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Differential Inhibition of Attachment and Eclipse Activities of HeLa Cells for Enteroviruses[1]

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Receptor activities of HeLa cells were evaluated for ability to both attach and eclipse enteroviruses after exposure of cells to acid or heat. A modified procedure of acid (*p*H 1.5) elution of cell-associated virus, as compared with other procedures, provided a general method for the optimal recovery of receptor-bound enteroviruses. With this procedure, eclipse of virus operationally was considered to be that amount of virus infectivity which was determined initially to be cell-associated and which was not dissociable from the cells. HeLa cells killed by heating at 56 C for 30 min could not attach or eclipse poliovirus T1, but they attached and eclipsed coxsackieviruses B1 and B3, and they attached echovirus 6 but did not eclipse it. HeLa cells treated at *p*H 2.5 for 10 min at 2 C could not attach or eclipse poliovirus T1, but they attached coxsackieviruses B1 and B3 and echovirus 6, although these viruses were not eclipsed. These results showed that, within the operational definition of virus eclipse, the eclipse activity of HeLa cells for some viruses can be irreversibly inactivated without impairing the activity of the receptors for attaching these viruses. The data provided additional evidence that HeLa cells possess specific receptors for the different enteroviruses.

Initiation of infection of susceptible cells by enteroviruses has been shown to be dependent upon the presence of specific receptors to attach virus (14, 21, 22) and an efficient mechanism for viral eclipse (5). Extensive studies have been performed in an attempt to characterize enterovirus receptors by the use of cellular homogenates (8, 9, 21), cell extracts (16, 19, 21), red blood cells (18), and living HeLa cells (1–4, 10, 23, 24) as a source of receptor activity. These studies have emphasized the diverse characteristics of the cellular receptors responsible for attachment of polioviruses, coxsackieviruses, and echoviruses. This paper describes the stability of the different enterovirus receptors of HeLa cells to acid and heat. The receptor activities of treated cells were evaluated for capacity both to attach and to eclipse virus. In these studies, the term eclipse (7) was used operationally to denote that amount of virus infectivity which was determined initially to be cell-associated and which was not dissociable from the cells by treatment with glycine buffer (6) at *p*H 1.5.

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MATERIALS AND METHODS

HeLa cells. Cells designated HeLa (JJH) were cultured as monolayers in growth medium containing calf serum (GMCaS10), as described elsewhere (23).

Virus strains. Coxsackieviruses B1 and B3, poliovirus T1, and the method of plaque assay used in this investigation were described previously (4). Echovirus 6, kindly supplied by M. Klein of Temple University Medical School, Philadelphia, Pa., was propagated and assayed in a fashion similar to the other enteroviruses.

Determination of receptor activity to attach and eclipse enteroviruses. Determination of the receptor activity of HeLa cells for attaching viruses was carried out at 37 C or at 2 C, as described elsewhere (23). The amount of virus which was attached to cells at a given time was determined by the difference between the amounts of free virus found and the input virus. In addition, in experiments conducted at 2 C, the amount of virus which was attached to cells was determined directly by elution of virus from the cells by use of glycine buffer at *p*H 1.5. A close correlation was found between the two methods used for determining the amount of attached virus provided the system was kept cold. Determinations of the ability of HeLa cells to eclipse virus were carried out at 37 C. Eclipsed virus (7) was considered to be that amount of virus infectivity which was determined initially to be cell-associated (by difference between free virus and input virus) and which was not dissociable from the

cell by treatment with 0.05 M glycine buffer (6) adjusted to *p*H 1.5.

Preparation of cell-associated virus (CAV). A sample of 5×10^7 washed HeLa cells was centrifuged at $225 \times g$ for 5 min, and the sedimented cells were resuspended in 5 ml of virus at 2 C. [Input virus multiplicity usually was approximately 10 plaque-forming units (PFU) for coxsackievirus B3, 50 PFU for coxsackievirus B1, and 100 PFU for poliovirus T1 and echovirus 6.] The cell-virus mixture was incubated at 2 C for 1 hr with frequent agitation. Virus attachment was stopped by diluting the cell-virus mixture 10-fold with cold balanced salt solution (BSS)–CaS_3 solution and centrifuging the cells at $225 \times g$ for 5 min at 2 C. The cells were readied for use by washing them four more times with cold BSS–CaS_3 solution (50 ml each wash) to reduce free virus. The CAV was determined by the acid reversal method described below.

