The Early Interaction of Coxsackievirus B3 with HeLa Cells (35732)

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Joklik and Darnell (1) showed that radioactive poliovirus not only attached to HeLa cells, but that at 37° a large proportion of the labeled virus eluted from the cells as nonproductive virus. Eluted virus, nevertheless, contained infectious ribonucleic acid and was found to have acquired a lighter buoyant density when centrifuged in a potassium tartrate gradient (2). Consequently, studies were initiated to determine whether an enterovirus representative of a different subgroup, coxsackievirus B3, would exhibit a different elution pattern from that of poliovirus. Alternatively, it was considered possible that coxsackievirus would not elute, since the group B viruses bind to specific receptors of HeLa cells which are separate and distinct from those which attach polioviruses (3–9).

Materials and Methods. Cells. HeLa cells were cultured as monolayers in Eagle's complete amino acid–vitamin mixture supplemented with 10% calf serum (CaS-10BME) or in suspension using 2X Eagle's minimal essential medium (MEM) (10) with 5% horse serum and 2% calf serum, and 2 μg/ml of insulin (11). Cells for virus plaque assays were grown either in plastic petri dishes (Falcon TCD, 60 × 15 mm) in a humidified atmosphere of 5% CO_2 in air, or in rectangular, 2-oz bottles sealed with rubber-lined screw caps. Mouse L fibroblasts, kindly supplied by Hans Diderholm, Uppsala University, Sweden were cultivated as indicated for HeLa cells.

Virus. Coxsackievirus B3, strain Nancy, (3) was plaque purified (5) and propagated in monolayer or suspension cultures of HeLa cells using an input virus multiplicity (PFU/cell) of 10–50, by methods described previously (5, 12). Virus labeled with ^{32}P-phosphate or with ^{14}C-valine was propagated in medium containing citrate (MEM-citrate) in place of phosphate or in Eagle's medium devoid of valine, respectively. For virus labeled with ^{32}P, dialyzed calf serum was added to a final concentration of 2%.

Virus purification. Virus labeled with ^{32}P-phosphate was purified by consecutive banding in two CsCl gradients, and ^{14}C-valine labeled virus was purified by use of ECTEOLA anion exchange resin followed by banding in CsCl (13). Assays of each 3-drop fraction of B3-^{32}P collected by puncture from the bottom of the CsCl gradients revealed only one major peak which contained both the infectivity and the radioactivity associated with virus. Usually two fractions comprising this peak were pooled for use. Preparations of purified coxsackievirus B3-^{32}P possessed an OD 260:280 ratio of 1.64, with approximately 500 particles/PFU as calculated by the method of Joklik and Darnell (1). Around 2% of the radioactivity in purified virus was acid soluble. Infection of 3 to 6 × 10^8 cells usually yielded 2 to 5 × 10^{10} PFU of purified ^{32}P- or ^{14}C-labeled virus. Purified virus was stored in concentrated form at −60° following dialysis against phosphate buffered saline without calcium or magnesium ions (PBSA) or else diluted 1:50 in BSS containing 0.1% bovine serum albumin (BSA) and kept at −20°. The plaque method used for assay of virus infectivity has been described previously (3) and was conducted in triplicate or quadruplicate.

Preparation of cell-associated virus (CAV).

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CAV was prepared at 0° at pH 7 as described in a previous report (9) with input virus multiplicities not in excess of 200 PFU/cell. The amount of CAV (PFU and cpm) was determined by dissociation of virus by acid pH (9) and/or SDS (14) and by assay of radioactivity in cell samples solubilized in 0.1 *M* NaOH.

Assay for "eclipsed" virus. "Eclipsed" virus (15) was considered to be that amount of virus infectivity or label which was determined initially to be cell associated and which was not released from the cells following treatment of washed cells for 1 min at 2° with 0.05 *M* glycine buffer adjusted to pH 1.5 (9). In some experiments, "eclipse" of virus infectivity was monitored by use of sodium dodecyl sulfate (SDS) by the method of Mandel (14).

Assay for radioactivity. Samples containing ^{32}P were dried on filter paper in planchettes and counted in a Tracerlab FD-1 gas flow counter fitted with a Monomol window or they were counted directly by Cerenkov radiation in 10 ml of 0.1 *M* NaOH with the ^{3}H setting in a Nuclear Chicago liquid scintillation spectrometer (16). Samples containing ^{14}C were solubilized in 10 ml of a toluene liquid scintillation mixture containing BIO-SOLV-BBS3 (Beckman Instruments, Inc., Fullerton, Calif.) and counted in a Beckman liquid scintillation counter, model LS-133.

