THE MAMMALIAN CELL-VIRUS RELATIONSHIP

VI. Sustained Infection of HeLa Cells by Coxsackie B3 Virus and Effect on Superinfection*

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The phenomenon of sustained infection of mammalian cells in continuous culture by several viruses has been reviewed recently by Ginsberg (1). Production of HeLa cell cultures persistently carrying Coxsackie B3 virus without obvious cytopathogenic effect is reported in this communication, together with viral interference effects associated with the carrier state. The findings are of interest in revealing more of the nature of mechanisms responsible for interactions of enteroviruses and human cells.

Materials and Methods

The Cell Origin and Cultivation.—Human esophageal epithelial cells of normal origin in continuous culture (EE, Minn. 55-12-1), parental line HeLa cells previously described (2), and derived HeLa lines were propagated in growth medium containing 10 per cent human serum in supplemented Hanks's balanced salt solution (BSS). The supplemented salt solution, basal medium Eagle complete (BMEC) contained 200 mg. per cent (*w/v*) glucose and a mixture of essential and non-essential amino acids and vitamins recommended by Eagle (3). The complete medium contained 100 units of penicillin and 100 mg. of streptomycin per ml. Cells were dispersed for transfer by treatment with 0.05 per cent trypsin (Difco 1:250). Cultures for virus plaque assay were prepared by inoculating approximately 1.2×10^6 EE cells into 30 × 60 mm. bottles, in BMEC containing 1 per cent human serum to aid spreading of cells and 10 per cent calf serum to promote adhesion of cells to glass. Satisfactory monolayers were obtained after 48 hours' incubation at 37°C.

Viruses.—Studied viruses included Coxsackie B1 (Conn.-5 strain from The American Type Culture Collection, Washington, D.C.), B3 (50531 strain from Dalldorf), B5 (53122 strain from Dalldorf), polioviruses (type 1 Mahoney, type 2 MEF-1, type 3 Saukett), vaccinia (Lederle vaccine virus), and a strain of herpes simplex virus isolated from liver tissue of a newborn child and identified by Tankersley of this department. Pools of these viruses were prepared as culture fluids from inoculated HeLa or EE cells.

Virus Assay.—For virus plaque assay, prepared monolayer cultures were rinsed with three 5 ml. volumes of BSS, drained dry and each of 2 cultures inoculated with 0.1 ml. of each dilution of virus. Inoculated cultures were agitated at intervals during incubation for 1 hour

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at room temperature, overlaid with 5 ml. of BMEC containing 5 per cent calf serum and 0.5 per cent Difco agar, and incubated at 37°C. for 40 to 48 hours. After plaque development, overlay medium was removed and monolayers stained with crystal violet (4). Virus concentrations were recorded as number of plaque-forming units (PFU) per ml. of original suspension.

For assay of Coxsackie B1 or B5 virus in the presence of B3 virus, an equal volume of diluted anti-B3 serum was added to each virus dilution. Tubes were agitated periodically while incubated at room temperature for 1 hour before inoculation into plaque assay cultures. Adequacy of antiserum content was verified by inclusion of control mixtures containing only Coxsackie B3 virus. When persistently infected and uninfected control cultures were used for comparative assay of challenge virus, antiserum to carried virus was added to dilutions of virus from control cultures as well as from carrier cultures.

Antisera.—Specific antiserums to Coxsackie B1, B3 and B5 viruses and to type 2 poliovirus were prepared mostly by repeated inoculation of monkeys with infectious virus; in some experiments, anti-Coxsackie B3 rabbit antiserum received from Microbiological Associates, Bethesda, was employed.

EXPERIMENTAL RESULTS

Sustained Infection of HeLa Cell Cultures with Coxsackie B3 Virus.—Parental-line HeLa cells in 25 ml. of BMEC containing 2 per cent calf serum were maintained in suspension in a 50 ml. Erlenmeyer flask by a spinning magnetic bar. This cell suspension was inoculated with Coxsackie B3 virus to a multiplicity of 10 (final cell concentration 1.25×10^5 per ml.), incubated for 6 hours at 37°C., and sampled for surviving cells by transfer of 1 ml. quantities of culture to tubes incubated on a slant. After 24 hours, a few cells were attached and spread on glass, more than 90 per cent having been destroyed. Maintenance medium and cell debris were removed and replaced with an equal volume of growth medium. Several morphologically typical HeLa cell colonies developed with continued incubation, and were expanded by continuous cultivation to yield a subline of cells propagable in 1 liter Blake bottles. At present, the line has been transferred more than 30 times during a 12 month period. Assay of fluid from each subculture at feeding or prior to transfer showed content of Coxsackie B3 virus from 10^4 to 10^6 PFU per ml. Thus a persistently infected cell line was obtained.

Relation of Human Serum to Perpetuation of Coxsackie B3 Virus-Carrier State of HeLa Cells.—At the 2nd routine passage of the carrier HeLa line, a subline was separated for propagation in medium containing 10 per cent calf serum in place of human serum. Growth capacity of these separated cells diminished until at the 7th passage inoculated cells failed to form monolayers and obvious cytopathogenic effects were seen. A final single surviving bottle culture was maintained 9 months without passage; cell propagation and destruction by virus alternated, depending on the feeding schedule, with eventual result in total cell destruction. Fluids removed from this maintained culture during the 9 month period contained Coxsackie B3 virus at concentrations of 10^5 to 10^6 PFU per ml. Human serum thus appeared essential for perpetuation

of the Coxsackie B3–carrier state of these cells, although the culture retained some capacity for propagation during the long period of maintenance in calf serum medium. As shown in Table I, pools of human serum employed for propagation of the carrier cells contained substances neutralizing Coxsackie B3 virus which were not present in the calf serum. Although the minimum amount of antibody required for carrier-state maintenance was not determined, in one experiment the carrier state was not perpetuated when medium containing 10 per cent calf serum was supplemented with 2 per cent human serum.