Reactivation of CAV by acid pH. The procedure of Fenwick and Cooper was modified and used (6). A known number of cells with associated virus was diluted 100-fold in cold (2 C) 0.05 M glycine buffer (*p*H 1.5). After treatment for 1 min, the sample was diluted 10-fold further into phosphate-buffered saline (PBS), devoid of calcium and magnesium, containing 3% calf-serum (PBSA–CaS_3), which was adjusted to *p*H 10.9 so that the final *p*H of the virus preparation was neutral. The amount of total virus recovered, as determined by plaque assay and corrected for the amount of virus remaining free prior to acid treatment, was considered to be the amount of infectious virus which was cell-associated and bound to receptors at the time of acid treatment. From a comparison of several methods used for dissociation of coxsackieviruses from cell receptors, this method was found to be the most effective (*see* Table 2).

Chemicals and enzymes. All chemicals were analytical reagent grade. Chymotrypsin (3 × crystalline) was purchased from Worthington Biochemical Corp., Freehold, N.J. Fresh enzyme solution was prepared for each experiment and used at a final concentration of 1%.

RESULTS

To study effectively the receptor activity of HeLa cells for attachment and eclipse of enteroviruses, we considered it important that the following preliminary conditions be met: (i) the viruses should attach and remain at the cell surface for extended periods of time; and (ii) a method should be used that will measure most accurately the amount of CAV prior to initiation of viral eclipse. The use of low temperature has proved to be the only efficient method for maintaining the attached viruses on the cell surface, and, as presented below, the method of acid reversal of CAV appeared to fulfill the second condition. Accordingly, experiments were performed in an attempt to follow the distribution of the total amount of input virus added to HeLa cells after incubation for 1 hr at 2 C.

Six separate experiments with coxsackievirus B3 and three separate experiments with poliovirus T1 were performed in which the total PFU of virus was determined in each of five consecutive wash fluids and in the CAV fraction (Table 1). The results showed that the major portion of the input virus remained in the fluid phase. The finding of almost complete recovery of the input virus inoculum attested to the efficacy of acid *p*H for recovery of total CAV under conditions which prevented viral eclipse.

Capacity of assorted reagents to dissociate CAV. Interest in the fate of cell-associated virus has produced many procedures for dissociating virus-cell complexes (6, 7, 12, 15, 18). These procedures were compared for their capacity to dissociate CAV under conditions which precluded viral eclipse. Earlier experiments had shown that the acid *p*H method used to dissociate virus-antibody complexes (11, 20) and CAV (6, 15) effectively released coxsackievirus B3 from the surface of HeLa cells (3). To determine the *p*H which would optimally reverse CAV at 2 C, a wide range of acid *p*H was tested.

Preparation of CAV by the use of poliovirus T1, echovirus 6, and coxsackieviruses B1 and B3

TABLE 1. *Distribution of input virus in the cellular and fluid phases of the HeLa cell suspensions after incubation for 1 hr at 2 C*

Phase[a]	Amt of input virus recovered[b]	
	Coxsackie B3	Polio T1
	%	%
Wash 1	82 ± 6	86 ± 3
Wash 2	3 ± 0.5	6 ± 3
Wash 3	<1	<1
Wash 4	<1	<1
Wash 5	<1	<1
CAV (*p*H 1.5)	13 ± 4	9 ± 5
CAV lost	1	<1
Total recovery	99 ± 10	101 ± 3

[a] The amount of free virus remaining in the fluid phase of the cell suspension used to determine CAV was <1 for both coxsackievirus B3 and poliovirus T1.

