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In vitro Interaction of Poliovirus with Cytoplasmic Dynein

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Key Words
Poliovirus · Dynein · Neuroblastoma cells · Axonal transport

Abstract
Objective: Poliovirus (PV) enters the host by the oral route and can infect the central nervous system (CNS) by two mechanisms: crossing the blood-brain barrier and traveling along the nerves from the muscle to the spinal cord. In the latter mechanism, the PV receptor, CD155, and the motor protein, dynein, have been implicated in the transport of PV to the CNS. In this work we analyzed the possible interaction of PV with dynein. ***Methods:*** PV was bound to a Sepharose 4B beads and they were used to analyze the interaction of PV with cytoplasmic proteins from neuroblastoma cells by affinity chromatography and Western blot. ***Results:*** The interaction with cytoplasmic dynein was observed only when the Sepharose beads bound to PV were used and not in the control ones, where proteins from uninfected cells were coupled. ***Conclusion:*** These preliminary results open the possibility that PV uses the dynein directly in its retrograde axonal transport.

The three serotypes of poliovirus (PV) belong to the *Picornaviridae* family [1, 2] and they are small, non-enveloped viruses with a single-strand and positive polarity RNA genome [1, 3]. Only 1–2% of the patients infected with any of the three serotypes of PV have paralytic poliomyelitis, mainly in the lower limbs caused by the destruction of motor neurons present in the central nervous system (CNS) [2, 4].

PV infection occurs through the oral route and the virus infects the digestive tract, especially the lymphatic tissue present in Peyer's patches and tonsils. Later, the virus reaches and replicates in the mesenteric and deep cervical lymph nodes and it can be spread to other tissues by the bloodstream [5].

There are two hypotheses to explain the CNS infection by PV. The clinical features suggest that PV can cross the blood-brain barrier (BBB) [5, 6], since the amount of PV present in the brain of both transgenic CD155+ (TghPVR) or non-transgenic mice inoculated intravenously is similar to the amount of anti-transferrin receptor antibody, suggesting that the transport to the brain is effective. This process is independent of the presence of the CD155 molecule or the PV strain [7] and its mechanism is still unknown.

The other hypothesis is based in the capability of PV to move through the nerves from the neuromuscular junction to the neurons of the spinal cord (SC) [8] as sug-

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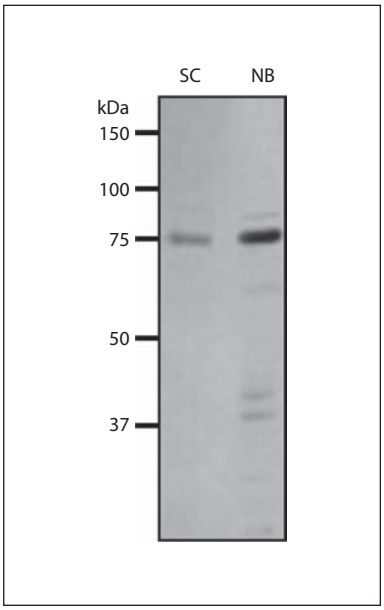

Fig. 1. Western blot with anti-dynein antibody. Total protein extracts from spinal cord of rat (SC) or S-10 protein extracts from neuroblastoma cells (NB) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and incubated with anti-dynein IgM antibody as described in Material and Methods. Migration of the molecular weight markers in kDa is shown on the left side.

gested for several studies: (i) the inoculation of the virus into the nerves of monkeys and Tg-hPVR mice causes the spread of the virus into the CNS in a progressive and ascendant manner [6, 9, 10]; (ii) the cordectomy prevents viral dissemination to the SC segments localized above the section [11]; (iii) the inoculation of PV in one limb causes the paralysis of that limb first [6], and (iv) freezing of the sciatic nerve with CO_2 before the intramuscular inoculation of PV prevents its dissemination [12]. To explain this mechanism, Ren and Racaniello [6] suggest that the virus, during the viremia, reaches and replicates in the muscle and, after interaction with receptors in the neuromuscular joint, can enter into the axons and move through the nerves toward the motor neurons present in the anterior horns of the SC, whereas PV can infect and destroy the cells. There, the new viral progeny can infect other cells and continue the ascendant transport to the brain [6, 8].

The velocity of the PV transport in the nerves is not more than 12 cm/day [9] and the treatment of sciatic nerve of PVR transgenic mice with vinblastine, an inhibitor of tubulin polymerization, reduces the capability of PV to reach the SC from the muscle [13], suggesting that the axonal retrograde transport is involved in viral dissemination to the CNS. In this mechanism, dynein has been described as one of the most important motor proteins [14]. In fact, the U_L34 protein of herpes simplex virus [15], the P protein of rabies and Mokola viruses [16, 17], and the E3 protein of adenovirus [18] are able to interact with the cytoplasmic dynein. Additionally, transport of adenovirus and herpes simplex to the nucleus is dependent of the integrity of the microtubule network and the dynein-dynactin complex [19, 20], and in canine parvovirus infection, the vesicles which contain the virus, require an intact microtubule network as well as the presence of dynein for their transport to the perinuclear region of the cell [21].

