Specific Cell-Surface Alteration by Enteroviruses as Reflected by Viral-Attachment Interference

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ABSTRACT

CROWELL, RICHARD L. (Hahnemann Medical College, Philadelphia, Pa.). Specific cell-surface alteration by enteroviruses as reflected by viral-attachment interference. J. Bacteriol. 91:198–204. 1966.—Exposure of HeLa cells to high levels of coxsackievirus B3 produced cells which were refractory to attachment of coxsackievirus B1, whereas poliovirus T2 attached normally. Under similar conditions, poliovirus T2 was found to interfere with the attachment of poliovirus T1 to HeLa cells without affecting the attachment rate of coxsackievirus B3. The data confirm earlier findings that the receptor sites on HeLa cells, which bind members of group B coxsackieviruses, are distinct from those for polioviruses. Quantitatively, coxsackieviruses B1 and B3 were found to be mutually exclusive in the attachment interference assay to suggest that they compete for the same receptors on the HeLa cell surface. The finding that input multiplicities of B3 virus which exceeded 500 saturated the homologous viral receptors of HeLa cells was unexpected, but was consistent with the results of interference assays. Excessive amounts of input virus did not, however, inhibit eclipse of homologous cell-associated virus. Attachment interference between enteroviruses occurred even though the interfering virus was eclipsed prior to addition of challenge virus. The finding that enterovirus attachment interference was reversible with acid pH suggested that attachment and eclipse of enterovirus does not result in a permanent alteration of the cell membrane and that these events occur at the cell surface.

HeLa cells chronically infected with either coxsackievirus B3 or B5 were shown to be resistant to superinfection by all members of coxsackie group B (3, 4). This acquired cellular resistance was of a specific nature, since the virus-carrier cells were fully susceptible to infection by polioviruses, vaccinia virus, herpes simplex virus, adenovirus T1, and to group A coxsackieviruses 13, 15, and 18. The resistance of the chronically infected cells to group B coxsackieviruses was found to be a result of altered cell surfaces, since superinfecting B viruses did not attach or eclipse normally. It was postulated that the surfaces of the virus-carrier cells were altered by virus-antibody complexes which blockaded specific viral-receptor sites. The results of studies to be reported here demonstrate that enteroviruses, in the absence of viral antibody, are capable of specific alteration of normal HeLa cells to render them refractory to attachment of closely related viruses.

MATERIALS AND METHODS

Viruses and virus assay. The origins of strains of coxsackieviruses B1, B3, and B5 and of polioviruses T1 and T2 with homotypic antisera used in this study have been described previously (4). Each virus was plaque-purified by five serial passages of virus from single plaques obtained at terminal dilutions by the method of Mosley and Enders (21). Virus to be used as the interfering agent was obtained in high titer, which exceeded 10^8 plaque-forming units (PFU) per milliliter, by collection of the intracellular yield of virus in small volumes of Hanks’ balanced salt solution (BSS) after infection, and incubation at 37 C for 10.5 hr. Infected cells were scraped from the glass and disrupted by four cycles of alternate freezing and thawing. The cell debris was removed by centrifugation at 900 X g for 10 min, and the supernatant fluid containing virus was frozen at —24 C until used. For...
some experiments, where indicated, coxsackievirus B3 was purified by differential centrifugation and passage through Ecteola according to the method of Levinthow and Darnell (18). Assay for virus was performed in quadruplicate by the modified plaque-count method of Holland and McLaren (14).

HeLa cells. A subline of HeLa cells obtained from J. J. Holland was routinely cultivated in medium containing Eagles’ complete amino acid-vitamin mixture supplemented with 10% calf serum by methods described previously (4).