Reagents. Carrier free ^{32}P-phosphate (35–70 mC/mg of phosphorus) was obtained from the Radiochemical Center, Amersham, England. ^{14}C-Valine was purchased from New England Nuclear Company, as 1.8 mC/mg. HEPES buffer (*N*-2-hydroxymethylpiperazine-*N*′-2-ethanesulfonic acid) was obtained from Sigma Chemical Co., St. Louis or from Calbiochem, Los Angeles.

Extraction and assay of infectious viral RNA. Eluted and native virus preparations were extracted with phenol for recovery of infectious RNA by the method of Oberg (personal communication). All procedures were performed at 4°. One ml of redistilled phenol saturated with water was mixed with 1 ml of virus preparation to which 0.05 ml of washed bentonite (0.58% in H_2O) was added. The mixture was shaken for 2 min, the aqueous phase was removed following centrifugation, and an equal volume of freshly opened ether was added. Following three successive ether extractions of the aqueous phase, nitrogen was bubbled through the final extract and the ether-free solution was assayed for infectivity.

For infectivity assay, the RNA preparations were diluted in Eagle's MEM solution containing 0.02 *M* HEPES, pH 7.4; and DEAE-dextran, 300 μg/ml (17); and 0.2-ml volumes were allowed to attach to HeLa cell monolayers at 23° for 30 min. The infected monolayers were washed, overlaid with medium containing 0.5% agar and incubated at 37° for 48 hr for plaque formation. As a control, RNAse (at 2 μg/ml) was incubated with portions of the RNA samples for 30 min at 23°, prior to assay. Based on the titer of the virus pool prior to extraction, the recovery of RNA infectivity was approximately 0.01%.

Results. An experiment was designed to determine the relationship between the attachment rates to HeLa cells of coxsackievirus B3 radioactivity (^{32}P) and infectivity (PFU) at 37°. Purified coxsackievirus B3-^{32}P was added to HeLa cells suspended at 5×10^6 cells /ml in PBSA (0.05 *M* HEPES buffer) pH 4.5 at 30 PFU/cell. The mixture was incubated at 37°; and at intervals samples were diluted 1:10 into PBSA, the cells were removed by centrifugation, and the fluids were assayed. The data showed that these two virus activities diverged somewhat from each other after the first few minutes (Fig. 1). A similar relationship between PFU and cpm was found when attachment of ^{14}C-labeled virus was measured. At 0°, virus label and infectivity were found to attach more slowly to HeLa cells but at comparable rates. The high divergence of attachment of poliovirus PFU and cpm at 37° at pH 7.0 has been attributed to elution (1) or sloughing (2) of a large fraction of the labeled virus population. The present results indicate, however, that most of the coxsackievirus B3 radioactivity remained cell associated at 37°. In this experiment, pH 4.5 rather than 7.0 was used, because attachment of coxsack-

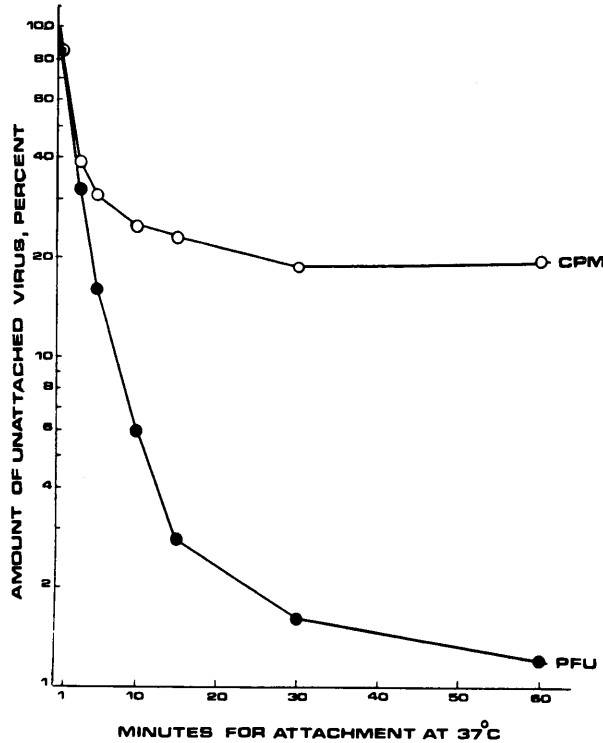

FIG. 1. The comparative rates of attachment at 37° of coxsackievirus B3 infectivity and radioactivity to HeLa cells at pH 4.5.

ievirus B3 infectivity to HeLa cells occurred at a maximum rate at pH 4.5 (18).

