Uncoating of Poliovirus by Isolated Plasma Membranes

V. F. CHAN¹ AND F. L. BLACK

Department of Microbiology, Yale University School of Medicine, New Haven, Connecticut 06510

Received for publication 4 November 1969

Poliovirus type I LSc strain labeled with ¹⁴C-uridine was adsorbed onto isolated plasma membranes and incubated with them. When membranes from Hep-2 or Vero cells were used, 22% of the label was converted to a trichloroacetic acid-soluble form, when trypsin or ribonuclease was added, the fraction rendered soluble was increased, and when the two enzymes were added in sequence, 85% or more of the label became trichloroacetic acid-soluble. This labilization of poliovirus could be reproduced when butanol-solubilized proteins from membranes were substituted for the whole plasma membranes, but it did not occur with membranes from poliovirus-resistant calf kidney or BHK-21 cells.

The enzyme-resistant capsids of the enteroviruses pose a major obstacle to uncoating. The fact that uncoating the genome by chemical means increases the host range of these viruses suggests that the uncoating principle may be a critical factor in determining the natural host range. The host-cell receptor of the enteroviruses is also found only on susceptible cells and it is possible, though by no means certain, that attachment of virus and modification of the capsid may be two functions of the same substance.

The receptor function has been extensively studied. Holland and McLaren (5) found virus adsorbing material on the surface of susceptible cells but also larger amounts in the microsomal fractions. On the basis of the nature of inactivating agents, they surmised that these receptors were lipoprotein. Later, Holland distinguished between the receptors from the two sites, showing that irreversible eclipse of the virus occurred only in association with the plasma membrane receptors (2). Phillipson and Lind (10) succeeded in solubilizing from human red-blood cells the receptor for the hemagglutinin of another enterovirus, ECHO 7, and showed it to be a lipoprotein.

The uncoating function is less well understood. Joklik and Darnell (7) showed that half the virus adsorbed to HeLa cells may be rejected again in a modified form unstable to high salt concentrations. It seems reasonable to hypothesize that this rejection represents the abortion of virus that has undergone the first steps of uncoating. Holland and Hoyer (3) found that label incorporated into viral ribonucleic acid (RNA) was released by

trypsin treatment when the virus was allowed to become irreversibly bound to the cell surface. Mandel (9) described a stage in viral penetration in which virus is not neutralizable but is recoverable as fully infectious virus by disruption of the cells. He suggested that this is virus that has been taken into viropeptic vacuoles, and this would seem to locate the uncoating process intracellularly rather than at the cell surface. It is possible, however, that Mandel's unneutralizable stage simply represents a close union of the virus with the plasma membrane without invagination.

It is the purpose of the present paper to present new data on the localization and mechanism of the uncoating process.

MATERIALS AND METHODS

Cell cultures. Cultures used were: (i) Vero line of stable green monkey kidney, kindly given by Sonja Buckley; (ii) Hep-2 from Microbiological Associates, Bethesda, Md.; (iii) BHK-21 from Microbiological Associates; and (iv) primary calf embryo kidney (CaEK).

CaEK cultures were prepared from the cortex portion of calf embryo kidneys after cell dispersal by a standard method of trypsinization.

All cultures were grown in roller bottles (no. 7000, Bellco Glass Inc., Vineland, N.J.) with Eagle minimal essential medium in Hanks balanced salt solution supplemented with 10% calf serum. An additional 2 mmoles of glutamine and doubled vitamins were used for Vero cells. Antibiotics were added to a final concentration of 1,000 µg/ml each of penicillin and streptomycin and 10 µg/ml of fungizone. Cultures were rotated at ½ rev/min except for the first day after seeding, when lower speeds were used. Once the cell monolayer was complete, the salt base was changed

¹ Present address: Institute of Hygiene, University of the Philippines, Ermita, Manila, Philippines.

from Hanks to Earle, and the calf serum concentration was reduced to 5%.