[b] Presented as the mean from six experiments with coxsackievirus B3 and three experiments with poliovirus T1 with the standard error of the mean, respectively. Washed HeLa cells (5×10^7) were suspended in 5 ml of virus at 2 C. Input multiplicity of virus was 10 PFU and 100 PFU for coxsackievirus B3 and poliovirus T1, respectively. After 1 hr of incubation at 2 C, the cell-virus suspension was diluted to 50 ml with BSS-CaS_3, and centrifuged at $225 \times g$; the supernatant fluid (wash no. 1) was assayed for virus infectivity. The cells were washed accordingly four more times, and the fluids were assayed for virus. After the fifth wash, the CAV was determined, as indicated in Materials and Methods.

was described in Materials and Methods. Cells with associated virus, kept at 2 C, were diluted 1:100 into cold 0.05 M glycine buffer at different *p*H values and held for 1 min at 2 C, after which the *p*H was brought to neutrality by a further 1:10 dilution into PBSA-CaS_3. The cells were sedimented by centrifugation and the supernatant fluids were collected and assayed to determine the amount of dissociated virus. The results of these experiments (Fig. 1) showed that optimal recovery of coxsackieviruses B1 and B3 and poliovirus T1 occurred at *p*H 1.5, whereas the recovery of echovirus 6 was accomplished equally well at all *p*H values tested. Determination of virus stability in the medium used showed that all of the viruses remained completely stable at *p*H 1.5 at 2 C for at least 3 min. At *p*H 1.0, poliovirus T1 was inactivated within 1 min, and coxsackievirus B1 and echovirus 6 lost close to 50% of their infectivity, whereas coxsackievirus B3 remained stable. The recovery of CAV remained constant irrespective of whether the duration of the cell treatment at *p*H 1.5 at 2 C was for 30 sec or for 5 min. Treatment of cells for 1 min at 2 C at *p*H 2.0 or 2.5 recovered only small amounts of the total CAV. However, by increasing the duration of treatment, at *p*H 2.0 or 2.5, to 5 min, progressive elution of CAV was found to approach the quantity of virus dissociated at *p*H 1.5. It can be concluded from these results and from those presented in Table 1 that, within the limits of the accuracy of the plaque assay, *p*H 1.5 yields total recovery of the receptor-bound virus.

In the next series of experiments, the efficacy to elute CAV at *p*H 1.5, *p*H 2.5 or with 8 M urea, 6 M lithium chloride (LiCl), 4% sodium dodecyl sulfate (SDS), 1% chymotrypsin, and alternate freezing and thawing was compared for each of the four viruses used previously. The cell-associated virus was prepared as described in Materials and Methods. The washed cells were taken up in a small volume of cold BSS-CaS_3, and counted with a Coulter electronic cell counter. The cell samples were diluted in cold BSS-CaS_3 to give a final concentration of 3×10^6 cells/ml for coxsackievirus B3, and 10^7 cells/ml for the other three virus preparations. The CAV was determined by further diluting each sample into acid solution, used for elution of CAV, as described previously. The reversal of CAV with 8 M urea, 6 M LiCl, and 4% SDS was performed by making a 10-fold dilution of each cell sample into the reagent followed by a 100-fold dilution into PBSA-CaS_3. The amount of free virus obtained by each of the methods tested was determined by the plaque assay. In these experiments, no attempt was made to account for the total amount of the input virus as was done in the experiments presented in Table 1.

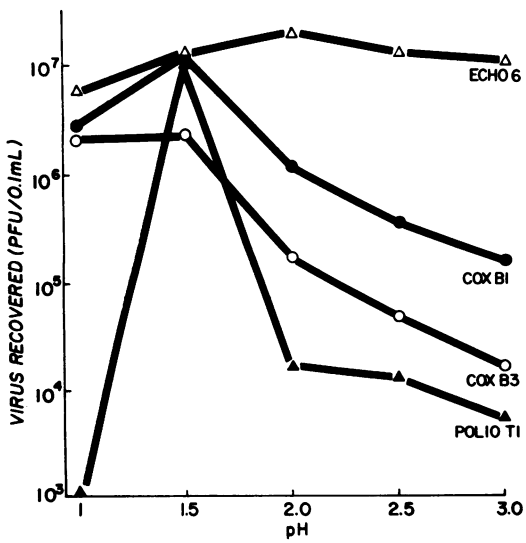

FIG. 1. *Effect of pH on the dissociation of enteroviruses from HeLa cells.*

The results of these experiments, compiled in Table 2, show that maximal recovery of coxsackieviruses B1 and B3 was obtained only by the method involving *p*H 1.5 and, therefore, the amounts obtained by this method were assigned values of 100%. The high salt concentration reversed only a small proportion, and SDS approximately 50%, of the coxsackieviruses available at the cell surface. In contrast, the poliovirus T1 receptor complex was dissociated equally well by the high salt concentration and *p*H 1.5, with SDS again being less efficient. The amount of recovery of echovirus 6 was approximately the same for all of the methods tested. In general, the procedure of alternate freezing and thawing was found to be an ineffective method for recovering CAV.

