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Antiviral Research 26 (1995) 27–35

Antiviral Research

Poliovirus infection without accumulation of eclipse particles

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Received 11 July 1994; accepted 12 September 1994

Abstract

Poliovirus type 1 (Mahoney) was treated with the capsid-binding pyridazinamine R 78206, followed by dialysis to remove free compound. Upon infection of HeLa cells by R 78206-pre-treated virus, the formation of intra- and extracellular modified particles was completely inhibited, except for a small amount of empty capsids. The synthesis of viral proteins and first cycle progeny virus was delayed by 1 h. The results suggest that poliovirus infection does not require intracellular accumulation of 135 S eclipse particles.

Keywords: Poliovirus; Eclipse particle; Antiviral; R 78206

1. Introduction

Picornaviruses are known to infect susceptible cells after first binding to a specific receptor on the cells plasma membrane. What happens after attachment and how the viral genome is delivered into the cytoplasm for translation is still poorly understood.

In poliovirus-infected cells, conformational alterations of the viral capsid take place, leading to the formation of eclipse particles. The cell-associated, intact 160 S virions are modified to intracellular 135 S; and a variable amount of 80 S empty capsids is always formed. RNA containing 110 S eclipse particles have also been described (Everaert et

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SSDI 0166-3542(94)00063-8

al., 1989). A substantial but variable amount of cell-associated virus elutes spontaneously from the cell surface as non infectious 135 S 'A particles' (Joklik and Darnell, 1961; Fenwick and Cooper, 1962). The role of the eclipse particles in uncoating still remains unsolved. This is indeed compounded by the small fraction of input virus (less than 1%) that gets productively uncoated.

There have been several models proposed for the uncoating of poliovirus. By one mechanism, the whole virion would enter the cell and release its genome. It has been suggested that the virus after binding to its receptor, progresses to multivalent tighter binding by nearby receptors and becomes inaccessible to extracellularly added antibodies. 'A particles' are formed, which are either eluted or enter and deliver the genome to the cytoplasm (Fenwick and Cooper, 1962; Mandel, 1967a; Mandel, 1967b; Lonberg-Holm and Whiteley, 1976). Other researchers propose that the plasma membrane-bound virus uncoats via a hypothetical 'infectosome' from which the genome penetrates the cell (Lee et al., 1993; Rueckert, 1991).

A receptor-mediated endocytotic pathway has been proposed (Habermehl et al., 1973; Madshus et al., 1984; Zeichhardt et al., 1985; Willingmann et al., 1989) This has been suggested mainly with the aid of pH perturbing agents like monensin and chloroquine, which moderately inhibit poliovirus infection. However, the importance of pH for conformational alteration (Gromeier and Wetz, 1990) and for productive infection (Kronenberger et al., 1991) has been questioned. Recently, Pérez and Carrasco (1993) indeed found that bafilomycin A1 (an inhibitor of vacuolar type ATPase) did not inhibit poliovirus infection and they suggested that uncoating could be at the level of the plasma membrane or from the endosomes. Using cell fractionation techniques, Kronenberger et al. (1994) also favoured uncoating from a prelysosomal site.

Whether poliovirus infection is via an endocytotic pathway or from the plasma membrane still remains controversial, but in the case of rhinovirus 2, the conformational changes thought to lead to infection were inhibited by bafilomycin A1, and uncoating was proposed to take place from early endosomes (Prchla et al., 1994).

The antiviral pyridazinamine R 78206 (ethyl-4-[3-[1-(6-methyl-3-pyridazinyl)-4-piperidinyl]propoxy]benzoate) has been shown to prevent poliovirus thermal degradation (Rombaut et al., 1991; Andries et al., 1994) and to prevent the entry of stabilized virus into lysosomes (Ofori-Anyinam et al., 1993). In our recent work, however, poliovirus was pretreated with R 78206 and then dialyzed to remove at least 99% of the unbound compound. It was decided to remove the unbound product, such that it could not interfere with the virus–cell interaction. Surprisingly, virus pretreated in this way was thermostable and fully infectious (Ofori-Anyinam et al., manuscript in preparation). We report here the effects of R 78206 pretreatment on the formation of eclipse particles, and on the synthesis of viral protein and progeny virus.

2. Materials and methods

^{35}S-labelled poliovirus type 1 (Mahoney strain) was used. Labelling and purification were as previously described (Everaert et al., 1989).