To further analyze the retrograde transport of PV, the possible interaction between dynein and PV was determined using an affinity chromatography assay. The SKY5Y, a neuroblastoma cell line, grown in DMEM (Dulbecco) (Gibco), pH 7.1–7.4, supplemented with 5.8 g/l Hepes (Gibco), 10% fetal bovine serum (Gibco), non-essential amino acids (Gibco), 2 mM of sodium pyruvate, 5,000 U/ml penicillin and 5 μg/ml streptomycin) at 37° with 4.5–5.5% of CO_2, was used to obtain a cytoplasmic protein extract (S-10) following the protocol described before [22]. On the other hand, the SC of Wistar rats was removed surgically, homogenized [23] and a total protein extract was obtained using the protocol reported previously [24]. The protein concentration of both extracts was determined by Bradford's method [25].

To demonstrate the presence of the dynein in the protein extracts, a Western blot assay was performed (fig. 1). A total of 15 μg of either neuroblastoma S-10 extract or total protein extract of SC were subjected to a 12% SDS-PAGE (Mini Protean System Bio-Rad) and transferred to a nitrocellulose membrane by using a semi-dry blotting apparatus (Bio-Rad) in 48 mM Tris-39 mM glycine-20% (vol/vol) methanol. Transfer efficiency was monitored by staining with 0.1% Ponceau red in 7% trichloracetic acid. The membrane was blocked at room temperature with 5% low-fat milk in PBS for 3 h and then incubated overnight at 4° with an IgM monoclonal antibody directed against the intermediate chain (IC) of cytoplasmic dynein (Sigma clone 70.1) diluted 1:2,000 in PBS. The membrane was washed 3 times with 0.5% PBS-Tween 20 and incubated at room temperature for 1 h with an anti-mouse IgM antibody conjugated to horseradish peroxidase (Zymed) diluted 1:8,000 in PBS. Then it was washed 4 times, for 5 min each, and once for 15 min with 0.5% PBS-Tween 20 at room temperature. The last wash was performed for 5 min at room temperature with PBS-50 mM

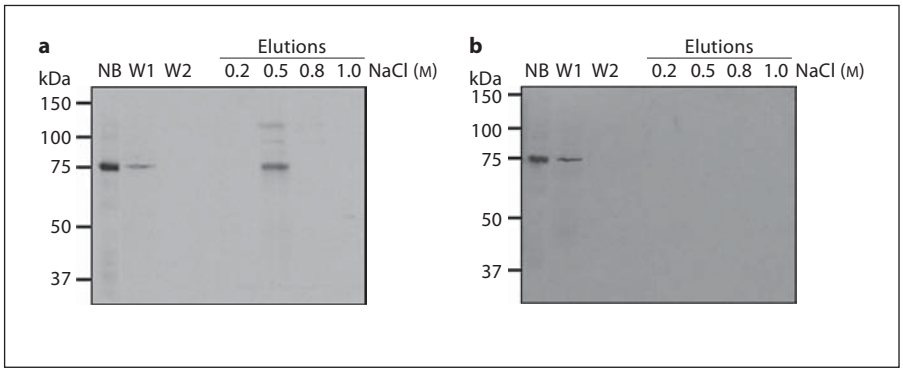

Fig. 2. Affinity chromatography using PV coupled to Sepharose 4B. S-10 protein extracts from neuroblastoma cells were reacted with Sepharose 4B beads coupled to PV (**a**) or to proteins from uninfected HeLa cells (**b**). The initial and final washes were collected (W1 and W2 respectively), and elution was performed with PBS with different NaCl concentrations (0.2, 0.5, 0.8 and 1 M concentrations). Samples of neuroblastoma S-10 extract were used as positive Western blot controls (NB). All fractions were analyzed by SDS-PAGE and Western blot as described before. Migration of the molecular weight markers in kDa is shown on the left side.

sodium chloride and finally the reaction was developed using the ECL chemiluminescence kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol and using X-Omat film (Kodak). The anti-dynein antibody detected a 74-kDa band that corresponded to the weight of the IC of cytoplasmic dynein, in both protein extracts [26]. Since the amount of cytoplasmic dynein was very low in the SC protein extract, the affinity chromatography assay with this extract could not be performed, however the neuroblastoma S-10 extract was used for the affinity chromatography assay.

