. We tested the neutralization activity of the 12F8 and 1E4 mAbs against a panel of patient-derived type 1 PVs that included WT strains, iVDPVs and an aVDPV (courtesy of Drs. Olen Kew and Merja Roivainen) (Supplemental Table S1). Fig. 2 shows that both mAbs had activity against all of the PV strains, but the 1E4 mAb had more consistent neutralization titers across the panel of strains than 12F8. These results suggest that a combination of these mAbs would provide a uniform protection against all strains. In addition, this observation points to a slightly different mechanism of binding for the two mAbs, suggesting that their use in combination may reduce the emergence of resistant strains.

To determine whether combining of the antibodies would improve their ability to neutralize poliovirus rather than lead to interference, we used representative strains from the wild-type and iVDPV PV panel as challenge viruses in the PV neutralization assay (Fig. 3). The strains were chosen according to the differences in neutralizing titers between the antibodies (even titers or different; Fig. 2). We compared 5 μg of single mAbs to 2.5 + 2.5 μg of the mAb combinations. Neutralization of the viruses with mixture of the two antibodies appeared to additive and consistently demonstrated no interference (Fig. 3), suggesting that the antibodies could be used together in therapeutic cocktails.

Table 4
Neutralization of escape mutant poliovirus strains by the monoclonal antibodies.[§]

Type 1	Escape mutants to monoclonal antibody								
	A12		H2			1o			Sabin 1
Monoclonal antibody	ES15-1/5	ES15-1/6	ES13/3	ES13/5	ES15/10	EX4-1	EX4-2	EX4-5	
8A12	>91	>91	>91	>91	>91	>91	>91	>91	>91
1E4	57	45	>91	>91	>91	>91	>91	>91	>91
12F8	36	29	>91	>91	>91	>91	>91	>91	>91
Control*	<1	<1	<1	<1	<1	<1	<1	<1	

Type 2	Escape mutants to monoclonal antibody							
	A12	F12		A6		B2		Sabin 2
Monoclonal antibody	ES15-6/3	ES17-4/3	ES17-4/10	ES15-9/1	Es15-10/3	Es15-13/2	Es15-13/5	
8F9	>91	>91	>91	<1	1	>91	>91	>91
12F8	>91	>91	>91	>91	>91	>91	>91	>91
Control*	<1	<1	<1	<1	<1	<1	<1	

§ The mAbs (10 μg/ml) were tested in the poliovirus microneutralization assay. The results are reciprocals of the highest antibody dilution that protects 50% of the cell cultures against challenge with 100 TCID50 of A12 escape mutant poliovirus strains (Table 3) or corresponding Sabin strains.
* Controls are antibodies that was used to generate respective escape mutant strain of poliovirus.

Table 5
Dual-reactive monoclonal antibodies neutralize A12 mAb escape mutant virus strains[§].

mAb	Type 1			Type 2	
	ES15-1/5	ES15-1/6	Sabin	ES15-6/3	Sabin
8A12	912	724	912		
1E4	57	29	287		
12F8	45	9	144	2299	287
8F9				5793	1825
A12	<1	<1	144	<1	181

§ The mAbs were tested (the highest starting concentration of 5 μg/ml) in the poliovirus microneutralization assay. The results are reciprocals of the highest antibody dilution that protect 50% of the cell cultures against challenge with 100 TCID50 of A12 escape mutant poliovirus strains or corresponding Sabin strains. The empty cells refer to tests not performed because the antibody does not react with respective serotypes of poliovirus.

Table 6
Human mAb escape mutant PV strains.

Antibody used for escape generation	Parental strain	Escape strain name	Mutation(s)
1E4	Sabin 3	HH2-1/12	VP1:M105S
12F8	Sabin 1	HH2-2/9	VP1:S233I, S233T, VP3:K234R
8F9	Sabin 2	HH2-1/13	VP1:R100I, S223I
7E2	Sabin 3	HH2-1/7	VP3:R98Y