TABLE I

Content of Coxsackie B3 Virus Inhibitors in Pools of Human Serum Reflected by Residual Virus Titer*

Serum pool	Virus titer
	10^4 PFU per ml.
Human	0.2
Human	1.6
Human	0.3
Human	2.7
Human	0.3
Calf	9400.0

* Each serum pool was collected from more than 10 donors. Equal volumes of virus dilution and serum diluted 1/10 were incubated for 1 hour at room temperature before inoculation of EE monolayers for assay of residual virus.

To assess influence of serum inhibitor removal on rate of endogenous B3 virus production, a monolayer culture of virus-carrier cells of the 14th cellular passage was rinsed with four 10 ml. changes of BSS, overlaid with 5 ml. of anti-B3 monkey serum diluted 1/10, and incubated for 1 hour at 37°C. to permit neutralization of extracellular virus. Subsequently antiserum was removed by similar rinsing, and the cells were covered with 10 ml. of growth medium containing 10 per cent calf serum as replacement for human serum. During incubation of the culture at 37°C., 1 ml. quantities of culture fluid were removed at intervals for assay of free virus and the fluid volume restored with more medium.

Results (Fig. 1) indicated increase in released virus to a maximum level attained by the 3rd day, when about one-third of the cell population had degenerated. No cytopathogenic effect was visible on the 2nd day. Despite precautions taken to reduce the amount of residual free virus in the system, a relatively high level of virus was present in the culture fluid at zero time. These results also suggested that the virus-carrier state depended on the presence of antiviral inhibitor in the culture medium.

Effect of Antiviral Antibody on Coxsackie B3 Virus–Carrier State of HeLa Cells.—In three independent experiments the 10 per cent human serum of the medium used for propagation of B3 virus–carrier cultures was supplemented with a 1 per cent concentration of serum from monkeys hyperimmunized with

B3 virus. Passage of carrier cultures in the antibody-supplemented medium for from 8 days to 5 weeks completely "cured" the carrier state, as shown by eventual failure of cells to produce virus in inhibitor-free medium. In one of the experiments, such propagation for 18 days was insufficient to eliminate carrier virus, but elimination was secured by further propagation of the cells for 3 weeks (4 culture passages) in medium supplemented with 5 per cent rather than 1 per cent of antiviral serum. A subline of HeLa cells thus freed of carrier B3 virus ("virus-cured" line) was maintained in continuous culture as control

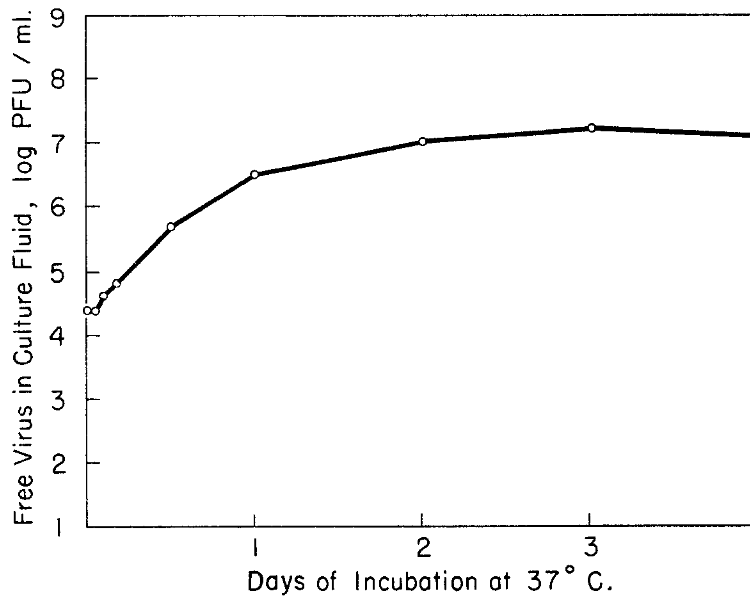

FIG. 1. Production of endogenous B3 virus by carrier HeLa culture in non-inhibitory medium.

for comparison with virus-carrier cells. Plaque assays of Coxsackie B3 virus with carrier-cured and parental HeLa cells and parental EE cells gave insignificantly differing results, to show that virus-resistant cells had not been selected from the parental HeLa line by production of the carrier state.

Proportion of Infectious Centers in Coxsackie B3 Virus-Carrier HeLa Cultures.—The course of production of endogenous Coxsackie B3 virus by carrier cells in inhibitor-free medium (Fig. 1) suggested that maximum virus yield was not attained after a single cycle of infection. Enumeration of culture cells yielding infectious virus was complicated by the necessity to remove or measure extracellular virus.

In one experiment, virus-carrier cells in monolayer culture were rinsed 4 times with 10 ml. changes of BSS at room temperature (these fluids being saved for virus assay), treated for 1

hour at 37°C. with 5 ml. per culture of anti-B3 hyperimmune monkey serum diluted 1/10, and rinsed 4 times with BSS after removal of antiserum. These wash fluids also were assayed for virus. Results from this experiment and a repetition are given in Table II.

Neither repeated rinsing nor neutralization with antibody was adequate for elimination of free virus from the carrier cultures.

Since extracellular virus could not be eliminated from the cultures for enumeration of virus-releasing cells as infectious centers, two methods were used to estimate the infectious center count less directly.

TABLE II

Effect on Concentration of Extracellular Virus in Coxsackie B3 Virus–Carrier HeLa Cultures of Rinsing before and after Culture Treatment with Anti-B3 Hyperimmune Monkey Serum

No. of 10 *ml.* rinses	Virus titer of wash fluids before antiserum treatment		Virus titer of wash fluids after antiserum treatment	
	Exp. No. 1	Exp. No. 2	Exp. No. 1	Exp. No. 2
	PFU/ml.	*PFU/ml.*	*PFU/ml.*	*PFU/ml.*
0	7.0×10^3	2.5×10^4	N.T.*	N.T.
1	1.6×10^3	1.0×10^4	1.0×10^1	8.0×10^1
2	5.9×10^2	6.0×10^3	2.0×10^1	9.4×10^2
3	2.6×10^3	3.2×10^3	1.0×10^1	4.4×10^3
4	2.2×10^3	2.5×10^3	1.1×10^2	2.4×10^4

* N.T., not tested.

