

Detection of persistent polioviruses in patients with the post-polio syndrome

Andreina BAJ, Giuseppe MACCARI, and Antonio TONIOLO

Laboratory of Medical Microbiology, University of Insubria Medical School
Viale Borri 57, 21100 Varese, Italy

Polioviruses (PVs) are the etiologic agents of acute paralytic poliomyelitis. Decades after being hit by the virus, polio survivors may develop the so-called “post-polio syndrome” (PPS), a progressive condition characterized by chronic fatigue and pain, new muscular weakness, and cold intolerance. The etiology and pathogenesis of PPS are unknown, thus empirical therapies are used. Current data suggest that PV genomes may persist for decades in the central nervous system of affected patients. Detection and sequencing of persisting PV genomes is pivotal for three reasons: a) investigating the hypothetical etiological link between virus persistence and PPS; b) setting up reliable molecular tests for diagnosis and evaluation of disease progression; c) developing etiologic treatments aimed at virus eradication. Using molecular tests, tissue culture studies and immunofluorescence with PV-specific antibody, PVs have been detected in 16/16 samples from PPS patients and in 0/16 samples from control patients. Genome sequence analysis revealed that the persisting PV isolates are markedly different from the reference PV strains. In addition, the activity of potentially effective antivirals has been investigated against type-1 PV.

Keywords: Etiology, Pathogenesis, Enteroviruses, Genome detection, Virus antigen, Antiviral drug

1. Introduction

One of the most relevant tasks of modern medicine is to understand whether some “idiopathic diseases” (i.e., diseases whose origin is unexplained) were of infectious origin [1]. Inclusion of a disease into the “infectious pathology group” would represent a significant progress, since this type of knowledge has been usually followed by enormous advancements in diagnosis, prevention and therapy.

Over the world, polio survivors are now experiencing increased physical hardship as they enter their middle and later years due to the onset of the Late Effect of Polio (LEP) symptoms that are also known as Post-Polio Syndrome (PPS). Foremost amongst these difficulties is reduced mobility, leading to problems in the performance of activities integral to a full life. In many cases, external assistance is required for personal care and mobility aids for performing basic tasks. It cannot be stressed enough that these are not age-related problems. Currently, PPS is included into the group of “idiopathic diseases”, since the development of fatigue and new neuromuscular symptoms in polio survivors could not be linked to any specific cause.

2. Polioviruses and the consequences of infection

2.1 Polioviruses: biological properties and current taxonomy

The three poliovirus (PV) serotypes (PV-1, -2, and -3) belong to the Enterovirus genus of the *Picornaviridae* family. Virions are non-enveloped icosahedral particles, 28 nm in diameter. As shown in Figure 1, the genome consists of a single-stranded, positive sense RNA of about 7.4 kb, with a 22-aa virus-encoded protein (3B, VPg) covalently linked to the 5' end. The 5' non-translated region (approximately 740 nt) has a complex secondary structure representing the internal ribosome entry site. The single open reading frame encodes a polyprotein of about 2,200 amino acids that is processed to yield four different capsid proteins (VP1, VP2, VP3, and VP4), a protease 2Apro, proteins 2B and 2C,

the 3BVPg precursor (3AB), the major viral protease (3Cpro), and the RNA-dependent RNA polymerase (3Dpol). The 3' non-translated region (approximately 70 nt) contains a poly-A tail of variable length [2].

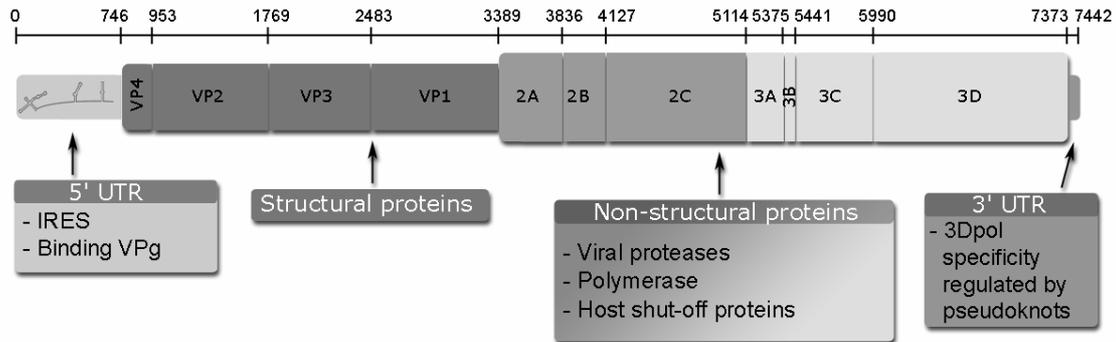


Figure 1. Schematic view of the poliovirus genome.

