SPECIFIC VIRAL INTERFERENCE IN HELA CELL CULTURES CHRONICALLY INFECTED WITH COXSACKIE B5 VIRUS[1]

RICHARD L. CROWELL[2]

Langbord Virus Laboratory, Department of Microbiology, Hahnemann Medical College, Philadelphia, Pennsylvania

ABSTRACT

CROWELL, RICHARD L. (Hahnemann Medical College, Philadelphia, Pa.). Specific viral interference in HeLa cell cultures chronically infected with Coxsackie B5 virus. J. Bacteriol. **86**:517–526. 1963.—The presence of large amounts of Coxsackie B5 virus in culture fluids of a HeLa subline, serially propagated over a 3-year period, provided evidence for attainment of a viral carrier state. Human serum in the growth medium of carrier cultures appeared prerequisite for maintenance of a stable virus-cell equilibrium. Virus was eliminated from HeLa cells by addition of B5 antiserum to the growth medium, whereas subcultivation in calf serum medium resulted in cellular degeneration by virus. HeLa cells, chronically infected by B5 virus, retained normal morphology in monolayer cultures and were found preservable by freezing. Persistently infected HeLa cells formed colonies with high efficiency in a medium containing B5 antiserum, to provide evidence that the majority of cells in carrier populations were not fatally infected. The significance of occurrence of small and large plaque variants of B5 virus in the carrier system remains to be determined. Coxsackie B5-carrier cultures were found specifically resistant to superinfection by all members of Coxsackie group B. This resistance, due to viral interference, was not extended to three immunological types of Coxsackie group A, poliovirus types 1 to 3, adenovirus T1, or vaccinia virus. Viral interference was found to be a consequence of altered surfaces of carrier cells, as reflected by decreased adsorption kinetics and cell penetration by Coxsackie group B viruses. The data suggested that Coxsackie group B viruses share a unique requirement, distinct from that of polioviruses, for reception and eclipse by HeLa cells. Interference between polioviruses and members of Coxsackie group B is discussed.

It was recently reported that HeLa cells are susceptible to persistent infection by Coxsackie B3 virus with a resultant acquisition of specific resistance to superinfection by selected members of Coxsackie group B (Crowell and Syverton, 1961). It was considered important to extend these observations to determine (i) whether additional group B viruses could be readily established in carrier states and (ii) if, in these systems, all Coxsackie B viruses (B1 to B6) would display reciprocal interference effects as a group. Results of studies to characterize viral interference properties of HeLa cell populations chronically infected with Coxsackie B5 are reported.

MATERIALS AND METHODS

Cells. Parental line of HeLa cells from the laboratory of the late J. T. Syverton and derived sublines were routinely propagated by methods previously described (Crowell and Syverton, 1961). In addition, a HeLa cell line grown in medium containing human serum was purchased from Microbiological Associates, Inc. (MBA), Bethesda, Md., and adapted to medium supplemented with calf serum to provide monolayer cultures for production of stock viruses and for routine plaque assays.