The effect of low pH on virus elution was determined. Cell-associated virus (CAV) labeled with ^{32}P or ^{14}C was prepared as described in Materials and Methods. Following washing, cells with attached virus were resuspended in saline–HEPES buffer (0.05 *M*) (^{32}P-labeled virus) or in BSS-BSA (^{14}C-labeled virus) at pH 7.0 or 4.5. The suspensions were incubated at 37° and sampled at intervals for assay of eluted radioactivity and infectivity. Samples of each of the respective cell populations were placed at 0° for control of virus elution. The results of a representative experiment depicted in Fig. 2 revealed that almost 60% of the coxsackievirus B3-^{32}P radioactivity eluted from HeLa cells at pH 7.0 in a manner similar to that found for poliovirus (1, 2). More than 90% of the radioactivity eluting was acid insoluble and confined to a particle with a lighter density than native virions (to be published). Attention is directed to the finding that pH 4.5 inhibited elution of virus radioactivity. In the control preparations at pH 7.0 at 0°, only 2–3% of the radioactivity was eluted. Recovery analysis revealed that radioactivity not eluted remained cell associated. Relative to the eluted label, there was no significant elution of infectivity at 37° from the cells when tested by the routine plaque assay. Experiments with ^{14}C-labeled virus revealed essentially the same findings. The greater inhibition of elution at acid pH found in this experiment compared to that implied by Fig. 1, may reflect the different procedures used.

In a control experiment, L cells were used to demonstrate that a resistant cell line would not attach radioactivity when exposed to coxackievirus B3-^{32}P. Results of previous studies had shown that L cells did not replicate or attach B3 virus when measured by plaque assay (unpublished observations). L-cells grown in suspension were washed and exposed to coxsackievirus B3-^{32}P in a manner similar to that described for the preparation of CAV at 0° using HeLa cells, except the

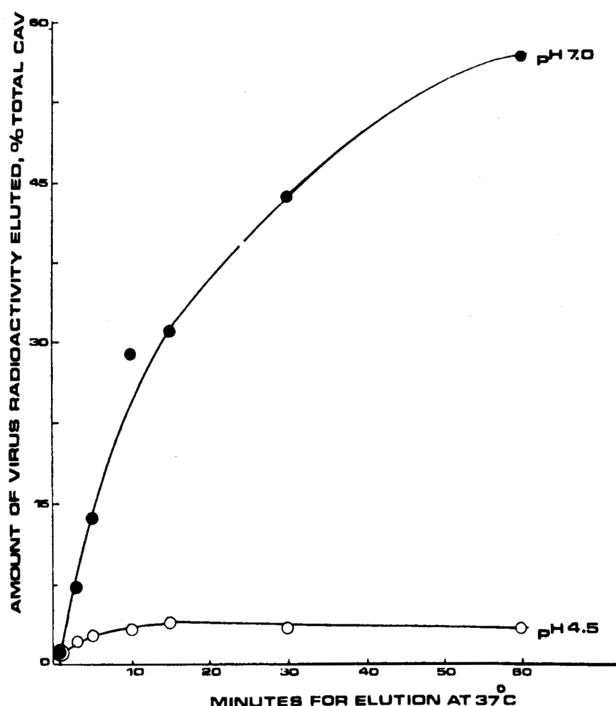

FIG. 2. The comparative rates of elution at pH 4.5 and 7.0 of coxsackievirus B3-^{32}P radioactivity from HeLa cells.

L-cell concentration was raised to 4 × 10^7 cells/ml to increase the chance of virus to attach. After 1-hr incubation at 0°, the cells were washed 3×; and the wash fluids and cells were assayed for radioactivity. Ninety-eight percent of the input label was recovered in the wash fluids with only 0.15% found to be cell associated. These results are in striking contrast to those found for HeLa cells, which attach approximately 90% of the input label under comparable experimental conditions.