Human enteroviruses (HEVs; at least 92 serotypes) have been recently reclassified, based largely on genome structure [3]. As shown in Table 1, the Enterovirus genus consists of five species: PVs, human enterovirus A (HEV-A), B (HEV-B), C (HEV-C), and D (HEV-D). Humans are the only recognized hosts of PVs. Person-to-person transmission occurs mainly through the fecal-oral route.

Table 1. Classification of human enteroviruses (HEVs) within the *Picornaviridae* family.

Genus	Species (No. of serotypes)	Serotypes
Enterovirus	PV (3)	PV serotypes 1, 2, 3
	HEV-A (17)	CVA serotypes 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16; EV-71, 76, 89, 90, 91, 92
	HEV-B (56)	CVB serotypes 1,2, 3,4,5,6, CVA9 Echo serotypes 1, 2, 3, 4, 5, 6, 7, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 24, 25, 26, 27, 29, 30, 31, 32, 33; EV-69, 73, 74, 75, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 93, 97, 98, 100, 101
	HEV-C (13)	CVA serotypes 1, 11, 13, 17, 19, 20, 21, 22, 24,, EV-95, 96, 99, 102
	HEV-D (3)	EV-68, EV-70, EV-94

Note: HEV-A: human enterovirus group A; HEV-B: human enterovirus group B; HEV-C: human enterovirus group C; HEV-D: human enterovirus group D; CVA, coxsackievirus A group; CVB, coxsackievirus B group; Echo, echovirus; EV, enterovirus; PV, poliovirus; SVDV, swine vesicular disease virus (a variant of CVB-5).

2.2 Acute PV infection: poliomyelitis

Poliomyelitis is an acute disease caused by infection with any one of the three PV serotypes. The virus multiplies in the pharynx and intestine for one to three weeks. In the majority of cases, virus spread is contained by a local immune response. Thus, over 95% of infections are either asymptomatic or characterized by flu-like symptoms. In 5% of the cases, a viremic phase occurs and virus reaches the central nervous system (CNS) through the blood. Patients may develop a meningitis-like illness characterized by fever with pharyngitis, myalgia, anorexia, nausea, vomiting, headache, and neck stiffness. The onset of spinal poliomyelitis is associated with myalgia and severe muscle spasms, with

the subsequent development of an asymmetrical (predominantly lower limb) flaccid weakness that becomes paretic within a few days. A purely bulbar form with minimal limb involvement may also occur. This form of polio has a particularly high mortality because of vasomotor disturbances and other complications (hypertension, hypotension and circulatory collapse, autonomic dysfunction, dysphagia, dysphonia, respiratory failure). In the epidemics of the last century, most paralytic cases were attributed to PV -1 [4]. Epidemics of polio occurred throughout the United States, including one severe outbreak from 1943 to 1956 in which 400,000 people were infected, resulting in 22,000 deaths. The introduction of Jonas Salk's inactivated polio vaccine (1955) and Albert Sabin's live oral vaccine (1961) dramatically reduced the number of cases. By 1965, only 61 infections were reported in the USA and by 1991 the disease was virtually wiped out in the Western Hemisphere. New poliomyelitis cases are now reduced to less than 2,000 per year. These cases are restricted to residual endemic areas such as parts of India, Nigeria, Pakistan and Afghanistan, and to areas that had interrupted transmission but were re-infected and now have continued circulation of wild poliovirus (Ethiopia, Somalia and Bangladesh) [5].