B3-carrier cells were washed 4 times, treated with antiserum, rewashed 4 times as before, then dispersed by application of trypsin, and counted with a hemocytometer. The suspended cells were diluted serially tenfold in BMEC with 10 per cent calf serum, added in 0.1 ml. amounts to duplicate monolayer EE cultures, and incubated at 37°C. for 2 hours to permit attachment of the added cells to the monolayers. The assay cultures were overlaid with semisolid agar and incubated at 37°C. for plaque development. A sample of undiluted cell suspension was centrifuged, the supernatant fluid was assayed for virus, and the reconstituted cellular sediment was disrupted by sonic vibration for assay of intracellular virus.

The difference between the number of plaque-forming units contained in unit volume of cell suspension (estimated by plating the cells) and the number of plaque-forming units representative of extracellular virus (estimated by plating the supernatant fluid of sedimented cell suspension) indicated that 1 in 110 carrier cells was a yielder of infectious virus. Since release of extracellular virus by cells during the 2 hour attachment period before overlayering with semisolid agar was not prevented, this estimate was considered maximal. Assay of the disrupted cell suspension showed 1 virus plaque-forming unit per 10 cells. This virus yielded by dissociated cells appeared to be cell-associated so as to be unavailable to neutralization by antiserum. In comparison, carrier cells rinsed 4 times with BSS but not treated with anti-B3 monkey serum, and assayed for cell-associated virus, in 3 independent experiments showed average values of 1.5 to 1.9 PFU per cell.

In another experiment, a monolayer of carrier cells was washed triply with calcium-free BSS; cells were dispersed by trypsinization, and the resultant cell suspension was diluted serially tenfold in cold medium containing B3 antiserum diluted 1/100. These mixtures were incubated for 30 minutes in an ice bath to permit neutralization of free virus, before 1 ml. samples of each suspension were centrifuged at 4°C.; sedimented cells were resuspended in 10 ml. of cold medium free of antibody and counted with a hemocytometer. Samples of 0.1 ml. of each suspension were distributed to 10 tubes, to which were added 0.9 ml. samples of a suspension of EE cells containing 200,000 cells per ml. These tube cultures were incubated slanted at 37°C. and observed periodically.

From observation of cytopathic effect in individual tubes and knowledge of the average number of carrier cells inoculated per tube, and from assumed Poisson distribution of infected cells in the samples it was calculated that about 1 in 160 cells released infectious virus. This estimate was comparable with the previous estimate of 1 infectious center per 110 carrier cells. Thus,

TABLE III

Colony Formation by HeLa Cells of Sublines Carrying and "Cured" of Persistent Infection by Coxsackie B3 Virus, in Medium Containing Anti-B3 Serum

Cells inoculated	Colonies formed by	
	Carrier cells	Cured cells
1000	316	285
100	46	41
50	29	27

only a small proportion of cells of the carrier culture was producing infectious virus actively. If these estimates were not grossly in error, it could be expected that the majority of carrier culture cells should undergo growth in a suitable medium.

To test this expectation, carrier cells in monolayer culture were dispersed by trypsinization, enumerated, and diluted in BMEC supplemented with a mixture of 10 per cent human serum, 5 per cent calf serum, and B3 monkey antiserum diluted 1/40. After incubation for 9 days at 37°C. in an atmosphere of 5 per cent carbon dioxide, Petri dish cultures prepared from the diluted cell suspensions were stained and the number of macroscopically visible colonies counted. As control, similar cultures had been prepared from cells of the virus-cured subline.

As seen in Table III, cells of the carrier and cured sublines exhibited similar efficiency of colony formation in virus-inhibitory medium. This observation supplied the conclusion that carrier cultures contained only a small number of virus-releasing cells.

Susceptibility of Coxsackie B3–Carrier HeLa Cultures to Infection by Coxsackie and Other Viruses.—Preliminary studies (5) showed that B3 carrier cells suffered typical cytopathic effects of infection by the 3 poliovirus types, by vaccinia

and herpes simplex viruses, but not by viruses of the Coxsackie B group. This apparent selective interference with Coxsackie virus superinfection by the B3-carrier state of HeLa cells was verified by evaluation of the course of production of the different viruses.

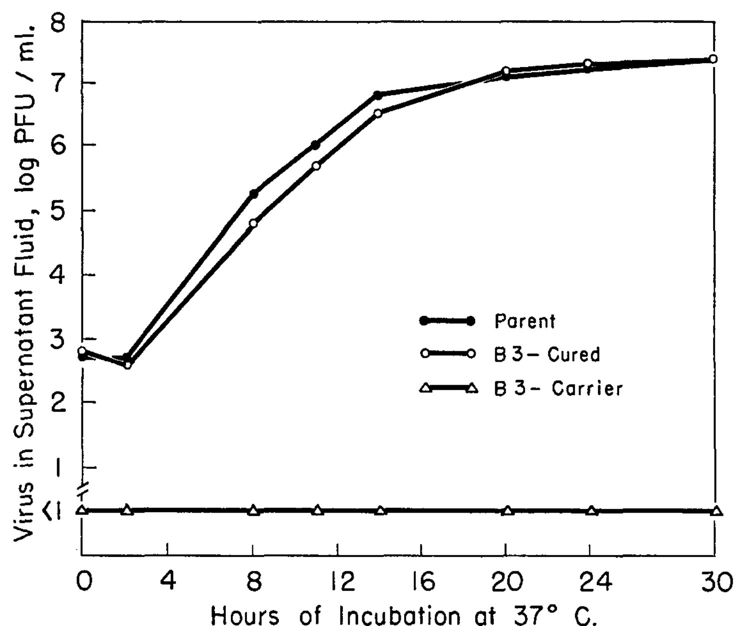

FIG. 2. Production of free Coxsackie B1 virus by inoculated HeLa cultures of parent, B3-carrier, and cured B3-carrier sublines.