Virus. To grow virus, 4- to 5-day-old Vero cell cultures were first treated with 5 μ g of actinomycin D/ml. One hour later, fluids were aspirated and replaced with 10 ml of medium containing 10^6 plaque-forming units (PFU) of the LSc strain of attenuated type I poliovirus and 1 μ g of actinomycin D/ml. After 30 to 45 min at 35 C, the inoculum was removed, the cultures were washed, and the media were replaced with Eagle minimal essential medium in Earle containing 1 μ C-uridine (specific activity, 51.5 mc/mmmole) and 1 μ g of actinomycin D/ml. When cytopathic effect became evident after about 11 hr of rotation, the cultures were frozen at -60 C.

Concentration and purification of 14 C-uridine-poliovirus. Infected cultures were frozen and thawed three times. Cell debris was removed by centrifugation at $1,500 \times g$ for 20 min. The supernatant fluid was concentrated 50-fold by overnight dialysis at 4 C under prechilled polyethylene glycol, 20 M. This concentrated virus was dialyzed for 72 hr against 2×10^{-2} M phosphate buffer (pH 7.4) with two changes of buffer every 24 hr. The resulting suspension was clarified by centrifugation at $11,000 \times g$ for 1 hr, and virus was pelleted by centrifugation at $104,000 \times g$ for 4 hr. The pellet was resuspended by means of a syringe and a 26-gauge needle in one-tenth the original volume with MST buffer [10^{-3} M $MgCl_2$, 0.25 M sucrose, and 0.02 M tris(hydroxymethyl)aminomethane (Tris), pH 7.4], and held overnight at 4 C. The virus was then layered on a 5 to 25% sucrose gradient and centrifuged at $70,000 \times g$ for 2 hr. A visible band of virus, 7 mm from the bottom of the tube, was drawn off and dialyzed against MST buffer. Portions were dispensed in vials, shell frozen, and stored at -60 C. The preparation had titers of 3×10^6 PFU and 5×10^6 counts per ml.

Isolation and purification of plasma membranes. The Tris method of Warren et al. (11) was used for the stabilization and isolation of the surface membranes. In the final centrifugation on a stepwise sucrose gradient, Warren et al. reported that the membranes were distributed on the top of the 45, 50, and 55% sucrose layers. In the present study only those membranes equilibrating on the top of the 45% sucrose layer were used, because some nuclei were found at the lower interfaces. Yields were about 1.0 ml of plasma membrane per 30 ml of packed whole cells. The product was washed three times in MST buffer and suspended in the same diluent.

Purity was checked by electron microscopy and by enzyme assays. Some rough endoplasmic reticulum appeared still associated with the membranes, but there was no evidence of cytochrome *c* oxidase that might suggest residual mitochondria and very low levels of ribonuclease (0.5×10^{-2} units/mg), indicating that lysozymes had been eliminated. The preparations contained residual proteolytic activity equivalent to 3 units of trypsin per mg that could not be removed by further washing.

Biochemical determinations. Protein was measured by the biuret method described by Kabat and Mayer (8), with bovine serum albumin used as a standard; ribonuclease was measured by the method of Zöllner

and Hobom (12) with yeast RNA as substrate; and trypsin was measured by the method of Hummel (6), with *p*-toluene sulfonyl-L-arginine methyl ester used as the substrate.

14 C trichloroacetic acid-precipitable counts were determined by the method of Billeter, Weissmann, and Warner (1).

Adsorption of virus to plasma membranes. To obtain maximal adsorption of virus to the membranes, each milligram of plasma membrane protein was mixed with 10^6 to 10^7 PFU in a modified MST buffer containing 0.05 instead of 0.001 M Mg^{2+} . The mixture was held for 2 hr at 0 C with shaking at intervals, and was then centrifuged at $1,200 \times g$ for 10 min. The pellets were washed twice with high Mg^{2+} MST buffer, and the washes were combined with the original supernatant fraction. Washed pellets are designated as plasma membrane-adsorbed virus (PM-AV) and the pooled supernatant fraction as unadsorbed virus (UAV). Approximately 25% of virus label was adsorbed under these conditions.

RESULTS

Effect of incubating poliovirus on plasma membrane. PM-AV, UAV, and virus that had not been exposed to plasma membrane were incubated at 37 C and tested at intervals up to 1 hr or more for trichloroacetic acid-precipitable radioactivity. The bulk of the UAV and control virus counts remained in macromolecular form; only about 10% became nonprecipitable. In the PM-AV preparation, 35% of the label was converted to a nonprecipitable form during incubation (Fig. 1A).