2.3 Persistent PV infection

To establish persistent infection, a virus must be able to reduce its cytopathic effects (i.e., its ability to kill or damage the infected cell), maintain its genome within host cells over time, and avoid elimination by the host immune system. Individuals with severe immunodeficiency support chronic PV infection, with production of large amounts of virus for years [6]. In vitro, persistent PV infection has been studied in neuroblastoma cells [7] and cultured fetal neuronal cells [8]. Persistence is a rather common event in enteroviral infections: in vitro, human glomerular mesangial and vascular endothelial cells support chronic enteroviral infection [9, 10]; in vivo, coxsackievirus infection has been documented in endomyocardial biopsies of individuals progressing from viral myocarditis to dilated cardiomyopathy. In vitro, persistence is associated to cellular secretion of a variety of cytokines and growth factors that modulate cell behavior (e.g., motility, adhesion, proliferation) and stimulate the inflammatory response [11]. In our hands, infection with PV-1 of primary cultures of human skeletal myoblasts was associated with the up-regulation of pro-inflammatory cytokines (IL1, TNF-beta, IL12) and cytokines supporting humoral immunity (IL4, IL5, IL15), as well as with increased expression and response to alpha- and beta-chemokines (manuscript in preparation).

Cell cultures persistently infected by a typical enterovirus show peculiar characteristics: a) only a small percentage of cells do express viral antigens, b) viral titers (i.e., the amount of infectious viral particles released by infected cells) are usually low [$\leq 10^3$ plaque-forming units/ml (PFU/ml)], c) cell-to-cell virus transmission probably occurs at intercellular junctions. By immunofluorescence, PV capsid antigens are localized into the nucleus rather than in the cytoplasmic compartment (personal observations). Persistent enteroviral infection is favored by the high genetic variation of these agents that is due to: a) high mutation rate of positive-strand RNA viruses [12]; b) recombination between different virus variants; c) immune defenses that appear to limit the production of highly cytopathic variants. So far, limited genomic changes of persistent enteroviral isolates have been documented in very few cases of natural infection in humans [6].

In mice, gene amplification and EM studies have shown that PVs are capable of producing persisting infection of the central nervous system [13, 14].

3. The Post-Polio Syndrome

Flaccid asymmetrical paralysis is the clinical manifestation of acute anterior poliomyelitis. After the acute episode, surviving patients experience a period of neurological and functional recovery, followed by a phase of almost complete stability [15]. As early as in 1875, survivors of paralytic poliomyelitis were described to develop new weakness, fatigue, and muscular atrophy [16]. Since 1980, there has been an increasing acceptance of PPS. The condition may develop 15 to 40 years after acute paralytic and nonparalytic disease in 20 to 78% of polio survivors. [17]. Common manifestations include generalized, central and peripheral fatigue, muscle weakness, musculoskeletal pain, and new disabilities that

sometimes involve the respiratory and the alimentary tracts [18]. Worldwide, there are over 20 million polio survivors, thus PPS represents the most prevalent motor neuron disease (www.post-polio.org). In spite of the numbers of affected patients, the etiology and pathogenesis of this syndrome are obscure and no effective therapy is available [15]. Current treatments are based on a conservative approach consisting of exercise, avoidance of muscular overuse, orthoses, and assistive devices. Diagnosis is based on medical history and clinical-instrumental examination, since no specific biomarkers are available.

3.1 Pathogenesis of PPS: PV persistence as a cause?

A number of hypotheses have been proposed to explain the pathological changes associated with PPS [17]. The Wiechers and Hubbell hypothesis [19] is summarized in Figure 2.