Effect of acid pH on the capacity of HeLa cells to attach and eclipse enteroviruses. The effective use of acid *p*H for dissociation of receptor–virus complexes prompted a study of the stability at low *p*H of the viral receptors of HeLa cells. HeLa cells were suspended in 5 ml of cold 0.05 M glycine buffer at *p*H 2.5 to a final concentration of 10^7/ml. At intervals during incubation at 2 C, 1-ml samples were withdrawn and diluted 10-fold in PBSA-CaS_3 to bring the *p*H to neutrality. The cells were sedimented by centrifugation for 5 min at $225 \times g$, washed, counted, and resuspended in BSS-CaS_3 to a concentration of 10^7 cells/ml. The treated cells were tested for their capacity to attach poliovirus T1 and coxsackievirus B3 during a 60-min period at 37 C. As a control, cells treated at neutral *p*H were tested concurrently.

TABLE 2. *Capacity of assorted reagents to dissociate enteroviruses from HeLa cells*

Reagents	Amt of virus recovered after 1 min at 2 C[a]							
	Coxsackie B1		Coxsackie B3		Polio T1		Echo 6	
	PFU/ml (10^8)	Per cent	PFU/ml (10^7)	Per cent	PFU/ml (10^7)	Per cent	PFU ml (10^8)	Per cent
0.05 M Glycine (*p*H 1.5)	1.07	100	1.34	100	3.9	100	1.26	100
0.05 M Glycine (*p*H 2.5)	0.058	5.4	0.03	2.2	0.05	1.3	1.34	106
8 M Urea	0.028	2.6	0.05	3.7	4.6	118	1.48	117
6 M LiCl	0.022	2.0	0.008	0.6	3.5	90	1.56	124
4% SDS in BSS[b]	0.57	53	0.4	30	2.7	69	1.38	109
1% Chymotrypsin[c]	ND[d]	ND	0.02	1.5	ND	ND	ND	ND
Freezing and thawing (5 times)	0.16	15	0.11	8.2	0.18	4.6	1.30	103
None[e]	0.0078	0.7	0.002	0.2	0.01	0.2	0.24	19

[a] Washed HeLa cells (5×10^7) were suspended in 5 ml of virus at 2 C. Input multiplicities of virus were 10, 50, and 100 PFU for coxsackievirus B3, coxsackievirus B1, and poliovirus T1 and echovirus 6, respectively. After incubation for 1 hr at 2 C, the cell-virus suspension was diluted to 50 ml with BSS-CaS_3 and centrifuged at 225 $\times$ *g*. The cells were washed accordingly four more times. After the fifth wash, the cells were adjusted to 3×10^6 cells/ml for coxsackievirus B3 and to 10^7 cells/ml for the other viruses. CAV was determined, as indicated in Materials and Methods. In these experiments, no attempt was made to account for the total amount of input virus.

[b] Due to the formation of a precipitate at 2 C, the temperature of the SDS solution was 15 C.

[c] Cells were treated for 30 min at 37 C at a concentration of 3×10^6/ml.

[d] Not done.

[e] The amount of virus in the fluid phase of the cell suspension prior to treatment.

The results presented in Fig. 2 demonstrate that the receptor activity for coxsackievirus B3 remained stable to treatment at *p*H 2.5 for 10 min, whereas the receptor activity for poliovirus T1 was rapidly inactivated.