Viruses and virus assay. Origin of strains of Coxsackie viruses B1, B3, and B5, of polioviruses T1, T2, and T3 with homotypic antiserums employed in this study, and the method of assay were previously described (Crowell and Syverton, 1961). Additional Coxsackie viruses A13, A15, A18, B2, B4, and adenovirus T1 were obtained
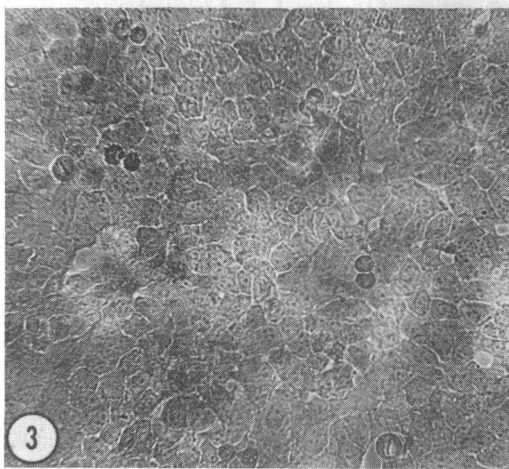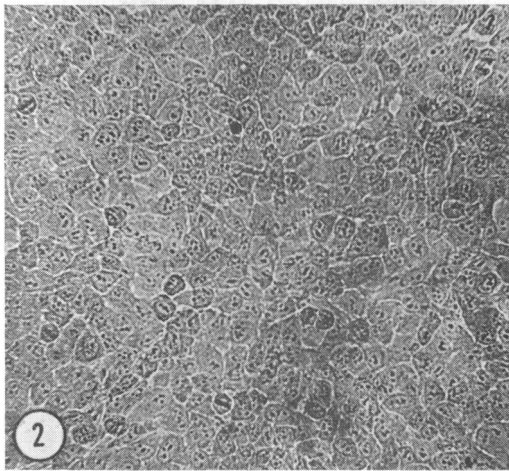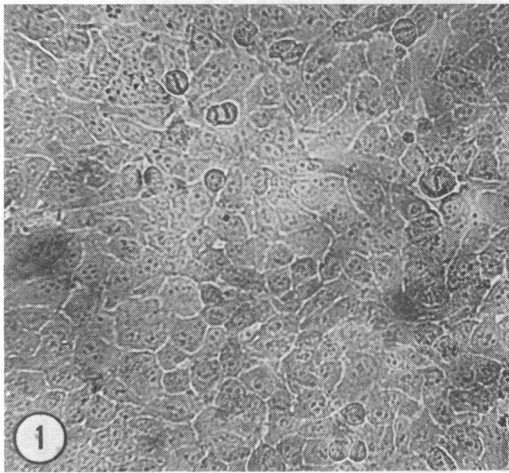
from the Minnesota collection. Coxsackie B6 virus was gratefully received as tissue-culture fluid from W. McD. Hammon. Vaccinia virus was derived from a vaccine ampule (Cutter Laboratory strain) by inoculation into HeLa cultures.

For assay of heterologous viruses in the presence of B5 virus derived from viral carrier cultures, an equal volume of diluted antiserum to B5 virus was added to each virus dilution. Complete neutralization of the B5 component was obtained during an incubation period (60 to 90 min) at room temperature prior to inoculation into plaque assay cultures. Adequacy of antiserum content was routinely verified in control assays. Cross-neutralizing activity of the B5 serum dilution used was shown absent for each virus under assay.

RESULTS

Development of HeLa-B5 carrier cultures. A population of HeLa cells in tube culture, which survived infection initiated by Coxsackie B5 virus at an input multiplicity of 5, was expanded in continous series in monolayer cultures by routine trypsinization for 3 years. Culture fluids from these normal-appearing HeLa cells (Fig. 1 to 3), which were routinely assayed at most passage levels, contained 10^4 to 10^7 plaque-forming units (PFU) per ml of Coxsackie B5 virus. The medium, with pooled human serum, used for propagation of B5-carrier cells was shown to contain only a minimal amount of inhibitory substance to B5 virus, as revealed by plaque-reduction assay (Table 1). In comparison, it is of interest that much higher levels of B3 virus inhibitory activity were found in several human serum pools used to propagate B3-carrier HeLa cells (Crowell and Syverton, 1961). Replacement of human serum with calf serum in the growth medium, however, resulted in almost total degeneration of the B5-infected cell population over a 15-day period; control noninfected cells thrived under similar conditions. These results suggested that, even though pooled human serum contained only a low amount of B5 inhibitor, it was essential to perpetuation of the viral carrier state.

FIG. 1 to 3. *Unstained preparations of monolayer cultures of HeLa sublines grown on cover slips. Photographed in bright field after BSS rinse. 120 ×. Figure 1 shows parent HeLa cells; Fig. 2, B5-cured subline; Fig. 3, B5-carrier subline. Monolayer cultures could not be differentiated by microscopic inspection.*

After 25 cellular transfers of the B5-carrier cells in series, a portion of the cell population was cured of B5 virus by cultivation for 5 days in growth medium supplemented with hyperimmune B5 monkey serum at 10% final concentration. The cell population was considered cured when B5 virus was no longer recovered in the supernatant fluids of monolayer cultures in inhibitor-free medium, and when the cells failed to demonstrate characteristic viral interference properties associated with the carrier state. The cells, once cured, have never spontaneously returned to the carrier state over a 2-year period of routine subcultivation. The cell population, cured of B5 virus infection, provided control cultures for comparative characterization of properties associated with the viral carrier state.