The antiviral compound R 78206 (ethyl-4-[3-[1-(6-methyl-3-pyridazinyl)-4-piperidinyl]propoxy]benzoate) (M.W. 383.49) was kindly provided by K. Andries (Janssen Research Foundation, Beerse, Belgium). R 78206 has a MIC value of 0.004 μM (Rombaut et al., 1991). A stock solution of 10 mg/ml R 78206 was prepared in dimethyl sulfoxide (DMSO) and diluted in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.3) to 10 μg/ml or lower concentrations.

Virus pretreatment was achieved by incubating 1.0 nM ^{35}S-labelled virus for 1 h at 30°C with the stated concentration of R 78206, followed by dialysis for 18 h at 4°C against 3 changes of PBS.

Virus was bound to HeLa cells suspended in Eagle's medium at a MOI of 30 PFU/cell (12 PFU in the experiment described in Fig. 5), for 2 h at 26°C in rotating 50 ml Falcon tubes. Unbound virus was washed off and cells were resuspended in an equal volume of medium as for binding and further incubated at 37°C. Cells were washed and resuspended in PBS (pH 8). Detergent lysis was performed by adding NP40 in PBS (pH 8) to a final concentration 1%. After 10 min on ice, the lysate was centrifuged ($9980 \times g$, 3 min). The postnuclear supernatant was layered onto a 15–30% sucrose gradient for centrifugation.

Rate zonal sucrose gradient analysis was performed using 15–30% sucrose gradients made in PBS (pH 8). Aggregates were collected on a cushion of 70% nycodenz at the bottom of the gradients. Centrifugation was for 2.5 h ($170\,000 \times g$). Fractionation of the gradient was by upward displacement.

SDS-PAGE was performed as described by Laemmli (1970) and Vrijsen et al. (1980).

3. Results

3.1. Inhibition of eclipse particles formation after R 78206 pretreatment

^{35}S-labelled poliovirus was pretreated with various concentrations of R 78206 as described in Section 2. HeLa cells were infected with the pretreated poliovirus for 2 h at 26°C, washed to remove unbound virus, and further incubated for 0.5 h at 37°C. When cell lysates were analyzed by sucrose gradient centrifugation, it was found that the formation of 80 S and 135 S eclipse particles was inhibited in a concentration-dependent way. Pretreatment with 10 μg/ml R 78206 totally inhibited the formation of intracellular 135 S eclipse particles, and severely reduced that of 80 S empty capsids (results not shown).

The experiment was repeated with longer incubation times at 37°C (Fig. 1). With control virus (left panel), most 160 S virions were modified to 135, 110 and 80 S particles after 1 h (tracing a). As the incubation was continued, the 135 S particles were further modified to 110 S (tracings b–d), in agreement with previous findings (Everaert et. al., 1989). Pretreatment with 10 μg/ml R 78206 (right panel) inhibited the formation of eclipse particles until 4 h after shift-up (tracings f–h). A small amount of 80 S was formed during the first hour at 37°C (tracing e).