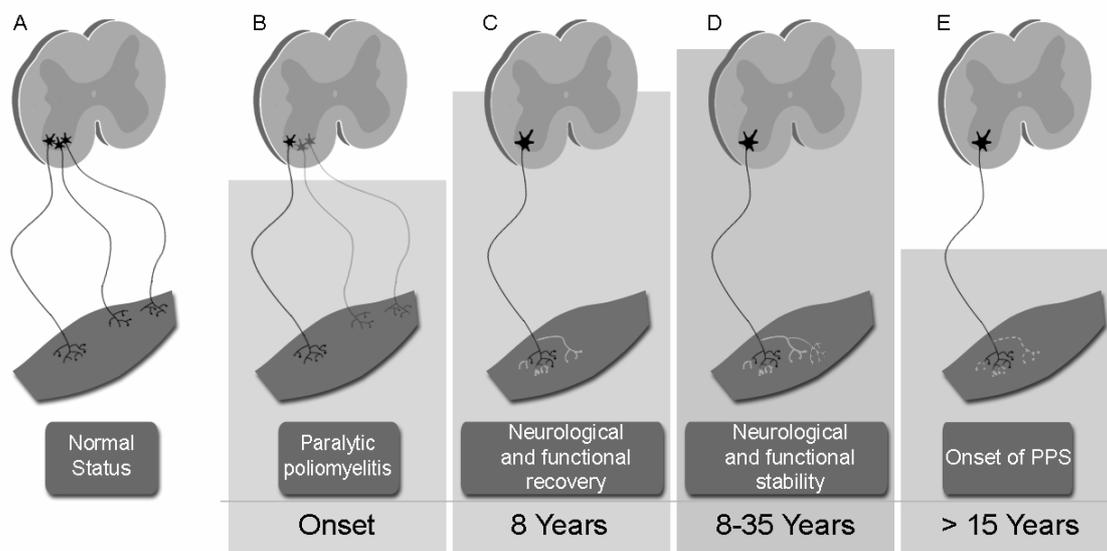


Figure 2. From acute paralytic poliomyelitis to the development of PPS. The structure of the normal motor unit is represented in panel A: in the spinal cord, a single anterior horn motor neuron does innervate numerous skeletal muscle myofibers (as an estimate, 200 to 2,000 myofibers per neuron). Panel B: when PV infects motor neurons, the cells are killed and do not regenerate. As a consequence of axonal degeneration, innervation of myofibers is lost and paralytic disease ensues. This event marks the onset of paralytic poliomyelitis. Panel C: neurological and functional recovery occurring during a period of several years is attributed to compensatory axonal sprouting of residual anterior horn motor neurons. This phenomenon markedly increases the number of muscle fibers innervated by residual motor neurons in the affected area. Extreme enlargement occurs in these motor units, reaching 7-8 times the normal innervation ratio of individual motor neurons. Panel D: functional stability goes on for 8-35 years, with little variation in strength and physical performance. Panel E: after years of stability, the late effects of polio develop and are interpreted as due to the degeneration of axonal sprouts in the enlarged motor units and/or to the loss of entire motor units (Wiechers and Hubbell hypothesis).

As shown in Figure 3, other pathogenetic factors may also contribute: a) the normal aging process, overuse myopathy and disuse muscular atrophy, and b) the establishment of a persisting PV infection.

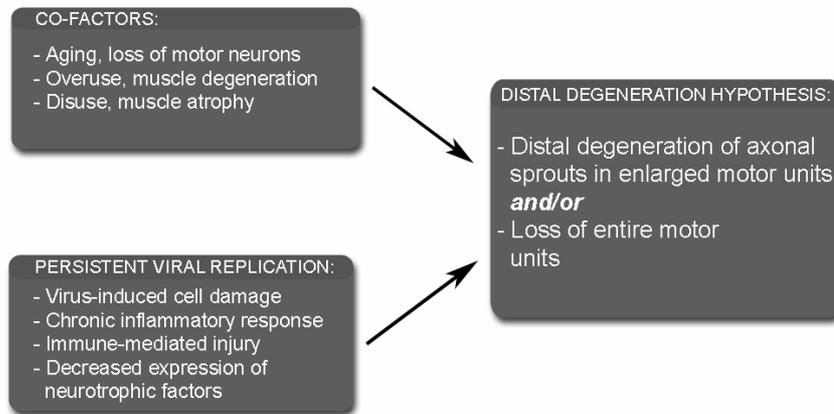


Figure 3. Pathogenesis of PPS. Schematic representation of the “distal degeneration hypothesis” proposed by Wiechers and Hubbel in 1981. The hypothesis avers that enlargement of motor units by axonal sprouting is not indefinitely stable and that the enlarged units undergo progressive loss of terminal axonal sprouts.

4. Detection of persisting PV in PPS patients

4.1 Detection of PV genomes

In support of a persisting PV infection, several authors have detected portions of PV genomes in cerebrospinal fluid (CSF) samples taken from PPS patients, with a prevalence of positives ranging from 10% to 65% in different studies [20]. Sequencing of amplified fragments allowed the presumptive identification of the three PV serotypes in different patients.