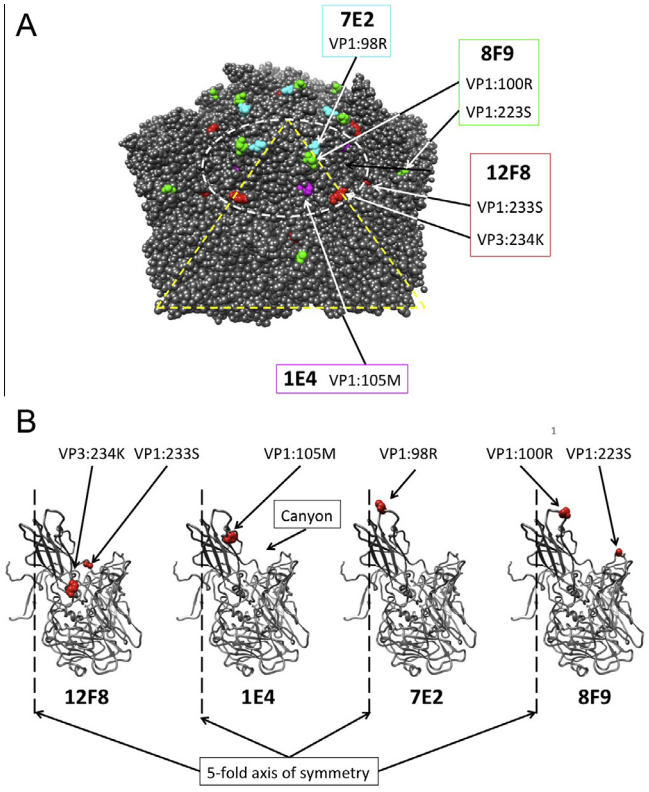

Fig. 1. Location of amino acids involved in binding of human mAbs to the surface of PV strains. Involved amino acids were identified by the generation and characterization of PV escape mutants. The single-serotype specific 7E2 and 8F9 mAbs interact with residues in antigenic site 1, where the cross-specific 1E4 and 12F8 mAbs bind residues in the canyon domain, which also interacts with the PV cellular receptor, CD155 (Belnap et al., 2000). Details of the interacting residues are shown here and summarized in Table 6. (A) A view of the face of the PV virion. The white circle shows the canyon and the yellow triangle connects the 5-fold axis of symmetry with the two 3-fold axes of symmetry. (B) A view of single domains within the 5-fold axis of symmetry, which clearly shows the relationship of the amino acids to the canyon.

3.6. Neutralization of strains resistant to anti-poliovirus compound V-073

One of the potential clinical applications of anti-PV mAbs is co-administration with antiviral small molecule drug(s) to treat chronic excretors of PV. It is important to determine whether strains that acquire resistance to drug also become resistant to neutralization by mAbs. One small molecule, V-073 (ViroDefense, Rockville, MD), has shown clinical efficacy against PV (Collett et al., 2008), although it may induce the emergence of drug-resistant variants. We tested several V-073 resistant strains of type 1 and type 2 PV for their ability to be neutralized by the human mAbs, measuring the reciprocal neutralization titer of a 5 μg/ml mAb solution (Kouiavskaia et al., 2011; Liu et al., 2012). As shown in Table 7, the drug-resistant phenotype had no effect on the neutralization titers of the 12F8, 6D11, 6D2, 7A1, 7E2, 8A12, 1E4, and 8F9 mAbs against these viruses, relative to their activity against the corresponding Sabin strains. This indicates that PV generates resistance to V-073 and our cloned mAbs through different mutations, supporting their use in combination therapy.

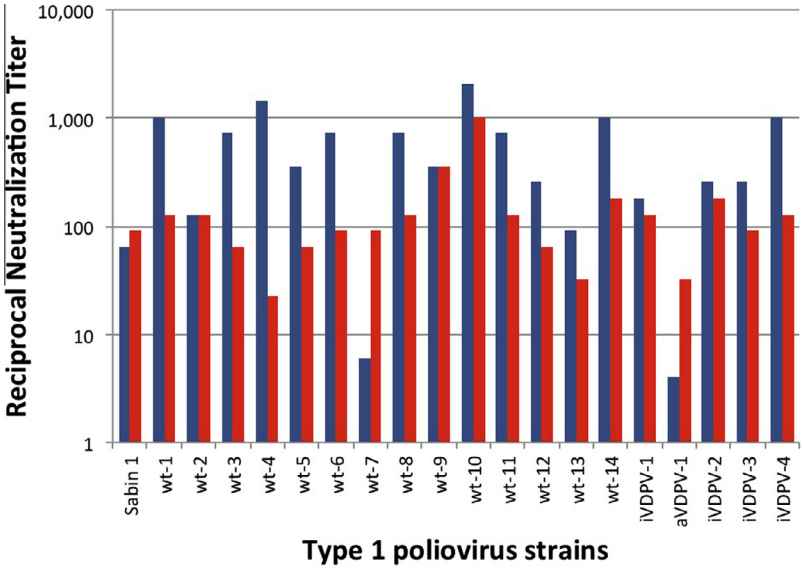

Fig. 2. Neutralization of a panel of type 1 poliovirus strains with 12F8 and 1E4 mABs. The reciprocal of the neutralization titer for the two mAbs are shown 1E4 (red) and 12F8 (blue). The identities of the viral strains are given in Supplemental Table 1.

