ADENOVIRUS UNCOATING: AN ADDITIONAL EVIDENCE FOR THE INVOLVEMENT OF CELL SURFACE IN CAPSID LABILIZATION

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The early stages in the infection process of permissive cell with adenovirus, namely adsorption, penetration and uncoating, have been examined in detail in several studies. The mechanism of uncoating has been investigated by electron microscopic observations [1–3] and by different biochemical approaches *in vivo* [4–7] and *in vitro* [8]. In these investigations the sequential events of attachment, uptake and uncoating leading to a viral deoxyribonuclease-accessible product were studied after cell disruption and fractionation, and indirect evidences were provided that adenovirus particles are uncoated as they are released from the plasma membrane into the cytoplasm. In the present study, the uncoating process was investigated *in situ*, and some additional arguments were presented for the role of permissive cell plasma membrane in adenovirus capsid labilization.

If host cell surface was involved in the uncoating process of adenovirus, a reduction of infectious viral progeny could be expected when infection occurred in the presence of deoxyribonuclease. Two series of KB cell monolayer cultures were maintained in parallel in 5 ml test tubes (1×10^5 cells per tube), under 1 ml Eagle's basal medium supplemented with 1% bovine foetal serum. Both series were infected with an aliquot of adenovirus inoculum, at low multiplicities of infection (2–5 particles per cell) in order to sensitize the assay. The adenovirus serotypes used were types 2 and 5, belonging to the same immunological subgroup III [9]. 200 μg/ml of deoxyribonuclease I (Sigma DNase EC) was added to one set of cultures simultaneously with adenovirus inoculum, the other serving as control. At the end of the multiplication cycle, the culture medium was sucked off and the cells resuspended in 1 ml hypotonic buffer (0.01 M NaCl, 0.001 M disodium EDTA, 0.01 M Tris–HCl pH 8.0). The content of tubes corresponding to the same dilution were pooled and cells disrupted with three cycles of quick freezing and thawing. The virus infectious progeny present in the cell lysates was titrated by counting cells containing virus-induced lesions after staining with Coriphosphin [10]. Such methods were shown to have the same accuracy and greater repeatability than plaque assay, as evidenced by standard errors of 0.05 or lower [11]. The activity of deoxyribonuclease was controlled by using commercial thymus DNA and ^{14}C-labeled adenovirus DNA as substrates under the same conditions, viz. in the same culture medium containing the same KB cell concentration: no deoxyribonuclease inhibition was detected. As shown in table 1, the virus progeny yielded by deoxyribonuclease-treated KB cells was constantly lower than that of control cells, with an average value of nearly 50% less, suggesting that a certain number of virus particles were rendered deoxyribonuclease-sensitive during penetration into the cell.

It was therefore of interest to explore the fate of viral DNA, immediately after cytoplasmic engulfment of the virus particles adsorbed onto cell membrane, in the presence and in the absence of deoxyribonuclease. Labeling virus DNA with [^{14}C]thymidine, 0.1 μCi/ml (specific activity 50 mCi/mM) was carried out for 28 hr during the multiplication cycle, commencing at 12 hr after virus adsorption. Virus was extracted with fluorocarbon and purified in CsCl gradient [12]. CsCl was eliminated by dialysis against 0.01 M Tris–HCl buffer pH 7.6, 1 mM disodium EDTA. The specific activity of the virus preparation herein used was

Table 1
Infectivity titration of adenovirus progeny yielded by KB cells treated with DNAase during the period of virus infection.

Adenovirus serotype	Multiplicity of infection (ICU*/cell)	Adenovirus progeny yielded ICU*/cell		
		Control KB cells	DNAase-treated KB cells	% under control
Type 2	2	1650	1200	27.2
	2	1500	1100	26.6
	5	4880	1800	63.2
	5	3000	1700	43.3
	5	3500	1800	48.5
Type 5	2	1200	540	55.0
	2	550	335	39.1
	5	1350	510	62.2
	5	3000	960	68.0
				Average 48.1

* Infectivity titers were determined with the coriphosphin method and the results were expressed as infecting cell units [10, 11]. Each value corresponded to 8 test tubes pooled at the end of the multiplication cycle. The standard error of the titration method was less than 0.04.

4–6 X 10^5 cpm/ml of viral suspension titration 5–10 X 10^{10} infecting cell units (ICU)/ml.