On several occasions, HeLa-B5 carrier-cell populations have been frozen in growth medium containing 10% glycerol and 20% human serum, preserved in liquid nitrogen for periods up to 8 months (the longest interval tested), and successfully recovered in an unaltered condition. Cell populations of parent, B5-cured, and B5-carrier HeLa sublines have thus been preserved, and provide a source of stock cells for comparative studies.

Status of B5-carrier virus. As found for B3-carrier HeLa cells, it was difficult to remove free B5 virus from chronically infected cells by washing. Nevertheless, assay of B5-carrier virus associated with cells, after cells were washed with balanced salt solution (BSS) and disrupted by three cycles of freezing and thawing, yielded on the average approximately 12 PFU of virus per cell. This figure, which has varied on different occasions from 2 to 12 PFU/cell, was considered a minimal value, since additional mature virus not detected by this procedure could also be present (Holland, 1962). To test this possibility, an experiment similar in design to that of McLaren, Holland, and Syverton (1960) was performed in which a B5-carrier culture was subjected to acidic conditions.

The fluid phase, composed of growth medium with 10% human serum, was removed from HeLa-B5 carrier cells in monolayer culture, and debris was removed by centrifugation at 2000 rev/min for 10 min in an International model UV centrifuge. Samples of the supernatant fluid were diluted equally with buffered gelatin, 1% gelatin in BSS buffered with 0.01 M tris(hydroxymethyl) aminomethane (tris)-HCl (TBG) at pH 7.5, or at

TABLE 1. *Content of Coxsackie B5 virus inhibitors in pools of human serum* reflected by residual virus titer*

Serum pool	Virus titer ($\times 10^6$ PFU/ml)
Human 1	18.5
Human 2	3.9
Human 3	3.2
Human 4	0.2
Calf 1	41.0
Calf 2	44.0

* Each human serum pool contained serum from 15 to 25 donors. Equal volumes of virus dilution and serum diluted 1:5 were incubated for 1 hr at room temperature prior to plaque assay of residual virus.

pH 2.5 with 1% gelatin in BSS buffered with 0.05 M phthalate-HCl (PBG), incubated at 37 C for 1 hr, and assayed for virus. The cellular phase of the culture was removed from the glass with 0.05% trypsin in calcium and magnesium-deficient phosphate-buffered saline, the cells dispersed, counted, and calf serum was added to 0.05% to neutralize residual trypsin. The cellular preparation was disrupted by three cycles of alternate freezing and thawing, and samples of the cell suspension were diluted equally with TBG at pH 7.5, with PBG at pH 2.5, or with original buffered culture fluid and incubated for 1 hr at 37 C. All virus preparations were serially diluted in TBG (pH 7.5) and assayed by routine plaque technique for B5 virus.

Results of exposure of carrier cells to low pH (Table 2) revealed that a fivefold increase in the amount of infectious B5 virus was recovered. These results are consistent with those of others in which a low pH environment was shown to dissociate virus from both antibody and cellular receptors (McLaren et al., 1960; Mandel, 1961). Thus, under routine conditions of cultivation, infectious B5 virus was considered to be complexed to viral inhibitor and surfaces of carrier cells, and thereby prevented from initiating fatal infection of cells.

Owing to inherent difficulties in assay procedures, a reliable enumeration of productively infected cells in B5-carrier cultures was not satisfactorily obtained. However, as with the B3-carrier system, active replication of infectious B5 virus was assumed to be restricted to a few HeLa cells in the carrier population. This assump-

TABLE 2. *Influence of pH on recovery of infectious B5 virus from a persistently infected HeLa cell culture*

Sample	pH	Virus titer (PFU/ml)	Fold increase at pH 2.5
		$\times 10^{6}$	
Fluid..............	7.4	2.3	1.0
Fluid..............	2.5	7.7	3.3
Cells..............	7.4	2.6	1.0
Cells..............	2.5	15.0	5.8
Fluid and cells....	7.4	4.4	1.0
Fluid and cells....	2.5	19.0	4.3

TABLE 3. *Colony formation by HeLa cells of B5-carrier and B5-cured sublines in medium containing antiserum to B5 virus*

No. of cells inoculated	Avg no. of colonies formed by	
	Carrier cells	Cured cells
200	231	183
100	119	96
50	63	54

tion was further tested by determining the ability of individual carrier cells to form colonies.