Evidence of even more extensive modification of the PM-AV was obtained by incubating the preparations with enzymes. When the preparations were treated with ribonuclease (Fig. 1B), nearly 60% of the label became acid-soluble. When the preparations were treated with trypsin (Fig. 1C), there may have been an effect on rate of conversion, but the fraction ultimately degraded was unchanged. When PM-AV was incubated consecutively, first with trypsin and then with ribonuclease, nearly all the label became nonprecipitable (Fig. 1D). UAV and control virus were virtually unaffected by the enzymes and their label remained precipitable by trichloroacetic acid. Only when both trypsin and ribonuclease were used did UAV show some evidence of modification.

Specificity of the labilizing effect of plasma membrane on poliovirus. If the phenomenon of virus labilization by plasma membrane is part of the natural uncoating mechanism; one would expect to find it on all susceptible cells and only on susceptible cells. To test this, plasma membranes from the other cell strains were investigated. The experiments given in the legend of Fig. 1 were repeated with each kind of plasma membrane.

Hep-2 plasma membrane (poliovirus-suscepti-

ble) produced a pattern of labilization similar to that of Vero plasma membrane, except that ribonuclease without trypsin had little effect (Table 1). Neither plasma membrane from BHK-21 nor CaEK, both poliovirus-resistant strains, had any effect on virus lability.

FIG. 1. Kinetics of degradation of ^{14}C -uridine-poliovirus by Vero plasma membranes (PM). Virus was incubated for 2 hr at 0 C with PM, and then centrifuged. Virus adsorbed to the PM (PM-AV), virus that remained in the supernatant fraction (UAV), and control virus unexposed to PM were then incubated separately at 37 C. Portions were removed at intervals as indicated. The PM-AV portions were recentrifuged, and the second supernatant fractions were combined with the UAV specimens taken at the same time. One set of samples (A) was processed for trichloroacetic acid-precipitable counts without enzyme added; other samples were incubated with 100 μg of enzyme per ml for 30 min at 37 C. B, Ribonuclease only; C, trypsin only; D, trypsin and ribonuclease, consecutively. Counts are plotted against time as per cent of the count at zero time. Symbols $\circ$, control virus; $\square$, PM-AV; Δ , UAV.

TABLE 1. Degradation of poliovirus by different kinds of isolated plasma membranes

Plasma membranes	Input virus adsorbed (%)	Adsorbed virus solubilized in 60 min (%)			
		No enzyme	With trypsin	With RNase ^a	With trypsin and then RNase ^a
Vero	27	22.0	36.5	59.6	86.8
Hep-2	26	21.8	26.8	33.9	85.0
BHK-21	1.6	6.6	4.3	3.9	4.8
CaEK	2.3	3.3	5.5	7.6	5.2
None (control virus)		6.4	3.2	4.9	4.9

^a Ribonuclease.

Solubilization of poliovirus labilizer from Vero plasma membrane. The labilizer may or may not be the same substance as virus receptor. There is no convenient test for isolated poliovirus receptor, but the work of Phillipson and Lind (10) suggested that the echovirus 7 receptor was not unlike the poliovirus receptor and provided a method for separating it from the membrane structure. We applied this method to the Vero PM plasma membrane and tested the product for labilizing activity.

From 25 to 50% of plasma membrane protein was solubilized in the aqueous phase after extraction with butanol, as in the Phillipson and Lind procedure. When 1 mg of solubilized plasma membrane protein was substituted for intact plasma membrane in the procedure, 18% of the virus was rendered directly nonprecipitable and 44% was enzyme-sensitive (Fig. 2). In this experiment, enhancing effect of the two enzymes in sequence was not as pronounced as in previous tests with whole plasma membrane.