To determine the *p*H at which the cellular receptors for each of four enteroviruses would be inactivated, replicate samples of HeLa cells (2×10^7 cells) were resuspended in 10-ml volumes of cold 0.05 M glycine buffer adjusted to different *p*H levels. After a 10-min treatment at 2 C, the cells were washed, resuspended in a small volume of BSS-CaS_3, and assayed for capacity to attach and to eclipse each of the viruses at neutral *p*H during a 1-hr period at 37 C. The amount of attached virus which could not be recovered by 0.05 M glycine buffer at *p*H 1.5 was considered to be eclipsed (7). The results presented in Table 3 show that the receptors for attaching coxsackieviruses B1 and B3 and echovirus 6 were stable over the *p*H range 1.0 to 7.0, whereas the receptors for poliovirus T1 were inactivated at a *p*H below 3.0. In contrast, the results presented in Table 4 demonstrate that the eclipse activity of HeLa cells for each of the viruses tested was inactivated by treatment below pH 3.0. It should be noted that such treatment had no effect on the attachment to HeLa cells of coxsackieviruses B1 and B3 and echovirus 6. Since HeLa-cell receptors for poliovirus T1 were inactivated below *p*H 3.0, the inactivation of eclipse below that level could not be demonstrated.

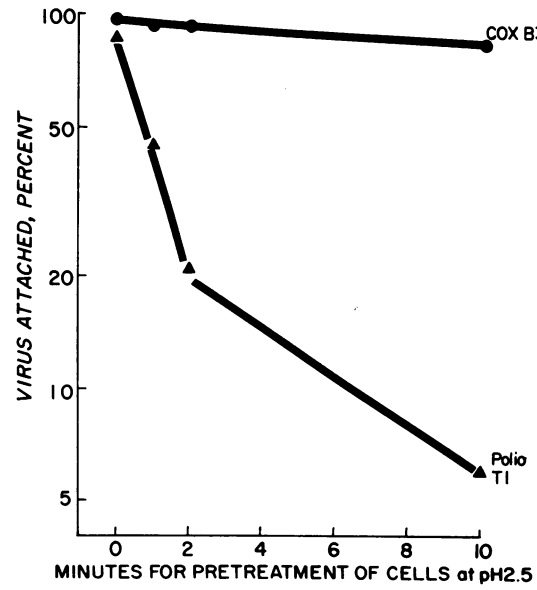

FIG. 2. *Comparative rates of inactivation, at pH 2.5, of the ability of HeLa cells to attach coxsackievirus B3 and poliovirus T1.*

Effect of temperature on the capacity of HeLa cells to attach and eclipse enteroviruses. The re-

TABLE 3. *Comparative stability of enterovirus receptors of HeLa cells to acid pH*

Pretreatment of cells[a]	Virus attached[b]			
	Coxsackie B1	Coxsackie B3	Echo 6	Polio T1
pH	%	%	%	%
1.0	99	97	74	2
2.0	96	97	86	7
3.0	99	99	80	54
4.0	98	99	82	65
5.0	98	97	76	55
6.0	99	97	77	57
7.0	98	97	80	64
BSS-CaS_3	98	99	75	67

[a] Replicate samples of 2×10^7 cells were suspended in 10 ml of cold 0.05 M glycine buffer at the pH indicated and treated for 10 min at 2 C. BSS-CaS_3 at pH 7.0 served as a control.

[b] Treated cells were sedimented, washed, resuspended in 1 ml of BSS-CaS_3, and counted. A sample of cell suspension containing 10^7 cells/ml was adjusted to volume of 0.9 ml, and 0.1 ml of respective virus was added. The input PFU/0.1 ml was 6.7×10^5 for coxsackie B1, 2.9×10^5 for coxsackie B3, 4.2×10^5 for echo 6, and 5.5×10^5 for polio T1. The capacity of the cells to attach each of the viruses was determined at neutral *p*H for 1 hr at 37 C.

ported differences in the heat stability of receptors for enteroviruses (18, 21) suggested temperature as a means for the selective inactivation of the capacity of HeLa cells to attach and to eclipse enteroviruses. Replicate samples of 2×10^7 HeLa cells were suspended in 1.9 ml of BSS-CaS_3 and heated at selected temperatures for 30 min. The cell suspensions were cooled prior to receiving virus inoculum, and the capacity of the heat-treated cells to attach and to eclipse coxsackieviruses B1 and B3, poliovirus T1, and echovirus 6 was determined at 37 C for 60 min. Replicate samples of cells which were not heated served as controls.