Determination of virus attachment and viral attachment interference. Monolayer cultures of HeLa cells were rinsed three times with 50-ml volumes of phosphate-buffered saline (PBS) free from magnesium and calcium ions, and the cells were removed from the glass by aid of ethylenediaminetetraacetic acid (EDTA) and a rubber scraper. The cells were sedimented at 300 g for 5 min, resuspended in cold PBS, counted in a hemocytometer, and samples containing 10⁶ cells were distributed to separate tubes. The cells were sedimented as before, the supernatant fluids were discarded, and the cells in one tube were resuspended in interfering virus diluted in BSS containing 3% calf serum (BSS-CaS3) to a final concentration of 5 x 10⁶ cells per milliliter with an input viral multiplicity of 1,000 to 10,000. Another tube, which served as control, received BSS-CaS3 without virus. The cell suspensions were incubated in a water bath at 37 C for 1 hr, except where indicated otherwise, and the cells were washed twice in BSS-CaS3 and tested for ability to attach a second (challenge) virus. HeLa test and control cells, respectively, were resuspended in challenge virus diluted in BSS-CaS3 to a final concentration of 5 x 10⁶ cells per milliliter at a viral multiplicity of 0.4. At intervals of incubation at room temperature (24 C), samples were withdrawn and diluted 100-fold to stop virus attachment, the cells were removed by centrifugation, and the amount of unattached challenge virus in the supernatant fluid was measured. The amount of virus which was attached to cells at a given time was determined by difference between the amounts of free virus found and the input virus. For assay of challenge virus in the presence of residual interfering virus, an equal volume of diluted antiseraum to the interfering agent was added to each virus dilution, and a neutralization period of 60 to 90 min was allowed at room temperature prior to inoculation into plaque-assay cultures (3). Adequacy of antiserum content was verified in control assays, and cross-neutralizing activity of the antiserum used was shown to be absent for each virus under assay.

Assay for eclipsed virus. Eclipsed virus (10) was considered to be that amount of virus infectivity which was determined initially to be cell-associated, and which was not dissociable from the cell by treatment of washed cells with 0.05 M glycine buffer (7) adjusted to pH 2.0.

RESULTS

Effect of coxsackievirus B3 treatment of HeLa cells on attachment of coxsackieviruses and polioviruses. In preliminary experiments, washed HeLa cells were allowed to react with coxsackievirus B3 at an input multiplicity of 2,000 PFU per cell for 1 hr at 37 C, as described in Materials and Methods. Normal cells and cells treated with B3 virus were tested for ability to attach coxsackievirus B1, and the results of a representative experiment are presented in Fig. 1. The kinetics of B1 virus attachment reveal that treatment of cells with B3 virus markedly inhibited the ability of the cells to attach coxsackievirus B1. Similar results were obtained on six different occasions, and with B3 virus which had been purified. In an experiment which served as control, no significant difference was found in the attachment rate of B1 virus to normal cells, and to cells treated with undiluted or diluted extracts of lyastes derived from normal HeLa cells. It was concluded that B3 virus was responsible for the inhibition of B1 virus attachment.

In experiments of similar design, HeLa cells were treated with coxsackievirus B3 at a multiplicity of 6,000 PFU per cell and were tested for ability to attach coxsackievirus B5 and poliovirus T2. The results given in Fig. 2 and 3 show that coxsackievirus B3 did not inhibit the attachment rate of poliovirus T2 and only partially reduced the rate of attachment of B5 virus. As control, a replicate sample of cells, which was treated concurrently with B3 virus, was found incapable of attachment of coxsackievirus B1 as described in Fig. 1. These results indicate that poliovirus binds to sites on live cells, which are distinct from those which attach coxsackieviruses B1 and B3. The significance of the reduced rate of attachment found for B5 virus remains to be determined,
although the results coincide with those observed for attachment of B5 virus to HeLa cells chronically infected with B3 virus (4).

Influence of multiplicity of interfering virus on viral attachment interference. To determine the amount of interfering virus required to render cells incapable of attaching B1 virus, decreasing concentrations of coxsackievirus B3 from 2,000 to 20 PFU per cell were tested. It was found (Fig. 4) that treatment of HeLa cells with as many as 20 PFU per cell gave no significant inhibition of B1 virus attachment, whereas the addition of more than 200 PFU per cell produced marked interference. Similar results were obtained from repeated experiments. Correspondingly, in experiments in which diminishing amounts of B1 virus were employed as the interfering virus, an amount of 200 PFU per cell was found to inhibit attachment of B3 virus (Fig. 5). These findings suggest that B1 and B3 viruses compete for the same sites for attachment to HeLa cells. Results of experiments of similar design revealed that treatment of HeLa cells with more than 90 PFU per cell of poliovirus T2 rendered cells unable to attach poliovirus T1 without reducing the ability of the cells to attach coxsackievirus B3 (Fig. 6).