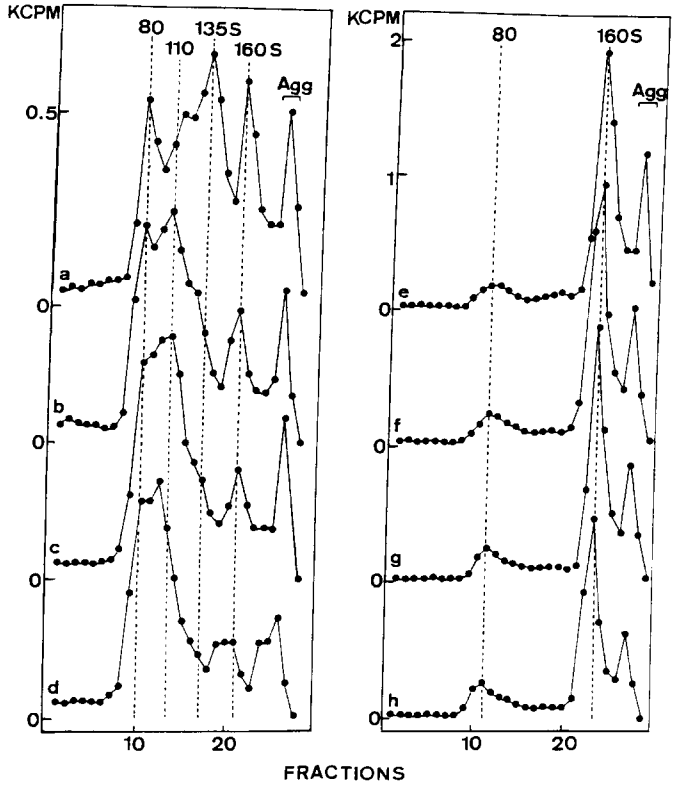

Fig. 1. Time course of eclipse particles formation, and effect of R 78206 pretreatment. ^{35}S-labelled virus was pretreated with 0.1% DMSO (left panel) or 10 μg/ml R 78206 + 0.1% DMSO (right panel). HeLa cells were incubated with 30 PFU/cell for 2 h at 26°C, washed and reincubated at 37°C for 1 h (tracings a and e), 2 h (tracings b and f), 3 h (tracings c and g) or 4 h (tracings d and h). Cell lysis and sucrose gradient centrifugation was as described in Section 2. Aggregates (Agg) were collected on a cushion of 70% nycodenz at the bottom of the gradients.

The kinetics of intracellular 80 S empty capsid formation were studied by collecting samples at short times after temperature shift-up from 26°C to 37°C. The bulk of 80 S particles was formed within the first 10 min (Fig. 2).

The possible influence of the multiplicity of infection on 80 S empty capsid formation was examined by using virus with a high specific radioactivity. Reducing the multiplicity of infection from the usual 30 to 4 PFU/cell did not change the amount of intracellular 80 S, relative to input (results not shown). The possibility was also examined that 80 S formation might be due to thermal degradation of virions; but when either untreated or R 78206 pretreated virus was incubated for 1 h at 37°C in cell-free Eagle's medium, no 80S particles were formed (results not shown).

The R 78206 pretreatment somewhat reduced the amount of cell-bound radioactivity that was eluted at 37°C, and completely changed the nature of the eluted particles. Elution following adsorption of untreated virus in our hands yielded 80 S and 110 S

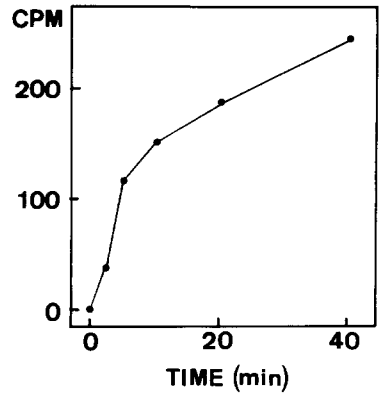

Fig. 2. Kinetics of 80 S formation. HeLa cells were infected for 2 h at 26°C with ^{35}S-labelled poliovirus, pretreated with 10 μg/ml R 78206. The cells were washed to remove unbound virus and further incubated at 37°C for the times stated. Cell lysates were analyzed by sucrose gradients to show eclipse particles. The total radioactivity of six fractions corresponding to the position of the 80 S particles were plotted against time of incubation at 37°C.