The results presented in Table 5 indicate that the receptors for attaching poliovirus T1 were inactivated at 56 C, revealing that they were the most labile of the receptors studied. The receptors for echo 6 were partially inactivated at 60 C, whereas a slightly greater temperature was required for inactivation of the receptors for coxsackieviruses B1 and B3. The results in Table 6 present the data pertaining to the thermostability of the capacity of HeLa cells to eclipse the four enteroviruses tested. These results demonstrated that, when HeLa cells were heated at 56 C for 30 min, they lost their capacity both to attach and to eclipse poliovirus T1. On the other hand, these cells attached but did not eclipse echovirus 6, whereas the attachment and eclipse mechanisms for coxsackieviruses B1 and B3 were affected minimally.

To confirm the observation that HeLa cells have a mechanism for eclipse of coxsackievirus B1 which could be separated from that for echovirus 6, an additional experiment was performed. HeLa cells were suspended in BSS-CaS_3 at a concentration of 10^7 cells per ml and heated at 56 C. At intervals, samples of cells were removed and tested for their capacity to attach and eclipse each virus as described previously. The results of this experiment (Table 7) confirmed the previous observation that attachment of both viruses occurred normally to the heated cells. However, the eclipse mechanism for echovirus 6 was rapidly inactivated whereas the cells were still capable of eclipsing at least 60% of the attached coxsackievirus B1 (Table 7). These results reinforce the previous observations and suggest that the mechanism which is involved in the eclipse of coxsackievirus B1 is distinct from that for echovirus 6.

TABLE 4. *Effect of acid pH on the capacity of HeLa cells to eclipse enteroviruses*

Pretreatment of cells[a]	Virus eclipsed[b]			
	Coxsackie B1	Coxsackie B3	Echo 6	Polio T1
pH	%	%	%	%
1.0	<10	<10	<10	ND[c]
2.0	<10	<10	<10	ND
3.0	76	31	56	80
4.0	89	45	97	94
5.0	85	62	100	98
6.0	93	62	100	92
7.0	91	70	95	97

[a] For conditions, see the footnotes in Table 3.

[b] After treating the cells at the *p*H indicated, the capacity of the cells to attach (*see* Table 3) and to eclipse each of the viruses was determined at neutral *p*H for 1 hr at 37 C. Eclipsed virus was considered to be that amount of input virus infectivity (*see* Table 3) which was determined initially to be cell-associated and which was not dissociable from the cells by treatment with 0.05 M glycine buffer (*p*H 1.5). The values of virus eclipsed were calculated from the following equations: (i) virus attached (PFU) = input virus − unattached virus; (ii) virus not eclipsed (PFU) = acid recovered virus − unattached virus; (iii) virus eclipsed (PFU) = virus attached − virus not eclipsed; (iv) virus eclipsed (%) = virus eclipsed (PFU) × 100/virus attached (PFU).

[c] Not done; the pretreatment inactivated the capacity of the cells to attach poliovirus T1.

TABLE 5. *Thermostability of enterovirus receptors of HeLa cells*

Pretreatment of cells[a]	Virus attached[b]			
	Coxsackie B1	Coxsackie B3	Echo 6	Polio T1
C	%	%	%	%
37	98	96	77	81
40	ND[c]	ND	80	74
45	ND	ND	74	78
50	ND	ND	67	62
56	99	98	69	15
60	98	97	42	<10
65	<10	<10	25	<10
70	<10	<10	<10	<10

[a] Replicate samples of 2×10^7 cells were suspended in 1.9 ml of BSS-CaS_3 and heated at the temperature indicated for 30 min.

[b] Cell suspensions were cooled prior to receiving the respective virus. The input PFU/0.1 ml was 1.98×10^6 for coxsackie B1, 2.5×10^7 for coxsackie B3, 1.38×10^6 for echo 6, and 1.16×10^6 for polio T1. The capacity of the cells to attach each of the viruses was determined at 37 C for 1 hr.

[c] Not done.

TABLE 6. *Thermostability of the capacity of HeLa cells to eclipse enteroviruses*

Pretreatment of cells[a]	Virus eclipsed[b]			
	Coxsackie B1	Coxsackie B3	Echo 6	Polio 1
C	%	%	%	%
37	72	73	68	83
40	ND[c]	ND	68	68
45	ND	ND	61	82
50	ND	ND	28	56
56	65	43	13	<10
60	63	39	<10	—[d]

[a] For conditions, see the footnotes in Table 5.