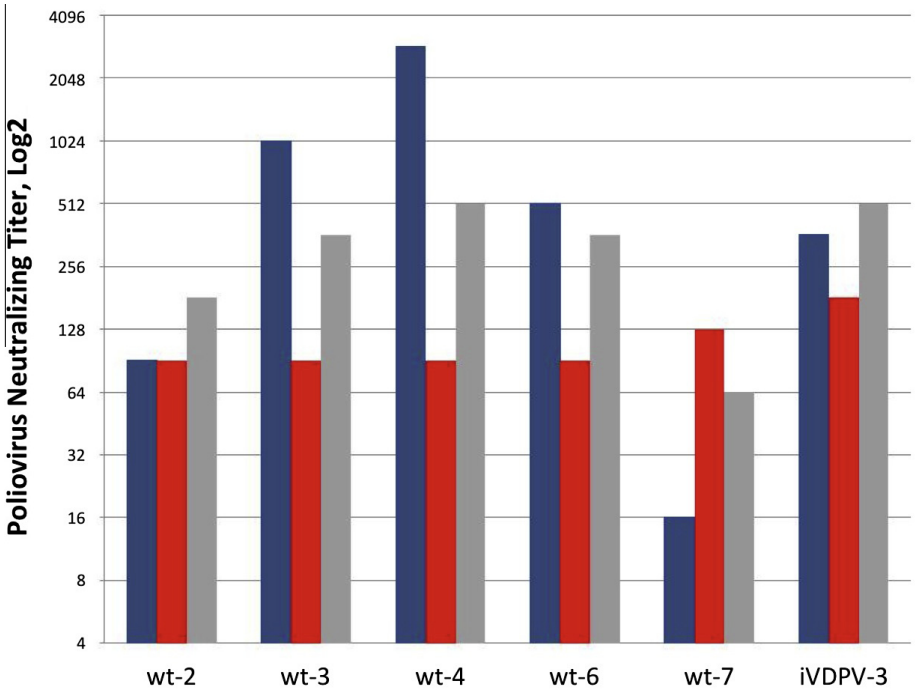

Fig. 3. Neutralization of representative wild type polioviruses by monoclonal antibodies 1E4 (red columns) and 2F8 (blue columns) and their mixture (gray columns). The antibodies were tested at a total of 5 μg/ml individually or as a mixture of equal amounts of the antibodies (i.e. 1 mAb at 5 μg/ml or 2 mAbs at 2.5 + 2.5 μg/ml). The results are presented as reciprocal of the highest antibody dilution protecting 50% of wells from poliovirus PV.

4. Discussion

We have cloned a panel of human IgG mAbs capable of neutralizing PV. They were cloned using peripheral blood B-cells of OPV-vaccinated individuals, following an IPV boost, with an improved human hybridoma method (Adekar et al., 2008). The mAbs had potent neutralizing activities against WT and Sabin strains of PV, and three of the mAbs, 12F8, 2H5, and 1E4, were reactive against more than one serotype (types 1 and 2 for 12H8 and 2H5 and types 1 and 3 for 1E4). This observation is in line with previous finding that cross neutralizing, anti-PV antibodies may be more prevalent in primates that in rodents (Chen et al., 2013; Uhlig and Dernick, 1988).

It appears that the cross-neutralizing mAbs bind the same general region that was previously shown to contain the epitope of the cross-neutralizing chimpanzee/human mAb, A12, as well as the cellular PV receptor CD155. Antibodies targeting the receptor-recognition site can be expected to have broader specificity, because this region is relatively conserved among all PVs to enable binding to the same cellular receptor. Despite that, the binding mechanism

Table 7
Neutralization of V-073-resistant polioviruses by human monoclonal antibodies. Results expressed as a reciprocal neutralization titer of 5 μg/ml antibody solution.

Monoclonal antibody	Type 1				Type 2			Type 3			
	10235	10235.1	10235.3	Sabin 1	10230.4	10230.8	Sabin 2	10805	10805.1	10805.3	Sabin 3
6D11	<1	<1	<1	<1	<1	<1	<1	724	512	1024	724
6D2	<1	<1	<1	<1	<1	<1	<1	32	45	45	64
7A1	<1	<1	<1	<1	<1	<1	<1	512	256	362	512
7E2	<1	<1	<1	<1	<1	<1	<1	1448	1024	362	1024
8A12	724	1024	1448	362	<1	<1	<1	<1	<1	<1	<1
1E4	256	362	256	128	<1	<1	<1	362	512	256	362
8F9	<	<	<	<	1448	1448	1024	<1	<1	<1	<1
12F8	181	256	256	181	181	181	256	1	1	1	1

for these cross-neutralizing mAbs may be different, resulting in incomplete functional overlap of their epitopes. Thus, escape mutants generated against A12, which had completely lost the ability to be neutralized by A12, could still be neutralized by 12F8 and 1E4. Further evidence of the differences between the epitopes comes from the results on neutralization of a panel of WT and vaccine-derived PV strains by mAbs specific to types 1 and 2 and types 1 and 3. The patterns of sensitivity to these mAbs differed, and strains that had diminished ability to be neutralized by 12F8 were readily neutralized by 1E4, suggesting that their use in combination could protect against a wide range of strains, and potentially minimize the emergence of resistant strains.