The amount of interfering virus which attached to the cells in the preceding experiments was determined by assay of free virus remaining in the supernatant fluid after treatment of cells with virus for 60 min at 37 C. Unexpectedly, in light of Fogh’s experience (8), the determinations revealed that a relatively low percentage of the virus became cell-associated when high multiplicities were used. In additional experiments, the relationship between input B3 virus multiplicity and
attached multiplicity was determined for HeLa cells incubated at 37°C for 60 min at a final concentration of $5 \times 10^4$ per milliliter. The results presented in Fig. 7 show that the per cent of virus which attached to cells decreased progressively when input multiplicities exceeded 50, and that effective viral receptor saturation was approached at input-virus multiplicities greater than 500. It is apparent that the values obtained for saturation of cellular receptors for homologous virus are of the same order of magnitude as that found for attachment interference between related heterologous viruses.

To determine more directly the ability of B3 virus to establish autointerference in the system described previously, HeLa cells were treated with virus which had been inactivated by ultraviolet (UV) irradiation. Preliminary determinations of the rate of UV inactivation of coxsackievirus B3 revealed linear inactivation kinetics to a survival of 0.01%, after which the rate of inactivation became progressively slower. In the experiment to be described, 2.5 ml of a B3 virus preparation containing $2.2 \times 10^8$ PFU per milliliter were placed in an open plastic petri dish (60 x 15 mm) and exposed for 10 min, with constant agitation, to UV irradiation from a 140-w Hanovia Lamp at a distance of 38 cm. This amount of irradiation reduced the virus titer to $7.7 \times 10^4$ PFU per milliliter or to a survival of 0.003%. HeLa cells, prepared in suspension as previously, were treated for 1 hr at 37°C with irradiated virus, washed, and measured for ability to attach fresh B3 virus at an input multiplicity of 0.2. The relatively small amount of residual infectious B3 virus contained in the irradiated virus preparation and which remained after washing of the cells accounted for less than 1% of the virus under assay. The results of this experiment showed that UV-inactivated virus can produce significant autointerference of attachment of virus. These results are in contrast to those of Drake (6) in which viral interference between polioviruses was eliminated by irradiation of interfering virus with UV light. The differences may be reflected in the amount of denaturation of viral protein which occurred during irradiation in the respective systems.

Nature of the cell-surface alteration produced by coxsackievirus B3. In the previous experiments, interfering virus was allowed to react with cells for 1 hr at 37°C. These conditions were shown to be sufficient for eclipse of 95% of attached virus when virus was used at low input multiplicities. Since viral attachment interference was dependent upon high input multiplicities, experiments were performed to determine the influence of virus concentration on eclipse of virus. To account for the observed interference, it was important to establish whether infectious virus remained at the cell surface.

Increasing amounts of coxsackievirus B3 were added to replicate samples of washed HeLa cells to give a final cell concentration of $5 \times 10^4$ per milliliter, respectively. The virus-cell mixtures were incubated at 37°C for 1 hr, and the amount of free virus in each sample was determined. The cells were washed three times in BSS-CaS2 to reduce residual-free virus, and the amount of cell-
associated virus, which was released by dilution into glycine buffer at pH 2.0 (virus not eclipsed), was measured by plaque assay. The results of a representative experiment (Table 1) show that virus eclipse occurred (87 to 95%) over a wide range of input-virus concentration. It was concluded, therefore, that the viral-eclipse mechanism of the cell was not saturated, and that viral-attachment interference occurs under conditions which permit eclipse of the interfering agent.

Finally, experiments were carried out to determine whether viral-attachment interference could be reversed. The possibility existed that specific viral-receptor sites on the cell surface were made unavailable to bind challenge virus, because (i) attachment and eclipse of the interfering agent resulted in a permanent change in the receptor structure, or (ii) attached virions or their subunits remained fixed to these sites to preclude further receptor activity. If the latter possibility were correct, then acidification of the virus-cell com-

**Table 1. Influence of virus multiplicity on eclipse of coxsackievirus B3 by HeLa cells as measured by recovery of infectious virus after incubation for 1 hr at 37 C**

<table>
<thead>
<tr>
<th>Input-virus multiplicity</th>
<th>Attached-virus multiplicity</th>
<th>Virus not eclipsed (PFU/cell)</th>
<th>Virus eclipsed (PFU/cell)</th>
<th>Per cent of virus eclipsed</th>
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<tr>
<td>5,000</td>
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<td>36</td>
<td>244</td>
<td>87</td>
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<td>4,000</td>
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**Fig. 8. Inhibition of attachment of coxsackievirus B3 to HeLa cells treated with ultraviolet-irradiated B3 virus.**