The finding of inhibition of elution of coxsackievirus B3-^{32}P radioactivity from HeLa cells at pH 4.5 prompted a more detailed investigation of the effect of pH on elution of virus. An experiment was performed similar to that described by Fig. 2 except that replicate samples of cells with attached virus were resuspended in saline-containing 0.1 *M* HEPES buffer adjusted to different pH values with HCl or NaOH and incubated at 37° for virus elution. The pH levels tested ranged from 4.5 to 8.0, at 0.5-unit intervals. The amounts of radioactivity eluted at 30 min are presented in Fig. 3. As found previously, low pH markedly inhibited elution of radioactivity, whereas a pH range from 6.5 to 8.0 permitted maximal elution. These findings confirm and extend those of Fenwick and Cooper (2) who reported little effect of pH on elution of poliovirus ^{32}P radioactivity over the pH range of 5.5 to 7.9.

An attempt was made to determine if virus elution was irreversibly affected at low pH levels. Virus was adsorbed to cells at 0° for 1 hr at neutral pH. The cells were washed free of unattached virus; and cell aliquots were resuspended in buffered saline at different pH values for elution of virus at 37° for 30 min as in the preceding experiment. The cells were then sedimented, resuspended in buffered saline at pH 7.0, and tested at intervals over the next 30 min for elution of radioactivity at 37°. In those cell samples which had been preincubated at pH 4.5, only a small increase in eluted virus was found, suggesting that incubation of cells at this low pH had damaged, irreversibly, the cellular activity giving rise to eluted virus. The con-

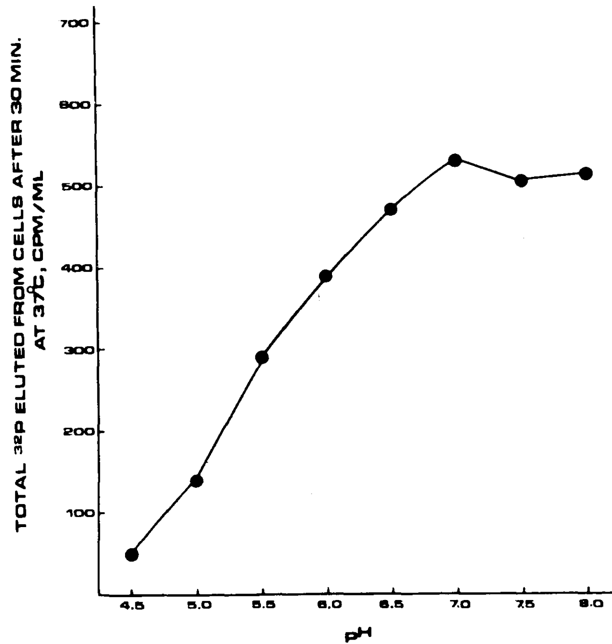

FIG. 3. The effect of pH on elution of coxsackievirus B3 ^{32}P from HeLa cells.

trol samples, which had been preincubated at pH 7.0 for 1 hr at 0°, eluted to a maximal level when placed at 37° in a manner similar to that described for poliovirus (1).

The observation that pH 4.5 provided maximal attachment of coxsackievirus B3 to HeLa cells with minimal elution of radioactivity directed our attention to evaluate whether low pH would permit "eclipse" of viral infectivity. In an extension of the experiment described in Fig. 2 each of the cell–virus suspensions was tested for recovery of virus infectivity following exposure to glycine buffer at pH 1.5 for 1 min at 2° (9). This procedure for dissociating virus from cells was found to be an effective method for measuring cell-associated virus which had not undergone "eclipse" (9). The data (Fig. 4) revealed that the virus remained infectious when the virus–cell complex was incubated at