HeLa-B5 carrier cells in monolayer culture were dispersed by trypsinization, enumerated by aid of a hemacytometer, diluted, and plated in triplicate in growth medium supplemented with a twofold concentration of Eagle's amino acid-vitamin mixture, 10% calf serum, 10% human serum, and 2% antiserum to B5 virus. After incubation for 7 days at 37 C in an atmosphere of 5% carbon dioxide, the petri dish contents were stained, and the number of macroscopically visible colonies was counted. As control, similar cultures were concurrently prepared from cells of the virus-cured subline.

Cells of the carrier and cured sublines exhibited equivalent efficiency of colony formation in virus inhibitory medium (Table 3). Since no evidence is available to indicate that cells remain viable and produce Coxsackie virus, these results strengthen the assumption that B5 virus synthesis was limited to a low percentage of cells in the chronically infected population.

Selection of viral plaque variants. Plaque assay of B5 virus, derived from early serial passages of carrier cultures, showed approximately equal numbers of large and small plaques on indicator HeLa cells. Assay for virus of fluids obtained from carrier cultures at the 22nd cellular passage

level, however, revealed a predominance of minute plaques (B5(m)), less than 1 mm in diameter after 48 hr of incubation. All subsequent passages of B5-carrier cultures were found to contain only B5(m) virus in repeated plaque assays. To determine the stability of B5(m) , virus, which was derived from the 40th serial passage of carrier cells, was transferred, undiluted, in series for five passages in indicator HeLa cells free from viral inhibitors. The resulting viral population was found to contain approximately 99.9% small plaque formers which were not altered by addition of 50 μg/ml of diethylamino-ethyl cellulose-dextran to the agar overlay (Liebhaber and Takemoto, 1961). Recently, large plaque virus was found to predominate in all fluids of carrier cultures after the 42nd serial passage at the time a new human serum pool (no. 4) was incorporated into the growth medium. These results suggested that viral inhibitors in serum may have selected for given variants present in the viral population (Takemori et al., 1958). It is of interest that viral interference characteristics associated with the carrier state were not detectably altered when either large or small B5 plaque variants predominated.

Specific viral interference by HeLa-B5 carrier cultures. A unique property of HeLa-B3 Coxsackie carrier cultures (Crowell and Syverton, 1961) was the remarkable failure of the cell population to undergo cytopathic response when challenged by high doses of virulent members of Coxsackie group B. In preliminary comparative experiments of similar design, cell populations of HeLa parent, B5-cured, B5-carrier, and MBA sublines in replicate monolayer cultures were exposed to assorted viruses to test both their range of viral susceptibility and their relative sensitivity as measured by cytopathic response.

Cells of the respective HeLa cell lines were transferred to plaque bottles to provide monolayers for comparative challenge by assorted viruses. When confluent growth was attained, the monolayers were rinsed free of human serum medium with three washes of BSS, and 0.1 ml of respective virus dilution added in quadruplicate to dry monolayers. A 1-hr interval was allowed for adsorption of virus after which agar-containing overlay was added to permit plaque development.

HeLa B5-carrier cultures were specifically resistant to superinfection by all members of Coxsackie group B (Table 4). It can be seen

that this resistance was not extended to three representative Coxsackie group A viruses, poliovirus types 1, 2, and 3, adenovirus T1, or vaccinia virus, since comparable end-point titers were obtained for control and chronically infected cells. It is of interest, however, that the B5-cured cell population exhibited a degree of resistance to Coxsackie B viruses as typified by failure of virulent virus to produce clear plaques. In control cultures of B5-carrier cells maintained under standard agar overlay lacking viral inhibitor, no viral plaques developed, although the cells eventually degenerated after several days.

Specific interference of B5-carrier cultures as reflected by viral growth curves. To further demonstrate viral interference in B5-carrier cultures, comparative growth curves of challenge viruses were determined.