PV 6.1 [27] was propagated at a multiplicity of infection of 100 in HeLa cell monolayers grown in DMEM culture medium without non-essential amino acids and sodium pyruvate at 37° with 4.5–5.5% of CO_2. After 15 h of infection, medium was recovered and clarified at 11,760 *g* for 20 min at 4°. The virus present in the supernatant was purified by a cesium chloride gradient as described by Minor [28]. The viability of viral particles was determined before and after the procedure by a plaque assay in HeLa cells [29] and the amount of protein was quantified using Bradford's method [25] and diluted in a coupling buffer (NaCl 0.5 M, $NaHCO_3$ 0.1 M, pH 8.3) to a final concentration of 1 mg/ml. Then, virus was incubated overnight at 4° with gentle shaking with Sepharose 4B beads activated with cyanogen bromide (Sigma) (500 mg) previously activated with 5 ml of 1 mM HCl. The Sepharose beads were washed 3 times with blocking buffer (0.1 M Tris-glycine, pH 8.6) followed by 3 times with acetate buffer (0.1 M acetic acid, 0.5 M NaCl, pH 4). Finally beads were washed 3 times with PBS (15 mM KH_2PO_4, 15 mM K_2HPO_4, 0.5 M NaCl, pH 7.4) and loaded into a 3-ml disposable syringe. To confirm the specificity of the interaction, another affinity column was prepared using proteins isolated following the same procedure to isolate virus but from non-infected HeLa cells and it was used as a negative control.

A total amount of 2.7 mg of S-10 protein extract from neuroblastoma cells was incubated with the Sepharose 4B beads coupled to PV for 40 min at room temperature. The column was washed with 40-bead volumes of PBS-NaCl 0.1 M and the proteins associated with PV were eluted using different concentrations of NaCl (0.2, 0.5, 0.8 and 1 M) in PBS. All fractions were precipitated with cold acetone for 1 h at –20° and the proteins were then recovered by centrifugation at 9,500 *g* for 10 min at 4°. The pellets were resuspended in PBS and subjected to 12% SDS-PAGE and Western blot assay as described before. The anti-dynein antibody recognized a protein present in the first wash and in the 0.5 M NaCl elution fraction when the PV-Sepharose beads were used (fig. 2a). This protein was also detected in the first wash fraction in HeLa-Sepharose beads but not in any of the elution fractions (fig. 2b). These results strongly suggest that PV interacts specifically with dynein.

CD155 is the cellular receptor of PV and it is a member of the immunoglobulin superfamily, and has three extracellular domains, a transmembrane region and a cytoplasmic tail [30–34]. Mueller et al. [26] and Ohka et al. [13] have demonstrated the interaction of amino acid sequence SKCSR present in the cytoplasmic tail of CD155α and δ with Tctex-1, a component of the cytoplasmic dy-

nein present in the sciatic nerve, neurons and other cells. Ohka et al. have well documented that the interaction of PV with CD155 at the neuromuscular junction induces the formation of vesicles that are transported along the microtubule network in the axons due to the interaction of the cytoplasmic tail of CD155 with the dynein. When these vesicles reach the neuron body, the viral particle is uncoated and the genomic RNA starts the translation and replication processes in order to produce the new viral progeny [13]. In agreement with this theory, the anti-CD155 antibodies are able to block the neural dissemination of PV in a dose-dependent manner and the virus purified from the nerves is the 160S particle [9]. It is well known that the interaction of PV with its receptor causes some conformational modifications in the 160S viral particle that induces the uncoating process [35, 36]. Curiously the particles purified from nerves were 160S suggesting that, even the entry of PV to the nerves is mediated by CD155, the conformational changes do not occur and the virus is transported to the neural body as a 160S particle. In M-like cells it has been shown that PV can be transported from the apical to the basolateral side by transcytosis without any conformational change, even when CD155 is expressed in the apical side of the cells [37]. Whether there is some factor or another protein associated to CD155 that prevents this change it is still unknown. On the other hand, it has been observed that PV is able to move along the axons retrogradely in non-transgenic mice [5] and naked RNA PV replicons inoculated in the muscle of rats are able to move towards CNS [38], suggesting that there can be a transport process at the synapse that is not mediated by CD155.

The evidence presented here about the interaction of PV with dynein could open a new possibility to explain the retrograde transport of the 160S particle in non-transgenic mice. Although this attractive mechanism could be functioning in vivo, it is clear that several additional experiments are required to demonstrate it. Unfortunately, as a consequence of PV eradication program, we will not be able to perform any additional assay with PV in our laboratory.

In this scenario, PV could be using two mechanisms to be transported along the nerves and in both mechanisms cytoplasmic dynein is involved. The first mechanism might require the direct interaction between PV and the dynein and the second one requires the presence of the CD155 as an intermediate in the interaction between PV and dynein. Both mechanisms have been demonstrated in other viruses supporting the possibility that PV can be transported retrogradely by a direct interaction with dynein [16–21].

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