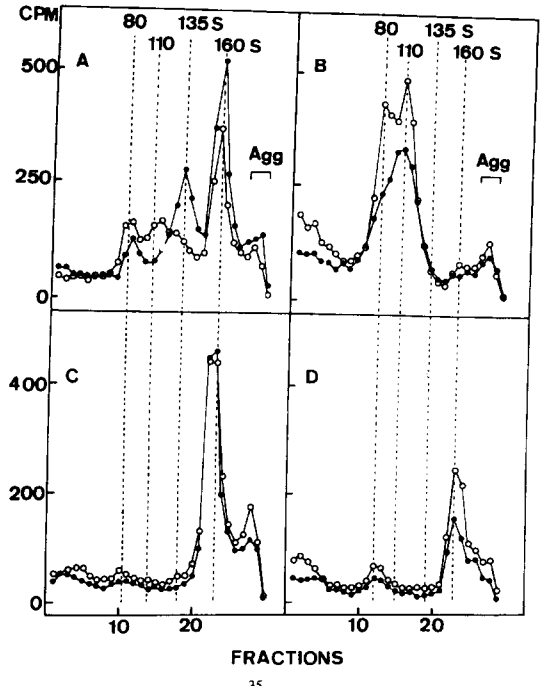

Fig. 3. Effect of R 78206 pretreatment on elution. ^{35}S-Labelled virus was pretreated with 0.1% DMSO only (panels A and B), or with 10 μg/ml R 78206 + 0.1% DMSO (panels C and D). HeLa cells were incubated with 30 PFU/cell for 2 h at 26°C, washed and reincubated at 37°C. After 0.5 h (●) or 1.5 h (○), samples containing 10^6 cells were collected and centrifuged to pellet the cells. The supernatants were collected for sucrose gradient centrifugation (panels B and D) and the cells (pellet) were washed and lysed with 1% NP40. The postnuclear lysates were layered onto sucrose gradients for centrifugation (panels A and C).

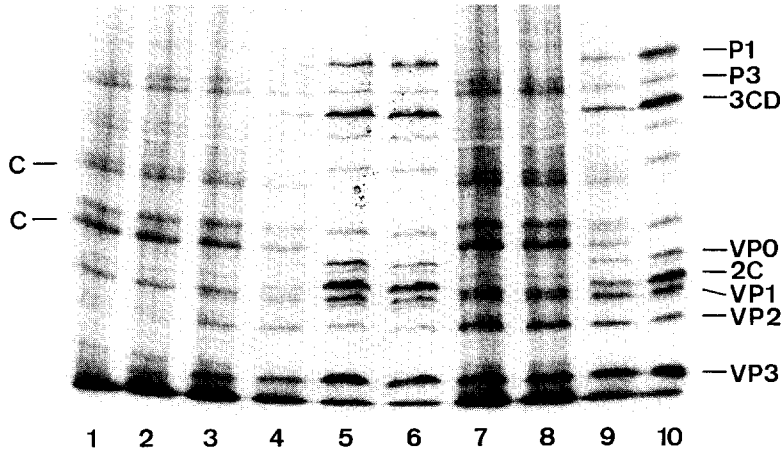

Fig. 4. Delaying effect of R 78206 pretreatment on viral polypeptide synthesis. During an experiment similar to that reported in Fig. 1, cells were sampled at various times after temperature shift-up, pulse-labelled for 0.5 h with [^{35}S]methionine (2.5 μCi/ml), and analyzed for labelled polypeptides by SDS-PAGE. Lanes 1–2: mock-infected cells pulse-labelled at 1 and 4 h after shift-up. Lanes 3–6: cells infected with virus pretreated with 0.1% DMSO only, and pulse-labelled at 1, 2, 3 or 4 h after shift-up. Lanes 7–10: cells infected with virus pretreated with 10 μg/ml R 78206+0.1% DMSO, and pulse-labelled at 1, 2, 3 or 4 h after shift-up. Two conspicuous cellular bands are marked 'C'.

particles (Fig. 3, panel B), rather than 135 S particles, as reported for the Sabin 1 strain by Fricks and Hogle (1990). When the virus had been pretreated with R 78206, however, a mixture of 160 S and 80 S particles was eluted (panel D). Thus, pretreatment prevented the formation of both intracellular 135 S and extracellular 110 S particles, but still allowed some formation of intra- and extracellular 80 S empty capsids.

3.2. Effect of R 78206 pretreatment on the synthesis of viral polypeptides and infectious progeny

Viral protein synthesis was monitored in an experiment similar to that of Fig. 1. Cells collected from 1 to 4 h after temperature shift-up to 37°C were pulse-labelled with [^{35}S]methionine, and the labelled polypeptides exposed after SDS-PAGE (Fig. 4). Lanes 1 and 2 show the cellular polypeptide pattern in mock-infected cells. In cells infected with control virus, newly synthesized viral polypeptides (P1, P3, 3CD, VP0 and 2C) began to appear after 2 h (lane 4). (Note: the labelling of VP1, VP2 and VP3 originates from the ^{35}S-labelled input virus and is of no account). Even heavily labelled, cellular polypeptides (marked 'C') were no longer synthesized 3 h after the temperature shift-up (lane 5). With the pretreated virus, the viral pattern emerged only at 3 h (lane 9), showing that the onset of viral polypeptide synthesis had been delayed by about 1 h. The synthesis of cellular polypeptides also subsisted for 1 h longer than after infection with the control virus (compare lanes 4 and 9).