Rate of accumulative production of free virus was determined by rinsing of human serum medium from replicate monolayer cultures of HeLa sublines grown in 200-ml square bottles, inoculation of rinsed cultures with 1 ml of challenge virus (input multiplicity of infection approximately 50), incubation of cultures for 90 min at 37 C to permit virus attachment and penetration, reduction of unadsorbed inoculum virus by three successive rinses with 10 ml of BSS, and incubation for 30 min with antiserum to challenge virus followed by three rinses with 10 ml of BSS each to remove antiserum. Finally, treated cultures were overlaid with 10 ml of medium containing 10% calf serum and incubated at 37 C. At intervals, 1-ml samples of culture fluid were removed and replaced with fresh medium. Cellular debris was removed from the samples by centrifugation, and the supernatant fluids were frozen for assay of free virus. This procedure was followed with cultures of HeLa parent, B5-cured, and B5-carrier sublines inoculated with Coxsackie viruses B1, B3, or A18, or poliovirus type 2, respectively. Production of endogenous B5 virus by carrier cells during the course of superinfection was measured in the presence of homotypic antiserum to challenge virus.

The experimental results (Fig. 4) show limited production of B1 virus by B5-carrier cultures. Similar results were obtained for B3 virus. In contrast, replicate chronically infected cultures challenged concurrently (as control) with poliovirus T2 or A18 virus were shown metabolically competent for virus replication (Fig. 5). Thus,

TABLE 4. *Comparative susceptibility of HeLa parent, B5-cured, B5-carrier, and MBA sublines, to assorted viruses as measured by end-point titration*

Virus	Virus titer in HeLa subline (× 10⁶ PFU/ml)*			
	MBA	Parent	B5-cured	B5-carrier
Coxsackie				
A13	96.0	3.5	0.5	0.4
A15	15.0	+	+	+
A18	80.0	11.0	3.8	8.4
B1	140.0	100.0	+†	−
B2	3.2	+	0.01	−
B3	270.0	190.0	62.0	−
B4	16.0	+	0.5	−
B5	16.0	14.0	11.0	−
B6	18.0	19.0	4.9	−
Poliovirus				
T1	360.0	65.0	55.0	31.0
T2	420.0	350.0	310.0	49.0
T3	400.0	10.0	20.0	10.0
Adenovirus T1	+	+	+	+
Vaccinia	5.0	7.0	7.0	6.0

* Symbols: + = definite cytopathic effect (CPE), titer not determined; − = no observed CPE or plaque formation, even at high multiplicity of infection.

† Plaques produced by Coxsackie group B viruses in B5-cured cells not as distinct and clear as found in other sublines.
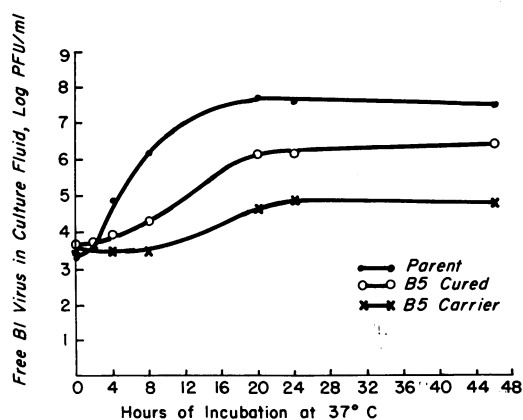

FIG. 4. *Production of free Coxsackie B1 virus by HeLa cultures of parent, B5-cured, and B5-carrier sublines.*
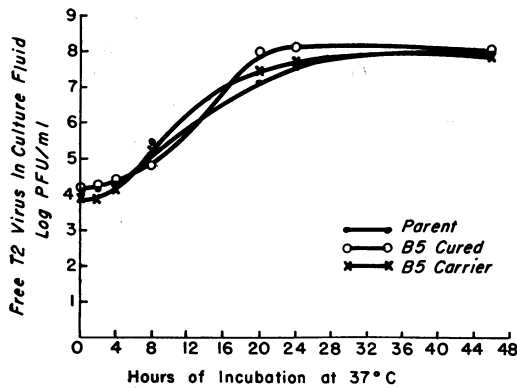
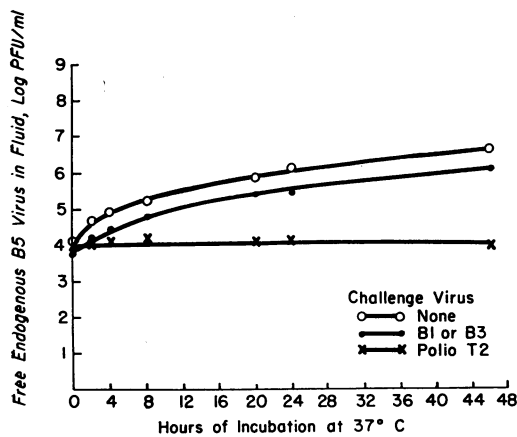FIG. 5. *Production of free poliovirus T2 by HeLa cultures of parent, B5-cured, and B5-carrier sublines.*