Two KB cell cultures were maintained in parallel at 0°C in nutrient medium at a concentration of 3 X 10^6 cells/ml (total cell number per spinner flask 7.5 X 10^7), and infected with 300 µl aliquots of a freshly prepared adenovirus inoculum, at a multiplicity of infection of 200–400 ICU/cell (total counts 150 000). Adenovirus adsorption was allowed to proceed for 2 hr at 0°C temperature at which neither viral penetration nor uncoating could occur [4]. Deoxyribonuclease I (200 µg/ml) was then added to one of the two cultures and the temperature was rapidly increased to 37°C and cultures maintained at this temperature for 15 min, since it has been demonstrated that the membrane-bound adenovirus has a half-life of about 15 min [7] and that some virus can be found deep in the cytoplasm as early as 15 min after penetration in cell [1]. At 15 min the cultures were quickly cooled in ice, centrifuged in the cold and the cell pellets resuspended in 5-fold vol of 0.14 M NaCl, 0.01 M Tris–HCl buffer pH 7.6 containing 10 mM disodium EDTA. Viral DNA was then analyzed on alkaline sucrose gradients, the cells being placed intact on top of the gradients, according to a modification of the method of Horwitz [13]. Over a 0.5 ml cushion of CsCl in water (ρ = 1.8) a linear 15 ml gradient was formed by mixing 5 and 20% sucrose prepared in 1.0 M NaCl, 0.19 N NaOH, 0.01 M disodium EDTA, 0.1% sodium lauryl sarcosinate (Sarkosyl, Ciba-Geigy). A 0.5 ml amount of 3% sucrose solution in 0.01 M Tris–HCl buffer pH 7.6 containing 0.01 M disodium EDTA, 0.5% sodium deoxycholate, 1% sarkosyl, 1 mg/ml pronase (Pronase free of nucleases, Calbiochem) was placed over the preformed gradient. Cells in 1 ml of buffered 0.14 M NaCl, 0.01 M disodium EDTA were placed intact on the gradient. Centrifugation was performed at 18°C for 16 hr at 18 000 rpm in the SW 25.3 rotor of a Spinco ultracentrifuge. For control 50 µl aliquots of untreated and DNAase-treated [^{14}C]thymidine labeled adenovirus suspension were centrifuged in the same conditions. Gradients were fractionated by pumping 0.4 ml aliquots through a probe placed 0.5 cm above the bottom of the tube, and radioactivity counted in Bray's scintillation fluid. The results are presented in fig. 1. The denatured DNA from control and DNAase-treated type 2 adenovirions sedimented in alkaline sucrose density gradient in one symmetric peak (fraction 18) corresponding to intact DNA strands. The same pattern of intact molecules was obtained with adenovirus DNA extracted from KB cells early after uptake of virions in the absence of DNAase. When infection was conducted in the presence of DNAase, most of the viral DNA was cleaved