The course of free virus production was ascertained by rinsing of human serum medium from replicate monolayer cultures of HeLa cells, inoculation of rinsed cultures with 1 ml. amounts of challenge virus, incubation of cultures for 1 hour at 37°C. to permit virus attachment and penetration, treatment of cultures to eliminate unadsorbed inoculum virus by sequential rinsing, incubation for 30 minutes with homotypic antiserum to eliminate free virus, and repeated rinsing with salt solution to remove antiserum. Finally, treated cultures were overlaid with 10 ml. of BMEC with 10 per cent calf serum and incubated at 37°C. At intervals, 1 ml. samples of culture fluid were removed and replaced with fresh medium (the resultant dilution not affecting the concentration of released virus appreciably). Cellular debris was removed from the culture fluid samples by centrifugation, and the supernatant fluids frozen for assay of free virus. This protocol was followed with HeLa cells of the parental, B3-carrier and B3-cured sublines inoculated with Coxsackie B1 or B5 viruses, or type 2 poliovirus. Assays for produced Coxsackie B1 and B5 viruses were carried out by use of antiserum to neutralize B3 virus released by cells of the carrier subline.

Comparison of Figs. 2, 3, and 4 shows that parental, carrier, and cured subline cells were equally able to produce type 2 poliovirus, and thus could be considered metabolically competent for virus production. In contrast, cells of

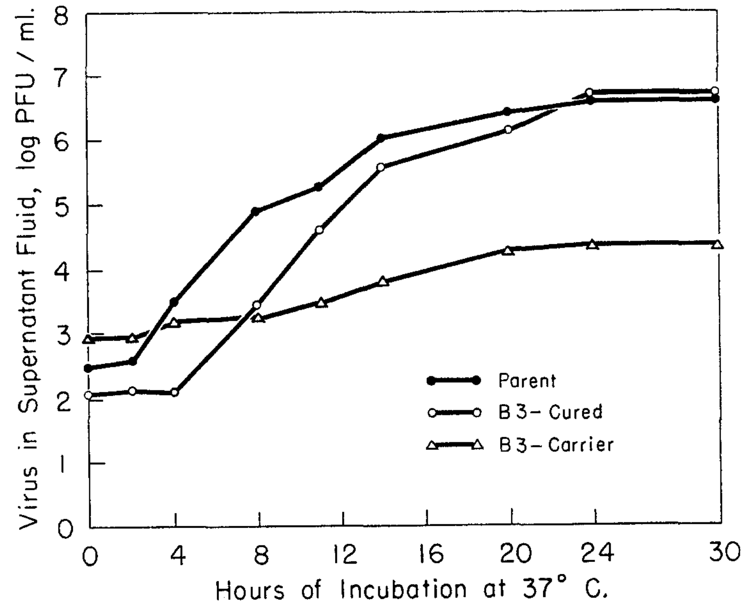

FIG. 3. Production of free Coxsackie B5 virus by inoculated HeLa cultures of parent, B3-carrier, and cured B3-carrier sublines.

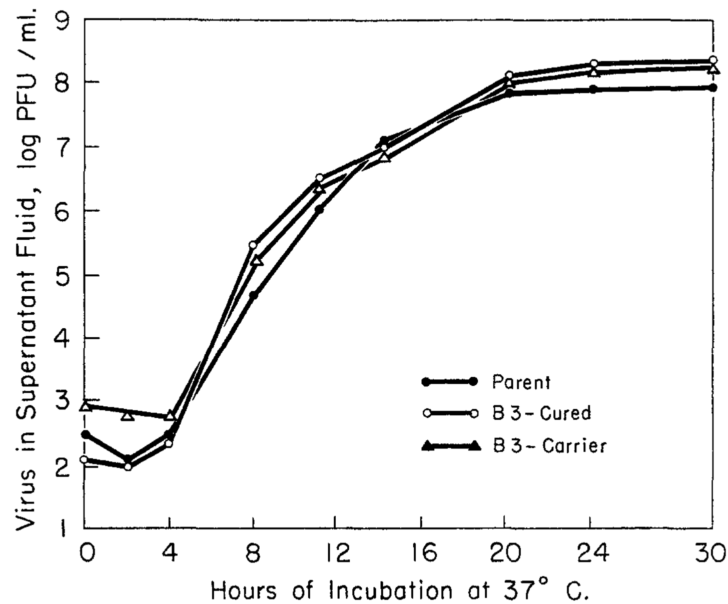

FIG. 4. Production of free type 2 poliovirus by inoculated HeLa cultures of parent, B3-carrier, and cured B3-carrier sublines.

the B3 carrier cultures did not produce significant amounts of challenging Coxsackie B1 virus and produced only minor amounts of challenging Coxsackie B5 virus, although in each instance infection was initiated at an infection multiplicity of 5. Existence of the B3 virus-carrier state in HeLa cells specifically interfered with multiplication and release of superinfecting heterologous Coxsackie viruses.

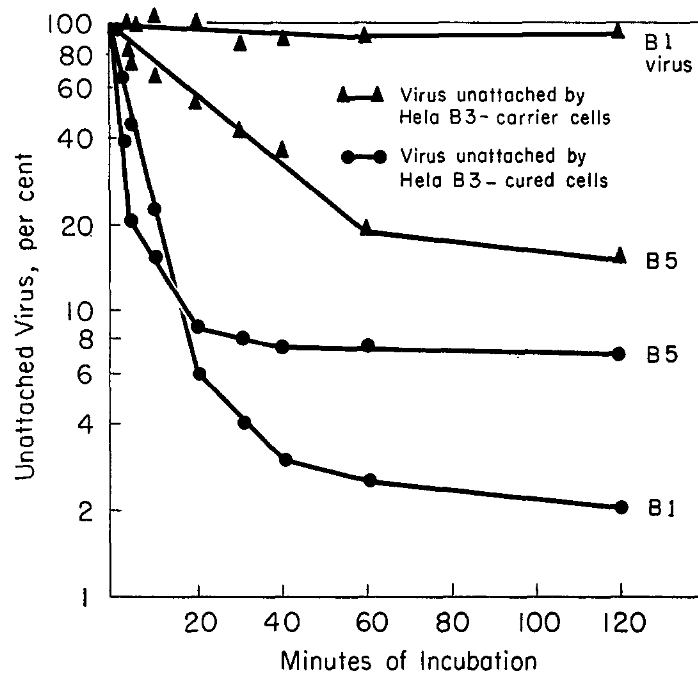

FIG. 5. Attachment of Coxsackie B1 and B5 viruses to HeLa (B3-cured) and HeLa (B3-carrier) monolayers as measured by plaque assay of residual virus.