FIG. 6. *Production of free Coxsackie B5 virus by B5-carrier cultures after challenge with heterologous viruses.*

failure of group B viruses to multiply readily in carrier cultures appears to explain why these viruses failed to produce an overt cytopathic effect (CPE) in the system. The B5-cured cell populations exhibited intermediate capability for Coxsackie B virus production, which is consistent with the observation of cloudy plaque formation by this system. Apparently B5 virus in the carrier state selected cells with increased resistance to Coxsackie viruses for propagation from the parental HeLa line. Interestingly (Fig. 6), endogenous B5 virus production by carrier cells was prevented as a consequence of superinfection with poliovirus T2. It is likely that inoculation of poliovirus in high concentration resulted in pre-emption and destruction of carrier cells before

infectious B5 virus was released from its bound state. As control, uninoculated B5-carrier cultures failed to show evidence of CPE at 48 hr.

To determine whether B5-carrier cells failed to produce free B1 or B3 virus was a reflection of failure of virus release mechanisms, an additional growth experiment of challenge virus was performed. This experiment was similar in design to those described, except that entire replicate cultures were removed at intervals to permit assay of both free and cell-associated virus and input multiplicity of infection was 100. By 10 hr after infection, intracellular virus yield had reached maximal and equivalent levels in both parental and cured cell lines, whereas less than 1% of this amount of virus was found either as cell-associated or as free virus in carrier cultures. Further incubation to 24 hr did not significantly increase the corresponding viral yield of carrier cells. Data from this experiment indicated that failure of B5-carrier cells to produce Coxsackie B virus during superinfection was not a result of inadequacy of cells to release virus, but that intracellular formation of challenge virus was actually impaired. To focus attention on mechanisms responsible for inhibition of group B virus replication in B5-carrier cells, viral attachment studies were performed.

Relationship of viral attachment to interference effects in HeLa-Coxsackie B5 carrier cultures. Repeated comparative determinations of attachment kinetics of challenge viruses have been performed with consistent results for cells of HeLa parent, B5-cured, and B5-carrier sublines in monolayer or suspension systems. Results of a representative experiment obtained with cells in suspension are given in Fig. 7.

Cells in monolayer were rinsed free of growth medium, removed from glass by aid of a rubber policeman and 0.02% ethylenediaminetetraacetic acid (in PBS without calcium and magnesium), collected by centrifugation, resuspended in PBS, and counted. Samples were distributed to separate tubes, cells were resedimented in the centrifuge, and the fluid phase was replaced with virus (in BSS without glucose) to give a multiplicity of 0.01 and a final cell concentration of 5×10^6/ml. Viral attachment was allowed to proceed at room temperature (26 to 28 C) during which, at intervals, 0.1-ml samples of virus-cell suspensions were diluted 1:100 in BSS with 3% calf serum to stop viral attachment, cells were removed by centrifugation, and the supernatant

fluid was frozen for plaque assay of unattached virus. Virus samples incubated without cells were removed correspondingly to provide control of virus stability.

Inspection of Fig. 7 reveals that B5-carrier cells have a limited capacity to adsorb B1 virus. Determination of rate of B3 virus attachment gave similar results. As control, uniform attachment kinetics were found for poliovirus T2 for the three cell lines under comparison. Thus, diminished attachment rates of group B viruses for carrier cells contributed to the observed interference characteristics. Since, however, 25 to 35% of total superinfecting virus did attach to carrier cells during a 1-hr period, it was evident that additional viral inhibitory mechanisms remained operative to account for the observed viral interference (Darnell and Sawyer, 1960; Holland, 1962).

Eclipse of superinfecting virus by HeLa-B5 carrier cultures. Attention was next directed toward determining the fate of superinfecting viruses which had absorbed to B5-carrier cells. Coxsackie virus types B1, B3, A18, and poliovirus T2 were added separately to replicate cultures of MBA, parent, B5-cured, and B5-carrier HeLa sublines in a comparative experiment designed to determine whether the process of viral eclipse proceeded normally in chronically infected cells.