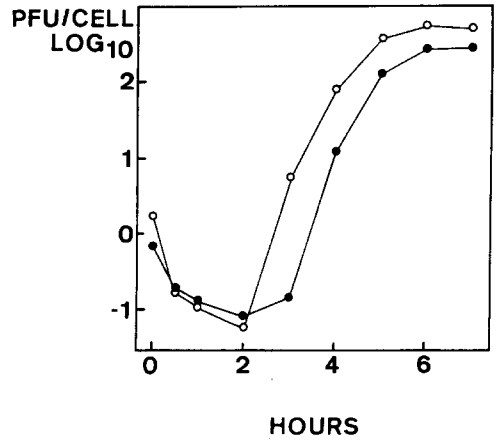

Fig. 5. Delaying effect of R 78206 pretreatment on single-cycle virus multiplication. ^{35}S-labelled virus was pretreated with 0.1% DMSO only (○), or with 10 μg/ml R 78206+0.1 % DMSO (●). HeLa suspension cells were infected with virus (12 PFU/cell) for 2 h at 26°C, washed and reincubated at 37°C. Samples were collected at times shown. The total cell suspension was lysed by 1% NP40, centrifuged (5 min, 12000× *g*) to remove nuclei and gross cellular debris, and stored at −20°C until virus titration by plaque assay (Vrijsen et al., 1993).

In a separate but similar experiment, the synthesis of infectious progeny virus was compared in HeLa cells infected with R 78206 pretreated and control virus. The pretreatment caused a 1-h delay in the production of progeny virus (Fig. 5).

4. Discussion

The formation of intracellular 135 S and 110 S eclipse particles and that of extracellular modified particles (in our hands, 110 S) was severely and permanently suppressed by pretreatment with 10 μg/ml R 78206. On the other hand, a small but significant amount of intracellular 80 S particles was rapidly formed after shift-up to 37°C. Synthesis of viral protein and first-cycle progeny virus production were only retarded by 1 h. The results show that no bulk formation of eclipse particles is required for infection, at least in multiply-infected cells. It is, however, possible that minute amounts of 135 S particles were formed that were not detectable on sucrose gradients.

The importance of eclipse particles in productive uncoating has recently been challenged in several reports. Chloroquine caused a massive redirection of the formation of 135 S to 80 S eclipse particle (Kronenberger et al., 1991). Other weak bases and the ionophore monensin similarly affected eclipse particle formation without reducing plaque titre (Kronenberger et al., 1994). WIN 52035-2 delayed by 1.5 h a single cycle growth curve of a 'compensation mutant' of rhinovirus 14, with inhibition of the formation of intracellular A-particles (Shepard et al., 1993).

The reverse situation, i.e., the failure of non-viable poliovirus and rhinovirus mutants to initiate infection despite the formation of normal amounts of eclipse particles, has

been encountered (Lee et al., 1993; Moscufo et al., 1993). The possibility of the viral RNA being injected into the cytoplasm from a hypothetical 'infectosome' to initiate infection has been suggested (Rueckert 1991; Lee et al., 1993). Taken together with these recent reports, our results cast doubt on the biological relevance of the bulk of the 135 S/110 S eclipse particles. On the other hand, the 80 S particles still formed from R 78206 pretreated virus may represent productive uncoating.

Acknowledgements

We thank K. Andries (Janssen Research Foundation, Belgium) for providing R 78206 and for helpful comments; B. Rombaut and P. Brioen for useful discussions, and M. De Pelsmacker, A. De Rees, R. De Bel and S. Peeters for their excellent technical and secretarial assistance. This work was supported by the National Fund for Scientific Research (Belgium). One of us (O. O.-A.) holds a grant from the Vrije Universiteit Brussel.

References

Andries, K., Rombaut, B., Dèwindt, B. and Boeyé, A. (1994) Discrepancy between infectivity and antigenicity stabilization of oral polio vaccine by a capsid-binding compound. J. Virol. 68, 3397–3400.

Everaert, L., Vrijsen, R. and Boeyé, A. (1989) Eclipse products of poliovirus after cold-synchronized infection of HeLa cells. Virology 171, 76–82.

Fenwick, M.L. and Cooper, P.D. (1962) Early interactions between poliovirus and ERK cell: some observations on the nature and significance of the rejected particles. Virology 18, 212–223.

Fricks, C.E. and Hogle, J.M. (1990) Cell-induced conformational change in poliovirus: externalization of the amino terminus of VP1 is responsible for liposome binding. J. Virol. 64, 1934–1945.