Mechanism of the Interference in Coxsackie B3 Virus-Carrier HeLa Cultures with Superinfection by Coxsackie B1 and B5 Viruses.—Effect of the B3-carrier state on virus adsorption kinetics was investigated first.

Replicate monolayers of HeLa cells of the parental, carrier, and cured sublines were rinsed free of human serum medium, inoculated with 0.1 ml. of test virus, and rocked at room temperature. After varying periods of time, 9.9 ml. amounts of cold BSS were added to the bottles, mixed with the virus inoculum fluid, removed, and treated with anti-B3 serum to neutralize B3 virus released by the carrier cells, and assayed for residual test virus by plaque count on EE monolayers. Plotting of per cent unattached virus against time of incubation revealed the adsorption kinetics shown in Fig. 5 for attachment of Coxsackie B1 and B5 viruses to HeLa cells of the 3 lines. Rates of virus adsorption by cells of the parental HeLa line are not shown because they did not differ significantly from rates with the virus-cured line.

Unlike cells of the B3-cured line, cells of the B3-carrier line failed to adsorb appreciable amounts of B1 virus, and adsorbed B5 virus at a considerably reduced rate. Similar results were obtained by 3 subsequent repetitions of the analysis. Apparently inhibition of B1 virus multiplication in B3-carrier cells could be attributed directly to failure of attachment; inhibition of B5 virus was not completely explained on this basis.

TABLE IV

Adsorption and Eclipse of Virus by HeLa (Parent), HeLa (B3-Cured), and HeLa (B3-Carrier) Cell Lines in Monolayer as Measured by Plaque Assay

No.	Virus	Multiplicity of infection	Hrs. at 37°	Virus attached to cells			Attached virus recovered from washed cells		
				Parent	Cured	Carrier	Parent	Cured	Carrier
				per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	B-5	2-5	1	n.d.*	80	86	n.d.	6	80
			4	n.d.	88	86	n.d.	4	>95
2	B-5	2-3	2	96	90	77	10	2	>95
			3	94	81	73	12	6	>95
3	B-5	0.25	2	94	93	75	2	1	67
			3	96	96	82	2	2	55
2	B-1	2-3	2	97	94	1	2	5	>95
			3	97	94	9	2	3	>95
3	B-1	0.25	2	97	96	10	2	1	84
			3	99	99	2	1	1	>95
2	Polio T2	2-3	2	86	83	79	48	49	51
			3	94	89	93	20	27	42
3	Polio T2	0.25	2	63	75	88	44	21	43
			3	88	92	94	7	3	10

* n.d., not done.

To learn the effect of the Coxsackie B3-carrier state of HeLa cells on penetration and eclipse of adsorbed B1 and B5 viruses, these viruses together with type 2 poliovirus as control were added separately to replicate cultures of the 3 sublines of HeLa cells.

Virus in 0.1 ml. volume was allowed to attach at 37°C.; unattached virus was diluted and assayed, and residual free virus and detachable cell-associated virus were removed from the monolayers by 4 successive rinses with cold BSS. Cell monolayers were disrupted by 4 cycles of alternate freezing and thawing in 5 ml. of BSS; cell debris was sedimented by centrifugation

at 4°C., and supernatant fluid was assayed for virus in the presence of B3 antiserum. This protocol was repeated with viruses inoculated at different multiplicities, and for different periods of attachment.

Results shown in Table IV indicate that B3-carrier cells were able to receive and eclipse type 2 poliovirus as effectively as cells of the parental and cured lines. Unlike cells of the latter lines, cells of the carrier line failed to eclipse the bulk of attached B1 or B5 virus, and additionally failed to receive the bulk of B1 virus by initial attachment. These findings confirmed the earlier indica-

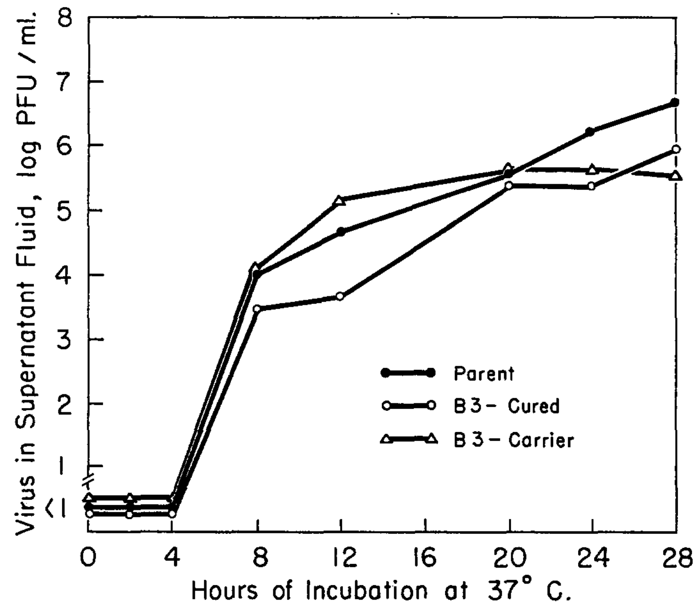

FIG. 6. Production of free Coxsackie B1 virus by HeLa cultures of parent, B3-carrier and cured B3-carrier sublines infected with B1 virus RNA.

tion that the interference with B1 virus superinfection could be attributed mainly to attachment failure; now it was evident that interference with B5 superinfection involved failure of penetration and/or eclipse.