Sequencing of escape mutants generated against the new mAbs showed that the mutations rendering virus resistant to neutralization were located in the canyon region, consistent with the location of the epitope for the previously published mAb A12 (Chen et al., 2013). In contrast to cross-neutralizing mAbs, human mAbs specific to just one serotype bind another region that is located at the tip of the so-called B-C loop in the VP1 region, which was previously identified as antigenic site 1, as well as the southern rim of the canyon (antigenic site 2). Therefore, the difference between cross-neutralizing and mono-specific mAbs may correlate with the depth to which they can penetrate the canyon. Direct studies conducted by cryo-EM could help identify the exact mechanism of neutralization by these mAbs.

Human mAbs could be used in attempts to stop chronically infected individuals from shedding PV. As indicated above, low concentrations of anti-PV antibodies supplied to these patients in the form of IVIG does not lead to viral clearance. The high specific potency of human mAbs suggests that their administration at moderate doses could lead to neutralizing titers in blood that are 100–1000 higher than titers achievable by immunization. This extremely high dose may help these patients to clear chronic infection.

Another potential application of human mAbs is for emergency prophylaxis of non-immune individuals exposed to PV. Our previous studies with the A12 and H2 mAbs, conducted in susceptible transgenic mice exposed to PV, demonstrated effectiveness even if the mAbs were administered after virus challenge (Chen et al., 2011). Studies conducted more than 60 years ago showed that administration of even small doses of antibodies can protect against paralysis (Hammon et al., 1954). However, the presence of serum neutralizing antibodies induced by IPV immunization does not fully protect vaccine recipients from PV infection and excretion. It would be interesting to study whether extremely high levels of neutralizing antibodies could protect individuals from infection.

Due to the rapid mutation rate of PVs, an antiviral strategy utilizing mAbs will require two or more non-cross-resistant mAbs for each serotype. The identification of non-cross-resistant mAb pairs that bind to multiple PV types could simplify the development of a PV mAb therapeutic. The current study has identified a candidate combination of the A12 and 12F8 mAbs, which are non-cross resistant and may be effective against type 1 and 2 WT or cVDPVs. This combination could potentially be effective against the majority of cVDPVs. We have also identified a number of mAbs with potent activity against type 3 strains (6D11, 7A1, 7E2, 1E4), from which a type 3 specific antiviral combination potentially may be constructed. In testing with a panel of patient-derived type 1 and circulating PV strains, we also found that the 12F8 and 1E4 cross-neutralizing mAbs could complement each other to broaden the spectrum of susceptible strains, arguing in favor of using these mAbs in the form of a cocktail that could neutralize all three serotypes of PV. When used together, 12F8 and 1E4 operate additively, rather than synergistically, and do not exhibit interference. Mutant strains resistant to the anti-PV drug V-073 were fully neutralized by the human mAbs. This suggests that a mAb cocktail could be used in combination with V-073 to reduce the emergence of drug-resistant strains.

Some of the most expensive drugs presently used in medicine are mAbs. However, dramatic improvements in the cost of mAb production, the high neutralization titers achieved with mAbs, and a development model that relies on non-profit organizations should make it possible to create mAb therapeutics affordable for use in the developing world. Nonetheless, significant questions need to be addressed to determine the utility of mAb therapeutics for control of PV in the post-OPV era. Will high doses of mAbs be able to clear chronic infection? Are mAb combinations able to prevent infection with WT and vaccine-derived PV and reduce shedding? What would be the ideal mAb format (IgG, IgA) and method of administration (parenteral or oral)? It is also important to find out how far into the incubation period the post-exposure prophylaxis by human antibodies can be effective. All these questions must be answered in clinical studies or in an appropriate animal model. In conclusion, we have cloned a panel of human mAbs that have potent anti-PV neutralizing activities. Two of these, 12F8 and 1E4, have cross-neutralizing activity and may be useful, in combination with A12 or other mAbs, to develop antiviral drugs to control PV in the post-OPV era.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.05.005.

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