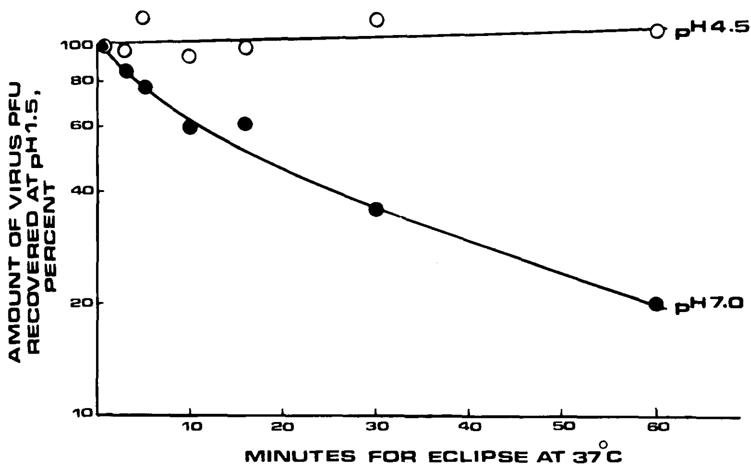

FIG. 4. Inhibition of "eclipse" of coxsackievirus B3 by HeLa cells incubated at pH 4.5.

TABLE I. Recovery of Infectious RNA from Native and Eluted Preparations of Coxsackievirus B3.

Preparation	Total (PFU)	
	Native virus	Eluted virus
Virions	1.0×10^9	1.6×10^5
Infectious RNA	9.2×10^4	1.9×10^5

pH 4.5 at 37°, whereas, incubation at pH 7.0 resulted in virus "eclipse." It is not possible with this technique to evaluate if the virus will penetrate the cell surface at pH 4.5.

The finding that both of the ^{14}C and ^{32}P labels were eluted in parallel from B3 virus-coated cells at pH 7 (37°), suggested that the eluted labels were a reflection of the structural integrity of the virions. However, negligible amounts of infectious virus were recovered. Experiments were performed to test whether infectious RNA could be extracted from the eluted preparations. RNA was extracted from native purified B3 virus and from eluted preparations by the cold phenol method outlined in Materials and Methods.

Results of the RNA infectivity assays given in Table I indicate that the eluted preparations contained infectious RNA. The eluted preparations thus contained large amounts of virons which had been rendered noninfectious following interaction with the cell surface. It was assumed that the efficiency of recovery of RNA infectivity from native and eluted virus was comparable (approx 0.01% of the original titer of native virus). These findings are in agreement with those found for poliovirus T1 (1). RNA extracted from native or eluted preparations yielded no plaques following incubation with RNAse (2 μg/ml) for 30 min at 23°.

Discussion. Elution of virus from the surface of host cells, as first described by Joklik and Darnell (1) for a poliovirus–HeLa cell system is an interesting phenomenon which has not yet been explained. The observation of this event has been confirmed by others (2, 19–21) although Taylor and Graham (22) did not find elution of poliovirus from monkey kidney cells. The results of the present study provide evidence that an enterovirus representative of a different subgroup also elutes from HeLa cells under conditions similar to those found for polioviruses. Thus, polioviruses, echoviruses (19), and a coxsackievirus of group B have a common property associated with virus elution. Virus elution probably reflects a cell membrane activity, since enteroviruses have not been shown to possess enyzme activity toward their receptor (5) and since a lipid-containing cellular component is considered to be associated with eluted virus [(2), unpublished observations also have revealed that eluted B3 virus has a lighter buoyant density in a tartrate gradient]. Elution is inhibited by glutathione (2); by low temperatures; and, as shown herein, by low pH; conditions which also inhibit the eclipse or uncoating of virus, leading to productive infection. It is tempting to speculate that eclipse and elution may be related activities of the cell membrane, each giving rise to altered virions which cannot be recovered as infectious particles. The reason why some particles are rejected by the cell while others result in productive infection remains to be determined (2). At present it is also difficult to understand why viruses, which attach to different receptors on the cell membrane, should be eluted in a similar way unless perhaps relatively large structures comprising several different virus receptors are sloughed together from the cell. Under conditions in which the experiments were performed, no significant amount of "soluble" receptor activity was found in the supernatant fluid from control cells to suggest that sloughing of viral receptors is not a normal cellular activity, unless the receptors were labile in this form and undetectable by biological assay (9). Experiments are in progress to further evaluate the elution phenomenon associated with enteroviruses and their host cells.

Summary. Purified coxsackievirus B3 labeled with ^{32}P- or ^{14}C-valine interacted with HeLa cells in a manner resembling that found by others for poliovirus. Radioactivity eluted from cells at 37°, pH 7, as noninfectious virus, but contained infectious RNA. Both elution and "eclipse" of virus were inhib-

ited irreversibly at low pH to provide further evidence that these processes may be related properties of the cell surface.

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