We have studied a few PPS patients diagnosed according to the criteria of the European Federation of Neurological Societies [15]. PV genome fragments were detected in 16/16 PPS cases. In contrast, the PV genome could not be detected in any of 16 “normal controls” (i.e., patients with CNS pathologies of non-infectious etiology). Direct sequencing of amplicons and alignment with deposited sequences of major PV reference strains (both wild type and vaccine strains) revealed that most cases were compatible with infection by PV-1 [20]. Sequence analysis of short sequences indicated that multiple deletions and mutations were present in the persisting PV strains. Thus, the reported findings indicate that modified PV genomes are able to persist for decades in the CNS of polio patients developing PPS.

The possible link between persistent PV infection and development of PPS remains, however, unclear. It is conceivable that continuous PV replication triggers chronic inflammatory responses, with pathological changes resulting both from direct virus cytopathology and/or immune-mediated injury. Potentially, the decreased production of neurotrophic factors is an additional factor.

4.2 Case histories

Based on the detection of PVs in CSF samples of PPS patients, we tried to characterize PV isolates obtained from different tissues of two PPS patients. A variety of methods have been used: tissue culture, immunofluorescence with PV-specific antibodies, genome cloning into *E. coli*, direct genome sequencing of virus produced in cell cultures.

Patient RR: 58 years old female who contracted acute paralytic poliomyelitis at 6 years of age. She was able to conduct a nearly normal life until the year 2000, when PPS symptoms started to develop. On the occasion of orthopedic surgery in 2006, bioptic samples were obtained from the quadriceps muscle and the ischiatic nerve. Samples were dissociated by mechanical and enzymatic treatment. Primary cell cultures were grown using media supplemented with growth factors and fetal calf serum. By

immunofluorescence, PV capsid antigens were detected in the cytoplasm of approximately 30% cultured cells (both myoblasts and nerve cells). Since low virus titers were detected in the cell culture supernatant ($<10^3$ TCID₅₀/ml), ultracentrifuged supernatants were used for total RNA extraction. After retrotranscription, cDNA was amplified using PV-specific primers directed to the following regions: 5'UTR, VP4/VP2, VP1, 2C/3A, 3C/3D. Amplicons were cloned into *E. coli* recipients and sequenced. Sequence analysis (Figure 4) showed that the "isolated virus" corresponded to a highly mutated strain of PV-1 containing numerous deletions and mutations in the 5'UTR, VP4/VP2, VP1, and 2C/3A regions. Notably, the 3D region (RNA-dependent RNA polymerase that is essential for genome replication) was conserved by over 98% as compared to wild type PV-1 (Mahoney strain and the vaccine strains Sabin-1 and Chat).