DISCUSSION

The results of these experiments are most easily explained if the uncoating of poliovirus is considered a two-step process. The first step appears

FIG. 2. Release of enzyme-sensitive RNA from ^{14}C -uridine-labeled virus after incubation with solubilized plasma membrane (PM) protein. The experiment described in Fig. 1 was repeated with solubilized protein instead of intact PM. Symbols: $\blacktriangle$, control virus after various times of incubation; $\circ$, control virus (average result after three methods of enzymatic treatment); $\square$, virus plus solubilized PM protein; $\bullet$, virus plus PM protein, trypsin treated; Δ , ribonuclease treated; $\blacksquare$, trypsin and ribonuclease treated.

to be attributable to a specific labilizer in the plasma membrane of susceptible cells. Incubation of the virus in contact with the substance renders it sensitive to proteases. In the second step, a tryptic enzyme, which may be added but which was always present in low concentrations in our preparations, acts on the labilized capsid opening it to transit by the RNA. From 30 to 60% of the viral RNA was shown to become ribonuclease-sensitive only after trypsin treatment, but it seems probable that much of the remaining virus passed through the same sequence of events through the mediation of the trypsin carried in the test system.

The results apply only to *in vitro* uncoating, but the fact that the labilizer was found only in membranes from poliovirus-susceptible cells suggests that it may be a natural phenomenon. If so, these results indicate that poliovirus is uncoated at the membrane without intervention of lysosomal enzymes. This does not preclude the possibility that the membranes may be invaginated to form vacuoles prior to uncoating *in vivo*, nor does it preclude the possibility of an alternative pathway through the mediation of lysosomes, but it does suggest that neither of these mechanisms is essential for uncoating *per se*.

Holland and McLaren (4) showed that virus RNA does not pass through a stage where it is exposed to extracellular enzymes. This does not, however, contradict the suggestion that uncoating occurs at the surface; the RNA may be discharged into the cell rather than outside it.

Holland and Hoyer (3) found virus receptors on both microsomal material and plasma membranes, but they found eclipsing activity associated only with the membranes. This leaves open the question of whether the two activities are manifestations of two different substances, with labilizer located only in the membranes, or whether the receptors from the two sites are in fact different. Our finding that methods used to

solubilize cell membrane receptors are also effective for solubilizing the labilizer suggests that the membrane receptor and labilizer are functions of similar if not the same substance; the microsomal receptor may be different.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-07554 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Billetter, M. A., C. Weissman, and R. C. Warner. 1966. Replication of viral ribonucleic acid. IX. Properties of double-stranded RNA from *E. coli* infected with bacteriophage MS2. *J. Mol. Biol.* 17:145-173.
2. Holland, J. J. 1962. Irreversible eclipse of poliovirus by HeLa cells. *Virology* 16:163-176.
3. Holland, J. J., and B. H. Hoyer. 1962. Early stages of enterovirus infection. *Cold Spring Harbor Symp. Quant. Biol.* 27:101-112.
4. Holland, J. J., and L. C. McLaren. 1959. The mammalian cell-virus relationship. II. Adsorption, reception and eclipse of poliovirus by HeLa cells. *J. Exp. Med.* 109:487-504.
5. Holland, J. J., and L. C. McLaren. 1961. The location and nature of enterovirus receptors in susceptible cells. *J. Exp. Med.* 114:161-171.
6. Hummel, B. C. W. 1967. Worthington enzymatic diagnostic reagents, p. 17. Worthington Biochem. Corp., Freehold, N.J.
7. Joklik, W. K., and J. E. Darnell, Jr. 1961. The adsorption and early fate of purified poliovirus in HeLa cells. *Virology* 13:439-447.
8. Kabat, E. A., and M. M. Mayer. 1961. Experimental immunochemistry, p. 559-560. Charles C Thomas, Publisher, Springfield, Ill.
9. Mandel, B. 1967. The relationship between penetration and uncoating of poliovirus in HeLa cells. *Virology* 31:702-712.
10. Phillipson, L., and M. Lind. 1964. Enterovirus eclipse in a cell-free system. *Virology* 23:322-332.
11. Warren, L., M. C. Glick, and M. K. Nass. 1966. Membranes of animal cells. Methods of isolation of the surface membrane. *J. Cell. Physiol.* 68:269-287.
12. Zöller, N., and G. Hobom. 1965. Ribonuclease, p. 793-799. In H. V. Bergmeyer (ed.), *Methods of enzymic analysis*. Academic Press Inc., New York.