**Fig. 9. Reversal of inhibition of coxsackievirus B1 attachment to HeLa cells by use of glycine buffer (pH 2.0).**

plex should dissociate interfering virus from the cell surface and allow the specific receptors to bind challenge virus. Preliminary experiments showed (Zajac and Crowell, unpublished data) that both the HeLa cell receptors for coxsackievirus B3 and the virus were stable when held for 1 min at room temperature in glycine buffer (pH 2.0). HeLa cells at a final concentration of 5 × 10^6 per milliliter were reacted with B3 virus at an input multiplicity of 5,000 PFU per cell for 1 hr at 37 C. The cells were washed free of unattached virus, diluted in glycine buffer (pH 2.0) for 1 min, neutralized, reconcentrated by centrifugation, and resuspended in coxsackievirus B1 at a multiplicity of 0.4 for determination of attachment kinetics of B1 virus. Normal cells, normal cells treated at pH 2.0, and cells treated with B3 virus, which were not exposed to acid pH, served as controls. The results of a representative experiment (Fig. 9) show that viral-attachment interference was almost completely reversed by pH 2.0 buffer. It should be mentioned that in several attempts to reverse viral interference, buffer adjusted to pH 2.5 was only half as effective as that found for pH 2.0. It is evident that the observed viral-attachment interference was due to the continued presence of virus particles at the cell surface and was not a consequence of an irreversible membrane alteration produced by attachment and eclipse of virus.
DISCUSSION

Interference between two viruses may result from a variety of effects, including interferon production, competition for intracellular sites essential to virus synthesis, and destruction or blockade of viral receptors located at the host-cell surface. The present study was concerned with elucidating events of the latter type of interference and served to extend the findings of Crowell and Syverton (4), Quersin-Thiry and Nihoul (26), and Crowell (3) in that specific interference of virus attachment was accomplished with the use of live cells in the absence of serum inhibitors to virus. These results continue to emphasize that major differences exist between cellular receptors for polioviruses and coxsackieviruses of group B as shown by assorted methods (9, 22, 25, 28).

Recent studies of interference between enteroviruses focused attention mainly on postadsorption events leading to macromolecular syntheses and to production of viral recombinants (1, 2, 6, 12, 16, 17, 24, 27). With the exception of the report by Pohjanpelto and Cooper (24), none of these studies showed that interfering doses of virus decreased significantly the attachment of challenge virus to host cells. The finding of viral attachment interference reported herein undoubtedly reflects the larger amounts of interfering virus used to treat the cells. Another variable which may influence the quantitative relationship between input-virus multiplicity and induced interference is the content of noninfectious virus particles present in the virus population. It was shown that noninfectious virus can initiate attachment interference, although it remains to be determined whether viral capsomers are effective.

The addition to HeLa cells of massive amounts of poliovirus was shown to result in cessation of cellular protein and nucleic acid synthesis as measured, respectively, by incorporation of labeled valine and phosphate (11). The mechanism of this inhibition was not determined. In this regard it is pertinent that comparable amounts of poliovirus used in the present study did not have any effect on attachment and eclipse of coxsackievirus B3. The question is raised as to whether saturation of different viral receptors of cells could influence specifically the uptake of selected amino acids. Use of the method of saturation of receptors by virus may provide a way for detection of specific functions of viral receptors of living cells. It is difficult to imagine that cells possess viral receptors for the single purpose of self destruction.

In view of the studies of Fogh (8), in which a constant percentage of input poliovirus was found to attach to monkey kidney cells over a wide range of virus concentration, it was unexpected to find that coxsackieviruses and poliovirus established interference of attachment of homologous and related heterologous viruses by a mechanism attributed to receptor saturation and blockade. No explanation is offered to resolve this apparent discrepancy other than that many aspects of the respective systems differed.

Eclipse of enteroviruses has been considered to occur either at the surface of the host cell (13, 15, 23) or at some intracellular location following engulfment of virus (5, 15, 19, 20). The results of the present study showed that attachment interference was established and maintained after eclipse of interfering virus, and that the effect was reversible by dissociation of attached virus by acid pH, which suggests a mechanism of continued surface blockade of specific receptor sites by interfering virus. Although the fate of interfering-virus ribonucleic acid was not determined, an active process of engulfment of virus by the cells would not be expected to be readily reversible. The results, therefore, are consistent with the concept that both attachment and eclipse of enteroviruses occur at the cell surface.

Acknowledgments

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some observations on the nature and significance of the rejected particles. Virology 18:212-223.