Since it has been established in these laboratories (6, 7) that susceptibility of animal cells to enteric virus infection is conferred by specific cell surface viral receptors whose influence can be by-passed by induction of infection with viral RNA, location of the interfering effect of the B3 virus carrier state of HeLa cells was investigated further by use of RNA. RNA from Coxsackie B1 and B5 viruses for these studies was prepared kindly by Dr. John Holland, University of Minnesota, Minneapolis.

In two separate experiments, monolayer cultures of the three HeLa cell lines were infected with B1 and B5 viral RNA preparations, and the usual procedures carried out for assessment

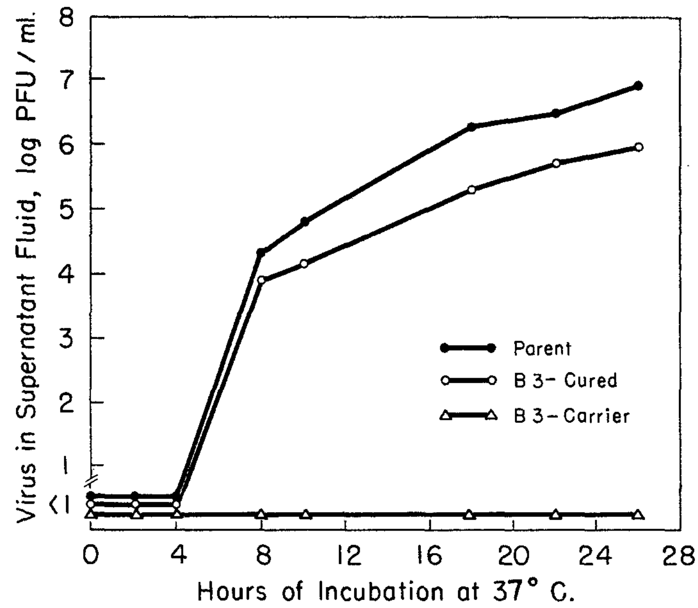

FIG. 7. Production of free Coxsackie B1 virus by HeLa cultures of parent, B3-carrier and cured B3-carrier sublines infected with whole B1 virus.

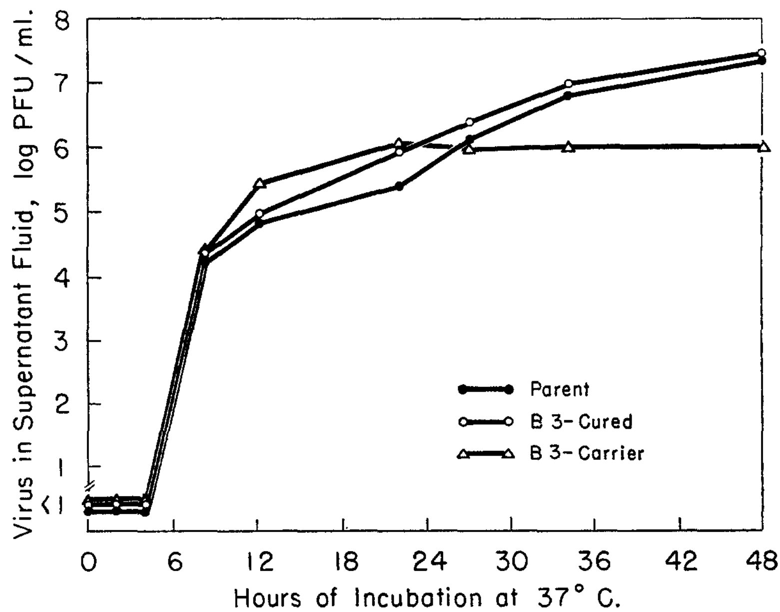

FIG. 8. Production of free Coxsackie B5 virus by HeLa cultures of parent, B3-carrier and cured B3-carrier sublines infected with B5 virus RNA.

of free virus production. Identity of free viruses resulting from RNA infection was determined by test for neutralization with homotypic antiserum. As controls, replicate cell cultures were infected with whole virus diluted to contain a concentration of infectious units approximately equivalent to the number of infectious units represented by the RNA preparations (infection multiplicity for whole virus approximately 0.01).

Figs. 6 to 9 show the contrasting courses of infection of the 3 HeLa sublines with Coxsackie B1 and B5 viral RNA preparations or whole viruses. The distinctive nature of the RNA-initiated infection of B3-carrier cells is re-

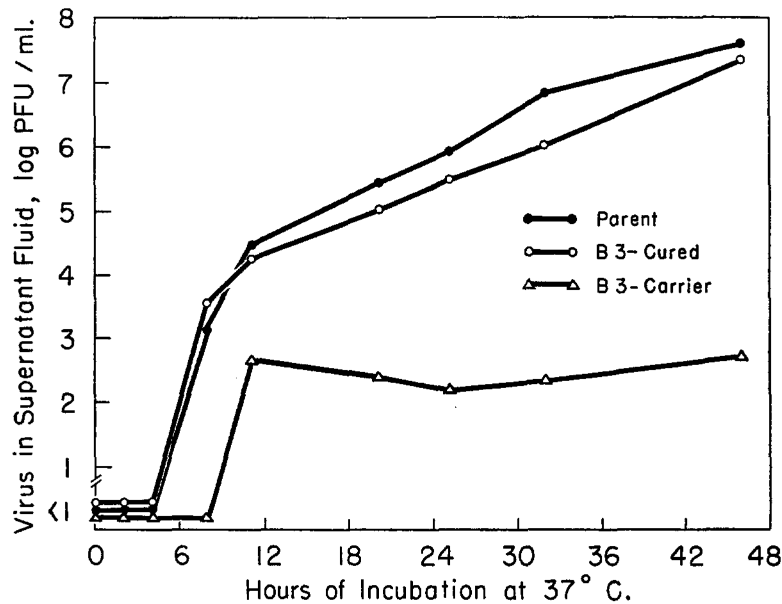

FIG. 9. Production of free Coxsackie B5 virus by HeLa cultures of parent, B3-carrier and cured B3-carrier sublines infected with whole B5 virus.

flected by the single cycle of virus production. These results would be expected because produced new virus would once more encounter the interference affecting whole virus seen in Figs. 7 and 9. The viruses produced by RNA infection were serologically identifiable as Coxsackie B1 and B5 respectively. It is evident that B3-carrier cells were as able to produce new virus when infected by viral RNA as parental or cured cells, and hence that the interfering effect of the carrier state involved sites of Coxsackie virus reception and penetration rather than replication.