Replicate monolayer cultures of each cell line in 2¾-oz. square bottles were rinsed free of medium containing human serum, were shaken dry, and 0.1 ml of respective virus dilution was added to give an input viral multiplicity of 0.25 to 0.5. Viral adsorption and penetration were allowed to proceed at 37 C during which, at hourly intervals, virus-cell interaction was stopped by addition of cold diluent (BSS with 3% calf serum) to give a 1:100 dilution of residual extracellular virus. The fluid phase from each culture was frozen for plaque assay of unattached virus. The cell monolayers were washed four times with 5-ml volumes of cold BSS to remove free virus, and the cells were disrupted by four cycles of alternate freezing and thawing. Cell debris was deposited by centrifugation, and the supernatant fluid, containing virus not eclipsed, was assayed in the presence of B5 antiserum.

The experimental results presented in Table 5 confirm and extend evidence for decreased attachment of B1 and B3 viruses to carrier cells. The finding that 51 and 86%, respectively, of B1 and B3 viruses, which had attached to carrier cultures
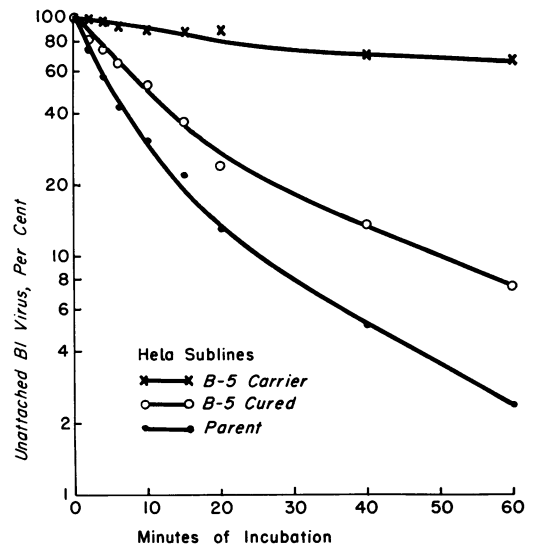

FIG. 7. *Attachment of Coxsackie B1 virus to suspensions of HeLa cells of parent, B5-cured, and B5-carrier sublines.*

TABLE 5. *Adsorption and eclipse of virus by HeLa cultures of MBA, parent, B5-cured, and B5-carrier sublines in monolayer as measured by plaque assay*

Virus	Time at 37 C (hr)	Virus attached to cells (%)				Attached virus recovered from washed cells (%)			
		MBA	Parent	Cured	Carrier	MBA	Parent	Cured	Carrier
B-1	1	96	97	94	26	0.08	0.08	0.70	51
	2	99	99	96	38	0.07	0.10	0.42	26
	3	99	99	99	46	0.35	0.31	0.49	20
B-3	1	90	91	86	35	0.20	0.30	3.5	86
	2	95	95	87	39	0.20	0.47	2.1	50
	3	95	95	89	42	0.28	0.56	2.0	51
A-18	1	84	71	61	60	32	44	27	38
	2	90	80	96	79	30	36	5	23
	3	92	91	99	93	25	25	1	13
Polio T2	1	67	64	67	70	25	36	34	32
	2	80	82	91	83	19	19	4	14
	3	81	92	96	92	18	5	1	4

during 1 hr of exposure, could be recovered as infectious virus from disrupted cells, suggested impairment in eclipse mechanisms for these viruses. These results take on increased significance when compared with eclipse values for the same

viruses by the three control HeLa sublines. Use of 8 M urea (Holland, 1962) failed to increase recovery of B1 virus from B5-carrier cells disrupted by freezing and thawing, suggesting that total infectious virus not eclipsed was made available for assay. Control viruses A18 and T2 demonstrated consistent, although surprisingly slower, rates of eclipse by the HeLa sublines tested. Thus, evidence of inhibition in the eclipse phase of viral infection for superinfecting Coxsackie B1 and B3 viruses by B5-carrier cells helps to explain the specific interference effects associated with the system.

DISCUSSION

A surviving population of HeLa cells infected with Coxsackie B5 virus was expanded and propagated for more than 50 serial transfers over a 3-year period. The finding that fluid from each cellular passage level contained high amounts of B5 virus provided evidence for attainment of another example of chronic infection of HeLa cells by Coxsackie group B viruses.