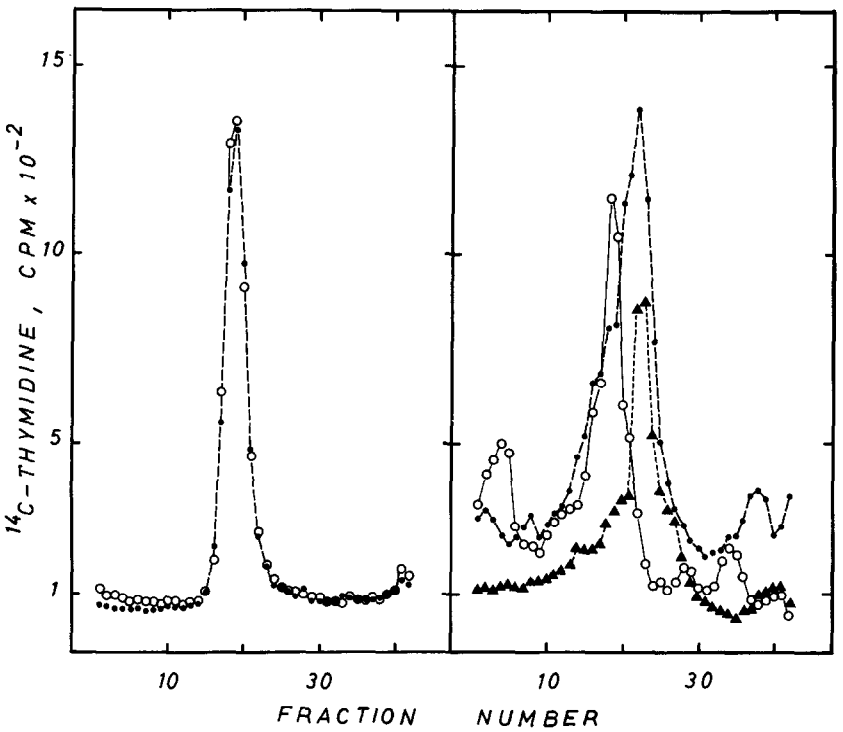

Fig. 1. Zonal sedimentation analysis of adenovirus type 2 DNA in alkaline sucrose density gradient. Left panel: 50 μl of untreated (o—o—o) and DNAase-treated (•—•—•) [^{14}C]thymidine labeled freshly prepared adenovirus suspension (25 000 cpm, 80 μg DNA) were layered onto a 15 ml linear 5–20% sucrose gradient prepared in 1.0 M NaCl, 0.19 NaOH, 0.01 M disodium EDTA, 0.1% sarkosyl, overlayered with 0.5 ml of 3% sucrose solution prepared in 0.01 M Tris–HCl buffer pH 7.6, 0.01 M disodium EDTA, 0.5% sodium deoxycholate, 1% sarkosyl, 1 mg/ml pronase, and centrifuged at 18°C for 16 hr at 18 000 rpm in the Spinco SW 25-3 rotor. Gradients were collected in 0.4 ml fractions; *Right panel*: 300 μl of [^{14}C]thymidine labeled adenovirus suspension (150 000 cpm, 500 μg DNA) were adsorbed onto 7.5 x 10^7 KB cells (200–400 infecting virus particles/cell) maintained in suspension in nutrient medium (3 x 10^6 cells/ml) for 2 hr at 0°C. At the end of the adsorption period, DNAase I (200 μg/ml) was added to one cell culture (•—•—•), the other serving as control (o—o—o), and temperature rapidly increased and maintained at 37°C for 15 min. At 15 min the cells were cooled, centrifuged, resuspended in 1 ml 0.14 M NaCl, 0.01 M Tris–HCl buffer pH 7.6, 10 mM disodium EDTA, and placed intact onto 5–20% alkaline sucrose gradient overlayered with the sarkosyl–deoxycholate-pronase solution. In a third experiment, cells were incubated with 200 μg/ml DNAase I, sedimented and resuspended in DNAase-free medium prior to adenovirus adsorption. Viral DNA from adsorbed adenovirions (125 000 cpm adsorbed corresponding to 400 μg DNA) was analyzed as above mentioned (▲—▲—▲).

and converted into smaller fragments with a broad distribution (fractions 21–28). Assuming a sedimentation coefficient of 34 S for denatured DNA [14, 15] a sedimentation coefficient value of 28 S was found for the peak of fragmented DNA, ranging from 20–30 S.

Inasmuch as the endogeneous endonuclease activity recently found associated with penton and virion of adenovirus type 2 [16, 17] could interfere with the exogenous DNAase added to the medium, same experiments were performed with adenovirus type 5, which was found devoid of endonuclease activity [18], giving similar patterns in alkaline sucrose gradients (not shown). These results supported the assumption the some critical event occurs at the cell surface, immediately after penetration through the plasma membrane, resulting in some alteration of the adenovirus capsid rendering the DNA accessible to deoxyribonuclease action.

However, it could not be excluded that DNAase, when used at the time of infection, could get into the phagocytotic vacuoles or even into the cytoplasm of the cell with the infection virion, and function within the host cell when the capsid in disrupted, rather than

acting only on the plasma membrane outside of the cells. To test this hypothesis, KB cells (3×10^6/ml) were incubated for 15 min at 37°C with 200 μg/ml of DNAase prior to virus adsorption, pelleted, and resuspended in DNAase-free medium. [^{14}C]thymidine labelled adenovirions were adsorbed onto the cells for 2 hr at 0°C, then the temperature was increased and maintained at 37°C for 15 min. Cells were sedimented, resuspended in isotonic buffer containing 10 mM disodium EDTA and viral DNA analyzed as described above. The sucrose gradient centrifugation patterns thus obtained showed an extensive breakdown of viral DNA (fig. 1), implying that DNAase absorbed into the cell was capable of acting on partially uncoated DNA. Our method of investigation permitted to establish that the first alteration of the adenovirus capsid occurs at the cell surface, but failed to determine where this phenomenon is located, on the outer or inner side of the plasma membrane, or in the superficial cytoplasm.

The results herein presented are consistent with previous results of *in vivo* investigations [1, 7] as well as with *in vitro* degradation of adenovirions by isolated KB cell plasma and nuclear membranes [19], which suggest that uncoating is a two-step process whose first stage, a capsid labilization, occurs at the plasma membrane, the complete uncoating being achieved at the nuclear membrane. Moreover, it has been recently reported [20] that DNA-labeled adenovirions extracted from lysosomes as late as 2 hr post infection, though remaining infectious, have become sensitive to pancreatic deoxyribonuclease. That would suggest that adenovirus particles adsorbed by phagocytosis and sequestered within lysosomes [1, 2] have been in some way labilized in their capsid. How infectivity may be resistant to lysosomal deoxyribonuclease after capsid labilization remains unelucidated.

Whatever its intimate mechanism is, this paper presents new evidence that the initial step in adenovirus uncoating occurs at the cell surface. In addition, it describes a simple procedure consisting of infecting cells at low multiplicity of infection in the presence of nuclease, and of subsequent titration of adenovirus progeny infectivity and analysis in alkaline sucrose density gradient of parental viral nucleic acid extracted early after adsorption, which can be used as a general method for a rapid cellular localization of the uncoating process and could be applicable to any species of virus.

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