DISCUSSION

Continued propagation of parental line HeLa cells surviving after heavy infection of cells in suspension yielded a continuous culture of morphologically unaltered cells which produced Coxsackie B3 virus persistently during each

of more than 30 serial cell passages. Sustained endogenous production of B3 virus appeared dependent on presence of antiviral substance in human serum growth medium (probably antibody), since carrier cultures degenerated eventually when propagated in non-inhibitory medium. This Coxsackie B3 virus-carrier system, previously unreported, appeared different from the Coxsackie A9–carrier cultures of Takemoto and Habel (8). Antiviral antibody was not required for maintenance of the A9 carrier state, and initial HeLa cell populations were relatively resistant to effect of the virus. As has been shown with other viral carrier systems (1, 8–11), the probability of lysogeny as a source of sustained infection in the present study was precluded by successful elimination of virus from carrier cultures by treatment with anti-B3 hyperimmune serum.

Continued release of Coxsackie B3 virus into the fluid phase of B3-carrier HeLa cultures after repeated treatment of the cultures by washing and antiserum neutralization over a 1 to 2 hour period to eliminate free virus suggested existence of a cell surface reservoir of mature virus. This idea was reinforced by results of assay of cell-associated virus in carrier cultures; although on the average at least one virus particle was associated with a cell, the number of cells capable of producing infectious virus was less than 1 per cent of the carrier cell population. Cells of the carrier HeLa line and a subline cured of the carrier state by antiserum treatment were equally able to form colonies on glass in presence of anti-B3 antibody, to indicate in agreement with the infectious center estimate that only a small proportion of the population at any time was productively infected with B3 virus.

Coxsackie B3 virus–carrier HeLa cultures were not visibly responsive to superinfection with high-titer Coxsackie B1 or B5 viruses. By assessment of the quantitative course of virus elaboration into the culture fluid phase, absence of a developing cytopathogenic effect was associated with failure or comparative failure of challenge B1 or B5 virus to multiply. Limitation of this interference effect was revealed by growth curves of poliovirus in carrier cultures which revealed normal virus production.

Analysis of the interference phenomenon showed differences in the mechanisms of interference with B1 and B5 virus infection by the B3-carrier state. Coxsackie B1 virus was shown unable to multiply in B3-carrier cells by reason of attachment failure primarily, and secondarily by lack of penetration and eclipse of the small proportion of challenging B1 virus which became cell-associated. In contrast, Coxsackie B5 virus was adsorbed readily, but remained mostly recoverable in the infectious state by disruption of cells. Thus, B5 virus attached but was not eclipsed. The slower course of B5 virus adsorption to carrier cells compared to its adsorption to controls suggested partial blockade of receptor sites for B5 virus. Productive infection of B3-carrier cells by ribonucleic acid prepared from B1 and B5 viruses showed that the blockading effect exerted by the B3-carrier state did not extend to the cellular mechanisms

responsible for reception of viral RNA and for replication and release of new Coxsackie virus.

Since the specific interference with Coxsackie virus superinfection was characteristic of cultures of B3-carrier cells, and since carrier cultures contained a relatively large quantity of cell-associated mature virus, it was reasonable to conclude that the specific interference resulted from loading of cellular mechanisms for attachment, penetration, and eclipse of superinfecting Coxsackie virus. Apparently, unrelated systems existed for productive reception of polio and other viruses, since the carrier cells responded normally to infection with these viruses. It could therefore be postulated that perpetuation of the virus-carrier state depended on existence at cell surfaces of antibody-virus complexes (perhaps in dynamic equilibrium of formation and dissolution), which masked or blockaded receptors specific for Coxsackie viruses. Without quantitative evidence, Ackermann (12, 13) has considered such a postulate to explain properties of a poliovirus-HeLa carrier system. More recently, Mandel (14) with poliovirus and Rubin (15) with Newcastle disease virus have established that virus exposed to minimal amounts of antibody may be adsorbed by receptive cells and remain at the cell surface until alteration of equilibrium by environmental conditions results in productive infection or cellular dissociation of virus. The probability that such a mechanism explains the Coxsackie B3–HeLa carrier system is strengthened by the attainment of productive infection of carrier cells following removal of viral-inhibitor–containing culture medium and, conversely, by successful elimination of the carrier state by cultivation of cells in the presence of specific viral antibody. No gross morphologic difference between colonies of carrier cells and B3-cured cells of the HeLa sublines was seen to suggest that viral selection of HeLa cell clones played a major role in the evolution of the carrier state. Such a factor in sustained infection has been implicated by Ackermann (13) and by Vogt and Dulbecco (16) for poliovirus, by Fernandez (17) for herpes virus and Cieciura (18) for Newcastle disease virus. Tests for presence of interferon (19) in the B3 carrier system as shown for other systems (20–22) have been negative. Since interference with viral infection mediated by interferon appears operative at the intracellular level (23), it seems unlikely that interferon has any role in the specific interference with Coxsackie virus superinfection reported here, which was shown to result from surface effects.

The findings reported here amplify the picture of structural mechanisms of HeLa cells responsible for attachment, penetration, and productive reception of enteroviruses presented by the work of McLaren and Holland on cellular reception of poliovirus and enteroviral RNA (6, 7). The differing influence of what presumably is receptor loading with one Coxsackie virus on reception of other Coxsackie viruses, and the lack of effect on receptor systems for other enteroviruses, suggests a multiplicity of conveyer-like systems proceeding from the cell surface convergently toward sites of replication of viral RNA and

production of intact new virus. This picture raises an interesting question of the normal function of the cell receptor systems, unless it is supposed that cells naturally possess specific structural receptors for infection by viruses which they may or may not encounter.