Selection of a HeLa cell population, inherently more resistant to Coxsackie virus infection, with concurrent selection of B5 virus variants from the wild virus population undoubtedly made easier the development of this carrier state. This biological selection process would appear to be an expected event (Vogt and Dulbecco, 1958; Takemoto and Habel, 1959; Solovyov and Gulevich, 1960); however, it is interesting to note that this did not appear to be the case for the HeLa B3 virus carrier system (Crowell and Syverton, 1961). Factors controlling the diminished sensitivity of B5-cured cells for Coxsackie B viruses have not been determined.

Since HeLa cells persistently infected with Coxsackie B viruses have shown no abnormal morphology, and since these cell populations can be preserved in the frozen state and recovered without alteration to the viral carrier state, it is possible that cell lines contaminated by virus may escape ready detection (Coriell, 1962).

Undoubtedly one of the most interesting characteristics of Coxsackie group B carrier states in HeLa cells is the interference property found specific and limited to superinfecting homologous or heterologous members of Coxsackie group B. Results of experiments designed to determine mechanisms responsible for the observed interference effects revealed that (i) challenge virus failed to multiply significantly in carrier cultures to account for lack of viral CPE and (ii) inhibition of viral multiplication was a consequence of reduced viral adsorption kinetics and cell penetration. It is considered unlikely that interferon played a major role in the system described, since interferon has been shown to be nonspecific in viral interference and operative at the intracellular level (Ho, 1962; Wagner, 1963). Results of the studies reported herein and of those previously recorded (Crowell and Syverton, 1961; Habel et al., 1958; Quersin-Thiry, 1958; Quersin-Thiry and Nihoul, 1961) suggest that members of Coxsackie group B may have similar and unique requirements for cell reception and penetration. Thus, if viral receptors on susceptible cells are previously occupied or altered by a primary virus, a second related virus is excluded (Baluda, 1959). This hypothesis appears tenable in that large amounts of mature B5 virus were found produced by cells in carrier cultures, with the bulk of the virus being held at cellular surfaces, and that a very low percentage of the cell population was fatally infected.

The question of interference between polioviruses and Coxsackie group B viruses in diverse systems, as recorded in the literature, remains unsettled. Results of studies of Coxsackie group B carrier systems showed no evidence for viral interference between these agents and the three polioviruses, whereas numerous reports (Halpern and Sulkin, 1961; Sabin, 1959; Hsiung, 1961; Pacsa, 1961) suggest data to the contrary. These conflicting observations may not be as discrepant as they appear when one considers the characteristics of the different systems studied. If a cytopathic virus infects a susceptible host cell first, and pre-empts that cell for synthesis of its own kind, a second virus is unlikely to be replicated by the dying host. Numerous studies have shown the importance of proper timing for addition of superinfecting virus, and the influence of the relative rates of synthesis of the respective viruses to demonstrate interference. Within this concept, quantitative variation in interference of viral replication is likely a result of asynchronous infection of large populations of host cells by the primary virus. On the other hand, production of interferon, which is known to be produced by enteric viruses (Ho and Enders, 1959), may account for some instances of interference reported to occur between Coxsackie and polio-

viruses. It is pertinent to point out that in the present study polioviruses T1, T2, and T3, as superinfecting agents, pre-empted B5-carrier cells for poliovirus synthesis both in fluid medium and under agar overlay. Typical plaque production during a 48-hr incubation period indicated the occurrence of repeated cycles of poliovirus replication in the system, suggesting that endogenous B5 virus was released too slowly from its bound form at the surface of cells to preclude poliovirus synthesis. "Receptor substance," derived from HeLa cells and subjected to a variety of experimental conditions, was reported to manifest parallel activity for Coxsackie B1 and poliovirus T1 (Holland and McLaren, 1961). Although a B1 carrier system has not been examined, results of studies with Coxsackie B3 and B5 carrier cultures have served to help differentiate between Coxsackie group B viruses and polioviruses with respect to requirements for reception and penetration of HeLa cells. The relative resistance of Coxsackie group B viral "receptor substance" to heat-inactivation and to anticellular serum (Habel et al., 1958; Quersin-Thiry, 1958; Quersin-Thiry and Nihoul 1961) provide additional characteristics to distinguish Coxsackie B virus receptors from those of polioviruses. Thus, if true viral interference between these agents occurs, it is unlikely to result from competition for cellular receptors.

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