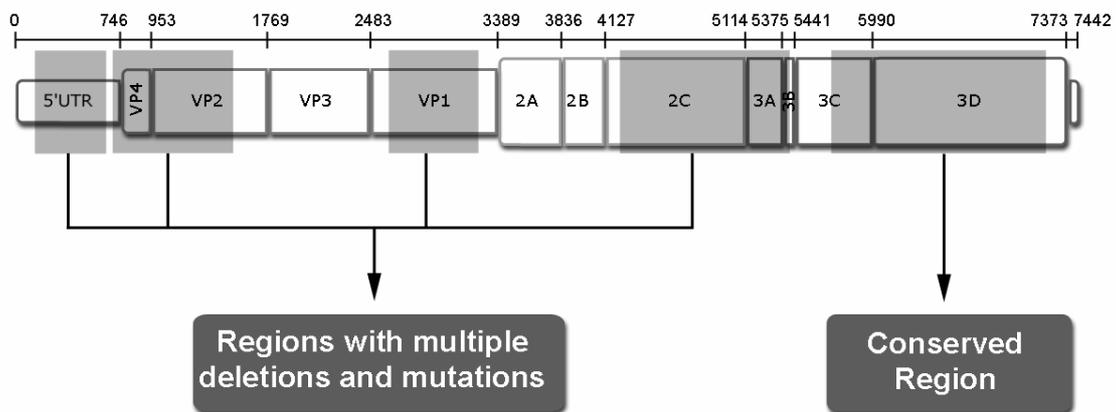


Figure 4. Patient LL: 74 years old female who contracted acute paralytic poliomyelitis at 1 year of age. She was able to conduct normal life and professional activity up to 71 years of age, when PPS symptoms started to develop. By RT-PCR, genomic fragments of PV-1 were detected in a CSF sample, but virus could not be isolated. Low titers of neutralizing antibody (1:16) were present in the serum against PV-1 and PV-3, but not against PV-2. PV-1 was isolated from peripheral blood leukocytes using as a target a mouse neuronal cell line that is very susceptible to PV-1 (the HpL-3.4 cell line derived from Prnp^{-/-} baby mice). By immunofluorescence, PV capsid antigens were detected in the cytoplasm of over 50% HpL-3.4 cells. Ultracentrifuged supernatants were used for total RNA extraction. After retrotranscription, cDNA was amplified using PV-specific primers directed to the following regions: 5'UTR/VP4, VP1, 2A, and 3D. As reported above, sequence analysis (Figure 5) showed that the "isolated virus" corresponded to a highly mutated strain of PV-1 containing deletions and mutations in the 5'UTR/VP4, VP1, and 2A regions. Again, the 3D region was conserved by over 98% as compared to reference PV-1 strains.

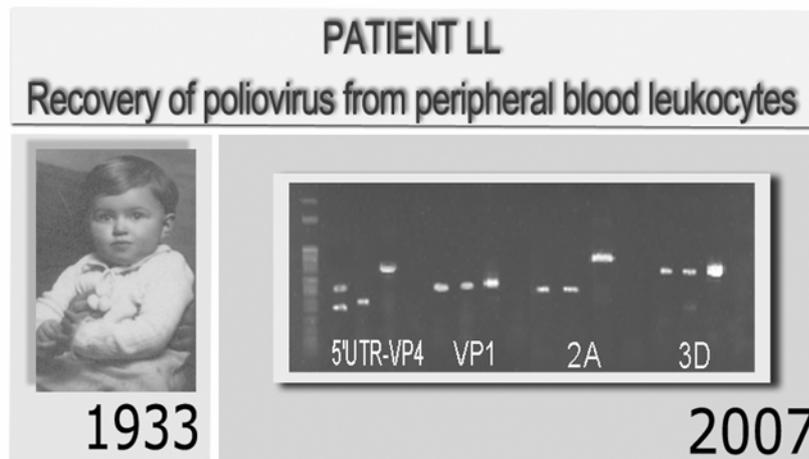


Figure 5. Patient LL contracted poliomyelitis in 1933 when she was 1-year-old. In 2007 (almost 74 years after the initial attack) a highly mutated PV-1 variant was isolated from peripheral blood leukocytes. Virus RNA was subjected to retrotranscription and amplification by PCR. Amplicons were separated by agarose gel electrophoresis (DNA fragments are separated on the basis of molecular size). For each genomic region, three lanes are presented. From left to the right: the first two lanes represent amplicons obtained from the RR virus isolate, the third lane contains amplicons obtained with the reference Chat strain. In all cases but one, amplicons of the RR strains show reduced molecular sizes as compared to the reference strain. The only exception is represented by amplicons of the 3D region whose molecular mass is equivalent to that of the reference strain.

Results obtained from the above two patients and data from other PPS patients show that:

- 1) Decades after acute infection, PVs remain present in the cerebrospinal fluid and in different cell types (muscle, nerve, peripheral blood leukocytes) of PPS patients. The finding shows that PVs are capable of establishing long persistent infections, at least in a portion of PV-infected patients.
- 2) Partial sequencing of PV-1 isolates revealed that persisting viruses are markedly different from wild type and attenuated vaccine strains, being characterized by numerous deletions and mutations. It is of interest that the region coding for the RNA polymerase is strongly conserved both in the RR and in the LL isolate. This makes the 3D region an interesting target for molecular diagnosis and antiviral therapy.

Sequencing of additional PV isolates obtained from PPS patients will allow to set up molecular tests capable of identifying the different PV variants associated with PPS [20]. Sequence data and infectivity experiments are also expected to clarify the mechanisms leading to PV persistence in hosts capable of developing effective immune responses.