SUMMARY

Sustained infection of HeLa cells by Coxsackie B3 virus, dependent on presence of viral inhibitor in culture medium, was achieved. Persistent treatment of carrier cultures with anti–Coxsackie B3 hyperimmune monkey serum eventually eliminated virus from carrier cultures indicating that a lysogenic virus-cell relationship was not operative.

Free virus was produced continuously by carrier cultures despite washing and neutralization with antiserum to eliminate free virus temporarily. In carrier cultures, about 1.5 to 1.9 plaque-forming units of virus per cell were cell-associated; approximately 6 per cent of this cell-associated virus was not neutralizable by antiserum. In growth medium containing anti-B3 antibody, cells from carrier cultures formed colonies as efficiently as cells from B3-cured cultures. Assays of carrier cultures for infectious centers indicated that less than 1 per cent of cells produced free infectious virus. The Coxsackie B3 virus–carrier state appeared to represent surface residence of B3 virus on the majority of carrier cells with restriction of productive infection to a small proportion of the population.

Coxsackie B3 carrier HeLa cultures, unlike control cultures, were not destroyed by challenge with Coxsackie B1, B3, or B5 viruses. The B3 carrier state did not interfere with superinfection by herpes, vaccinia, and types 1 to 3 polioviruses. In contrast to parental or B3-cured lines, B3-carrier HeLa cultures superinfected with Coxsackie B1 virus produced no significant virus, and cultures superinfected with B5 viruses produced new virus to a limited extent only.

Specific interference with Coxsackie virus superinfection by the B3-carrier state of HeLa cells was shown to be attributable to failure of attachment in the instance of Coxsackie B1 virus, and failure of penetration and/or eclipse in the instance of B5 virus. The interfering effect was circumvented successfully by superinfection of carrier cells with ribonucleic acid extracted from Coxsackie B1 and B5 viruses.

BIBLIOGRAPHY

1. Ginsberg, H. S., The significance of the viral carrier state in tissue culture systems, *Progr. Med. Virol.*, 1958, **1,** 36.
2. Syverton, J. T., and McLaren, L. C., Human cells in continuous culture. I. Derivation of cell strains from esophagus, palate, liver, and lung, *Cancer Research*, 1957, **17,** 923.
3. Eagle, H., Metabolic studies with normal and malignant human cells in culture, *The Harvey Lectures*, 1958–59, **54,** 156.

4. Holland, J. J., and McLaren, L. C., Improved method for staining cell monolayers for virus plaque counts, *J. Bact.*, 1959, **78,** 596.
5. Crowell, R. L., and Syverton, J. T., Specific interference between Coxsackie viruses in a HeLa cell—Coxsackie B3 carrier culture, *Bact. Proc.*, 1960, 95.
6. Holland, J. J., and McLaren, L. C., The mammalian cell-virus relationship. II. Adsorption, reception, and eclipse of poliovirus by HeLa cells, *J. Exp. Med.*, 1959, **109,** 487.
7. Holland, J. J., McLaren, L. C., and Syverton, J. T., The mammalian cell-virus relationship. IV. Infection of naturally insusceptible cells with enterovirus ribonucleic acid, *J. Exp. Med.*, 1959, **110,** 65.
8. Takemoto, K. K., and Habel, K., Virus-cell relationship in a carrier culture of HeLa cells and Coxsackie A9 virus, *Virology*, 1959, **7,** 28.
9. Chambers, V. C., The prolonged persistence of Western equine encephalomyelitis virus in cultures of strain L cells, *Virology*, 1957, **3,** 62.
10. Dinter, Z., Philipson, L., and Wesslen, T., Persistent foot-and-mouth disease infections of cells in tissue culture, *Virology*, 1959, **8,** 542.
11. Henle, G., Deinhardt, F., Bergs, V. V., and Henle, W., Studies on persistent infections of tissue cultures. I. General aspects of the system, *J. Exp. Med.*, 1958, **108,** 537.
12. Ackermann, W. W., Mechanisms of persistent and masked infections in tissue culture, *Ann. New York Acad. Sc.*, 1957, **67,** 392.
13. Ackermann, W. W., Certain factors governing the persistence of poliovirus in tissue culture, *in* Symposium on Latency and Masking in Viral and Rickettsial Infections (D. L. Walker, R. P. Hanson, and A. S. Evans, editors), Minneapolis, Burgess Publishing Co., 1957, 169.
14. Mandel, B., Studies on the interaction of poliomyelitis virus, antibody, and host cells in a tissue culture system, *Virology*, 1958, **6,** 424.
15. Rubin, H., Interactions between Newcastle disease virus (NDV), antibody, and cell, *Virology*, 1957, **4,** 533.
16. Vogt, M., and Dulbecco, R., Properties of a HeLa cell culture with increased resistance to poliomyelitis virus, *Virology*, 1958, **5,** 425.
17. Fernandez, C. G., Persistence of herpes simplex virus in HeLa cells, *Nature*, 1960, **185,** 268.
18. Cieciura, S. J., A virus carrier state in mammalian cells, *Bact. Proc.*, 1959, 78.
19. Isaacs, A., and Lindenmann, J., Virus interference. I. The interferon, *Proc. Royal Soc. London*, (*Series B*), 1957, **147,** 258.
20. Ho, M., and Enders, J. F., Further studies on an inhibitor of viral activity appearing in infected cell cultures and its role in chronic viral infections, *Virology*, 1959, **9,** 446.
21. Henle, W., Henle, G., Deinhardt, F., and Bergs, V. V., Studies on persistent infections of tissue cultures. IV. Evidence for the production of an interferon in MCN cells by myxoviruses, *J. Exp. Med.*, 1959, **110,** 525.
22. Cooper, P. D., and Bellett, A. J. D., A transmissible interfering component of vesicular stomatitis virus preparations, *J. Gen. Microbiol.*, 1959, **21,** 485.
23. Wagner, R. R., Viral interference. Some considerations of basic mechanisms and their potential relationship to host resistance, *Bact. Rev.*, 1960, **24,** 151.