Gromeier, M. and Wetz, K. (1990) Kinetics of poliovirus uncoating in HeLa cells in a non acidic environment. J. Virol. 64, 3590–3597.

Habermehl, K.-O., Diefenthal, W. and Buchholz M. (1973) Distribution of parental viral constituents in the course of poliovirus infection. In: Advances in the Biosciences 11 (Raspe, G., ed.), pp. 41–64, Pergamon press, Oxford.

Joklik, W.K. and Darnell, J.E. (1961) The adsorption and early fate of purified poliovirus in HeLa cells. Virology 13, 439–447.

Kronenberger, P., Vrijsen, R. and Boeyé, A. (1991) Chloroquine induces empty capsid formation during poliovirus eclipse. J. Virol. 65, 7008–7011.

Kronenberger, P., Vrijsen, R. and Boeyé, A. (1994) Intracellular proteolysis of poliovirus eclipse particles is abortive. J. Gen. Virol. 75, 573–578.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lee, W-M., Monroe, S. and Rueckert, R. (1993) Role of maturation cleavage in infectivity of picornaviruses: activation of an infectosome. J. Virol. 67, 2110–2122.

Lonberg-Holm, K. and Whiteley, N.M. (1976) Physical and metabolic requirements for early interaction of poliovirus and human rhinovirus with HeLa cells. J. Virol. 19, 857–870.

Madshus, I.H., Olsnes, S. and Sandvig, K. (1984) Mechanism of entry into the cytosol of poliovirus type 1: requirement for low pH. J. Cell Biol. 98, 1194-1200.

Mandel, B., (1967a) The relationship between penetration and uncoating of poliovirus in HeLa cells. Virology 31, 702–712.

Mandel, B., (1967b) The interaction of neutralized poliovirus with HeLa cells. II. Elution, penetration, uncoating. Virology 31, 248–259.

Moscufo, N., Yafal, A.G., Rogove, A., Hogle, J. and Chow, M. (1993) A mutation in VP4 defines a new step in the late stages of cell entry by poliovirus. J. Virol. 67, 5075–5078.

Ofori-Anyinam, O., Vrijsen, R., Kronenberger, P. and Boeyé, A. (1993) Effect of a capsid-stabilizing pyridazinamine, R 78206, on the eclipse and intracellular location of poliovirus. J. Virol. 67, 2367-2369.

Pérez, L. and Carrasco, L. (1993) Entry of poliovirus into cells does not require a low pH step. J. Virol. 67, 4543-4548.

Prchla, E., Kuechler, E., Blaas, D. and Fuchs, R. (1994) Uncoating of human rhinovirus serotype 2 from late endosomes. J. Virol. 68, 3713–3723.

Rueckert, R.R. (1991) Picornaviridae and their replication. In: Fundamental Virology, 2nd edition (Fields, B.N., Knipe, D.M., Chanock, M.S., Hirsch, M.S., Melnick, J.L., Monath, T.P. and Roizman B., eds.), pp. 409–449, Raven press, New York.

Rombaut, B., Andries, K. and Boeyé, A. (1991) A comparison of WIN 51711 and R 78206 as stabilizers of poliovirus virions and procapsids. J. Gen. Virol. 72, 2153–2157.

Shepard, D.A., Heinz, B.A. and Rueckert, R.R. (1993) WIN 52035-2 inhibits both attachment and eclipse of human rhinovirus 14. J. Virol. 67, 2245–2254.

Vrijsen, R., Brioen, P. and Boeyé, A. (1980) Identification of poliovirus precursor proteins by immunoprecipitation. Virology 107, 567–569.

Vrijsen, R., Mosser, A. and Boeyé, A. (1993) Postadsorption neutralization of poliovirus. J. Virol. 67, 3126-3133.

Willingmann, P., Barnert, H., Zeichhardt, H. and Habermehl., K.-O. (1989) Recovery of structurally intact and infectious poliovirus type 1 from HeLa cells during receptor-mediated endocytosis. Virology 168, 417–420.

Zeichhardt, H., Wetz K., Willingmann, P. and Habermehl, K.-O. (1985). Entry of poliovirus type 1 and mouse Elberfeld (ME) virus into HEp-2 cells: receptor-mediated endocytosis and endosomal or lysosomal uncoating. J. Gen. Virol. 66, 7368–7373.