5. Anti-enteroviral drugs

Since enteroviruses may be responsible of a variety life-threatening CNS and heart diseases, a number of anti enteroviral drugs are now under development. None is currently approved for clinical use. The reappearance of poliomyelitis in countries unable to meet the eradication goals set by WHO and the appearance of PPS in the aging population has increased this interest [3, 21]. We tested the activity of WIN63843 (a capsid inhibitor) [22, 23] and ABT538 (a protease inhibitor) against the Chat vaccine strain of PV-1. As shown in Figure 6, when each of the two drugs was employed singularly, virus titer was decreased by 0.5-1.5 log units. Interestingly, a titer reduction of 3 logs was observed when a combination of the two drugs was used. The data suggest that WIN6384 and ABT538 may have a synergistic effect against PV-1 when used at therapeutic doses (1 μ g/ml). We plan to use similar in vitro

methods for investigating the activity of other antiviral compounds against PV variants isolated from PPS patients.

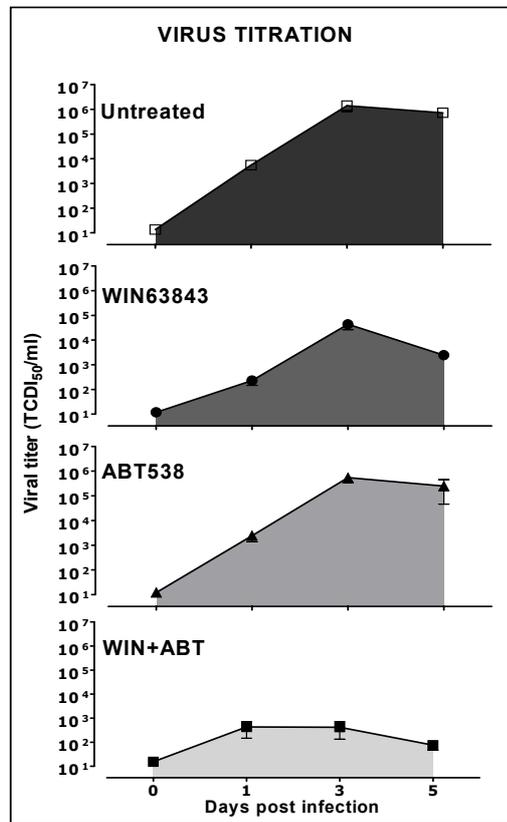


Figure 6. Effect of two antivirals on the replication of PV-1 (Chat strain) in cultured human AV3 cells. Briefly, confluent cell monolayers were treated for one-hour with WIN6384, ABT538 or a combination of the two drugs (each at the concentration of 1 μ g/ml). Monolayers were then incubated for one-hour with a small dose of PV-1 (multiplicity of infection = 0.01), washed extensively, and reincubated with complete medium supplemented with the indicated drugs. Samples of culture supernatants were taken at different times post-infection, cleared by centrifugation, and frozen at -70°C. Virus titers were determined in triplicate using HeLa cells. Titers are expressed as tissue culture infectious doses 50% (TCID₅₀).

6. Conclusion and perspectives

PPS is currently the most prevalent anterior motor neuron disease in developed countries. It is rarely fatal, but is associated with a progressive decline in muscular strength and quality of life. Thus, many disabled patients lose the ability to conduct autonomous lives [15]. Current empirical therapies are of limited help. One documented clinical trial based on the use of normal Ig preparations produced only marginal effects [24]. The virological studies summarized here will allow setting up reliable molecular methods for detecting the genetically-altered PV variants peculiar of PPS. Antiviral treatments will be considered for virus-positive patients, even if a direct link between persistent PV infection and PPS development is still undemonstrated. When the time will be ripe for clinical trials, methods are already available to assess treatment outcome. In the course of therapy, measurements of virus load in different tissues will help understanding the possible link between virus replication and neuromuscular symptoms.

Acknowledgements. We express our gratitude to the Agencies that have supported these studies over the past two years: University of Tokyo, Japan; Fondazione Comunitaria del Varesotto ONLUS (Varese, Italy); Ministry of the University (Rome, Italy); Fondazione Cariplo (Milano, Italy); Cattolica Assicurazioni (Verona, Italy). We also wish to thank the clinical colleagues who initiated us to this area of research: Drs. Salvatore Monaco, Gianluigi Zanusso, and Franco Molteni. Finally, our deepest appreciation goes to the PPS patients whose enthusiasm, patience, and suggestions made these investigations possible.