[b] After treatment, the capacity of the cells to attach (*see* Table 5) and to eclipse each of the viruses was determined at neutral *p*H for 1 hr at 37 C. Eclipsed virus was considered to be that amount of input virus infectivity (*see* Table 5) which was determined initially to be cell-associated and which was not dissociable from the cells by treatment with 0.05 M glycine buffer (*p*H 1.5). The values of virus eclipse were calculated from the following equations: (i) virus attached (PFU) = input virus − unattached virus; (ii) virus not eclipsed (PFU) = acid recovered virus − unattached virus; (iii) virus eclipsed (PFU) = virus attached − virus not eclipsed; (iv) virus eclipsed (%) = virus eclipsed (PFU) × 100/virus attached (PFU).

[c] Not done.

[d] Pretreatment inactivated the capacity of the cells to attach poliovirus T1.

TABLE 7. *Comparative rates of inactivation of the ability of HeLa cells to attach and eclipse coxsackievirus B1 and echovirus 6*

Pretreatment of cells at 56 C[a]	Coxsackie B1		Echo 6	
	Attached	Eclipsed[b]	Attached	Eclipsed[b]
min	%	%	%	%
None	96	78	88	68
5	97	61	81	5
15	96	61	72	<1
30	97	59	69	<1

[a] Replicate samples of 10^7 cells/0.9 ml of BSS-CaS_3 were heated. At time intervals indicated, the cells were removed and cooled prior to addition of virus. The input PFU/0.1 ml was 8.7×10^5 and 7.9×10^5 for coxsackie B1 and echo 6, respectively. The capacity of the cells to attach and eclipse each of the viruses was determined at 37 C for 1 hr.

[b] Values were calculated from the equations given in Table 4.

DISCUSSION

The comparative experiments described in this report represent but one phase of our continuing investigations directed toward characterizing the properties and functions of enterovirus receptors on intact HeLa cells (1–4, 10, 23, 24). The results of these experiments showed clearly that the receptor activity of HeLa cells for attaching poliovirus T1 was more labile to elevated temperatures and to low *p*H than were the receptor activities for binding echovirus 6 and coxsackieviruses B1 and B3. These findings, in part, confirmed and extended the results of Quersin-Thiry and Nihoul (21), who showed a difference between the thermostability of receptor activity of HeLa cell extracts for poliovirus T1 and coxsackievirus B4. Thus, additional evidence is provided which serves to reemphasize the differences among the receptors of HeLa cells for attaching the different enteroviruses.

To determine the thermostability and acid stability of HeLa cells to eclipse enteroviruses, we considered it important first to obtain a method to measure accurately the amount of virus which becomes cell-associated under conditions precluding viral eclipse. Thus, of all the methods tested, a brief exposure of CAV to buffer at *p*H 1.5 at 2 C provided a general method for recovery of maximal amounts of each of the viruses studied. By determining the amount of receptor-bound virus at a given time, it was possible to assess the capacity of HeLa cells to eclipse enteroviruses at 37 C after treatment of cells with acid or heat. The results showed that the eclipse activities

of cells (as defined in this paper) for coxsackieviruses B1 and B3 and echovirus 6 were inactivated irreversibly at *p*H 2.0 without impairing the activity of the receptors for attaching these viruses. In contrast, the finding that both of these functions were inseparable for poliovirus T1 suggests that different events may be operative during the early stages of infection by the different enteroviruses.

It was observed that even though heating of HeLa cells at 56 C resulted in loss of cell viability, as determined by trypan blue staining (*unpublished data*), such "dead" cells, which did not attach poliovirus T1, attached but did not eclipse echovirus 6; yet these cells both attached and eclipsed coxsackieviruses B1 and B3. The finding that heat-killed cells remained capable of attaching and eclipsing coxsackieviruses B1 and B3 raises again the question whether the uncoating of coxsackieviruses occurs within phagocytic vesicles (13) or at the receptors on the cell surface (3, 17). The improbability that heat-killed cells retained the ability of engulfing virus supports the latter possibility. Resolution of this question, however, requires a better understanding of the relationship between the various operational definitions of viral eclipse and of the uncoating of the RNA genome of each of the different enteroviruses.

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