References

- [1] C.A. Ludlam, W.G. Powderly, S. Bozzette, M. Diamond, M.A. Koerper, R. Kulkarni, B. Ritchie, J. Siegel, P. Simmonds, S. Stanley, M.L. Tapper, M. von Depka: *Lancet* Vol. **367** (2006), 252.
- [2] V.I. Agol: *Curr. Top. Microbiol. Immunol.* Vol. **299** (2006), 211.
- [3] G. Palacios, M.S. Oberste: *J Neurovirol.* Vol. **11** (2005), 424.
- [4] V.R. Racaniello: *Virology* Vol. **344** (2006), 9.
- [5] M.A. Pallansch, H.S. Sandhu: *N. Engl. J. Med.* Vol. **355** (2006), 2508.
- [6] C.F. Yang, H.Y. Chen, J. Jorba, H.C. Sun, S.J. Yang, H.C. Lee, Y.C. Huang, T.Y. Lin, P.J. Chen, H. Shimizu, Y. Nishimura, A. Utama, M. Pallansch, T. Miyamura, O. Kew, J.Y. Yang: *J Virol.* Vol. **79** (2005), 12623.
- [7] A.S. Gosselin, Y. Simonin, F. Guivel-Benhassine, V. Rincheval, J.L. Vayssiere, B. Mignotte, F. Colbere-Garapin, T. Couderc, B. Blondel: *J Virol.* Vol. **77** (2003), 790.
- [8] N. Pavio, M.H. Buc-Caron, F. Colbere-Garapin: *J Virol.* Vol. **70** (1996), 6395.
- [9] P.G. Conaldi, L. Biancone, A. Bottelli, A. De Martino, G. Camussi, A. Toniolo: *J Virol.* Vol. **71** (1997), 9180.
- [10] P.G. Conaldi, C. Serra, A. Mossa, V. Falcone, F. Basolo, G. Camussi, A. Dolei, A. Toniolo: *J Infect Dis.* Vol. **175** (1997), 693.
- [11] M.M. Zanone, E. Favaro, P.G. Conaldi, J. Greening, A. Bottelli, P.C. Perin, N.J. Klein, M. Peakman, G. Camussi: *J Immunol.* Vol. **71** (2003), 438.
- [12] M. Vignuzzi, J.K. Stone, J.J. Arnold, C.E. Cameron, R. Andino: *Nature* Vol. **439** (2006), 344.
- [13] S. Girard, A.S. Gosselin, I. Pelletier, F. Colbère-Garapin, T. Couderc, B. Blondel: *J Gen Virol.* Vol. **83** (2002), 1087.
- [14] J. Destombes, T. Couderc, D. Thiesson, S. Girard, S.G. Wilt, B. Blondel: *J. Virol.* Vol. **71** (1997), 1621.
- [15] E. Farbu, N.E. Gilhus, M.P. Barnes, K. Borg, M. de Visser, A. Driessen, R. Howard, F. Nollet, J. Opara, E. Stalberg: *Eur J Neurol* Vol. **13** (2006), 795.
- [16] V. Cornil, R. Lepine: *Gaz Me'd Paris* Vol. **4** (1875), 127.
- [17] D.A. Trojan, N.R. Cashman: *Muscle Nerve* Vol. **31** (2005), 6.
- [18] M. Fiorini, G. Zanusso, A. Baj, L. Bertolasi, A. Toniolo, S. Monaco: *Fut. Neurol.* Vol. **4** (2007), 451.
- [19] D.O. Wiechers, S.L. Hubbell: *Muscle Nerve* Vol. **4** (1981), 524.
- [20] A. Baj, S. Monaco, G. Zanusso, E. Dall'ora, L. Bertolasi, A. Toniolo: *Futute Virology* Vol. **2** (2007), 183.
- [21] P.D. Minor: *Nat. Rev. Microbiol.* Vol. **2** (2004), 473.
- [22] D.L. Barnard: *Current Pharmaceutical design* Vol. **12** (2006), 1379.
- [23] H.A. Rotbart, A.D. Webster: *Clin Infect Dis.* Vol. **32** (2001), 228.
- [24] H. Gonzalez, K.S. Sunnerhagen, I. Sjoberg, G. Kaponides, T. Olsson, K. Borg: *Lancet Neurol.* Vol. **5** (2006), 493.