Correlation Between Propagation of a Bovine Enterovirus and Development of Receptor-Like Material in Tissue Mince[1] (34170)

BRUCE C. CASTO[2] AND WILLIAM MCD. HAMMON

Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

During the course of investigations, designed to test *in vitro* methods for measuring the oncolytic potential for human tumors of several viruses of animal origin (1), it was observed that nontrypsinized minced fragments of freshly excised calf kidney were not susceptible to infection with bovine enterovirus type (BE-1) as evidenced by a decline in virus titer through a 48-hr period following inoculation of freshly removed kidney fragments. Incubation of the virus–tissue fragment mixtures for an additional 72 hr did, however, result in a substantial increase in virus titer in these inoculated minces. Thus, it appeared that susceptibility of the tissue mince to infection with the intact virus was acquired as a result of prolonged incubation *in vitro*.

Homogenates prepared from freshly removed calf kidney also failed to adsorb BE-1 from a test inoculum. The inability of certain fresh human and monkey tissue homogenates to adsorb poliovirus, in contrast to the adsorption of virus by cells cultured *in vitro* from such tissues, was described by Holland (2) and Quersin-Thiry (3). Their findings, however, are in contradiction to the observations of Kunin and Jordan (4) and of Kunin (5), who found that several noncultured monkey tissues were capable of adsorbing significant proportions of poliovirus type 1.

These variations in results allow for different interpretations as to the mechanism of the appearance of virus sensitive cells *in vitro* which arise from apparently virus insensitive tissues *in vivo*. Based on the available data, the more plausible hypotheses are: (i) virus-sensitive cells are present in small numbers *in vivo* which, after *in vitro* cultivation, comprise the bulk of the cultured cell population, as believed by Kunin (5); or (ii) virus sensitive cells arise during *in vitro* culture by virtue of the synthesis of virus receptor material which is repressed in cells *in vivo* by contact inhibition, as proposed by Holland (2).

The present studies, utilizing calf kidney tissue fragments and bovine enterovirus, were done in an attempt to answer, in part, the basic questions presented above.

Materials and Methods. Cell cultures. Cell suspensions of calf kidney were prepared by trypsinization of minced tissue fragments according to the method of Bodian (6). The packed cell volume was determined after centrifugation for 5 min at 150*g* following two washes in 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution (LAH). Cell cultures in 3-oz bottles to be used for plaque assay were seeded with 8.0 ml of a cell suspension containing 3.0 ml of packed cells/liter of medium. Outgrowth and maintenance medium have been described (7). The first and subsequent changes of medium, if necessary, were done 24–48 hr prior to the use of the cell cultures using 5 ml of medium/bot-

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[2] Present address: Institute for Biomedical Research, American Medical Association, Education and Research Foundation, Chicago, Illinois 60610.

TABLE I. Surviving BE-1[a] after 1 hr Incubation in Replicate Tubes of LAH.

Expt. no.		Tube no. 1	2	3	4	5
1	Mean plaque count	46.6	48.6	52.3	49.6	47.3
	Percentage of highest mean count	89.1	92.9	100	94.8	90.4
2	Mean plaque count	27.7	27.7	28.3	34.0	32.7
	Percentage of highest mean count	81.5	81.5	83.2	100	96.2

[a] All values are expressed as the mean plaque count obtained from triplicate assay bottles per sample.

tle. The change fluid contained 1% agamma calf serum which was substituted for regular calf serum.

Virus stock. The BE-1 was kindly supplied by T. Moll, State College of Washington. Prior to preparation of a working stock, this virus was passaged three times in calf kidney (CK) cell cultures and 22 times in KB cells in our laboratory. A dilution of the final passage fluid was made in LAH, so that each 0.1 ml of the stock virus would contain approximately 40,000 plaque-forming units (PFU) as determined by titration in primary CK. All virus stocks were stored at —60°.

Plaque assay. Appropriate virus dilutions were made in LAH and 0.1 ml of each dilution was inoculated onto cell sheets of each of three bottles per dilution. The virus was allowed to attach for 1 hr at 36°, after which 8.0 ml of agar overlay medium (8) was added to each bottle. After the agar had solidified, the bottles were sealed with rubber stoppers, placed in an inverted position and incubated at 36° for approximately 48 hr, at which time plaque numbers were determined.

Estimation of virus attachment to cell debris. Primary CK debris was prepared by scraping cells from the wall of 32-oz bottles on which heavy cell sheets had been allowed to accumulate over a period of 7–10 days. After removal from the bottles, the cells were washed two to three times by sedimentation in the centrifuge at 250*g* and resuspension in LAH. The packed cell volume was determined and a 20% cell suspension (v/v) was made in LAH containing 0.5 ml of 4% sodium bicarbonate/100 ml. One ml of the 20% cell suspension (approx 2×10^7 cells/ml) was transferred to each of several Pyrex tubes, which were then subjected to 7 cycles of repeated freezing and thawing to disrupt the cells (3). To either 0.5 or 1.0 ml of an appropriate concentration of cell debris was added 0.1 ml of a virus dilution containing 1000–2000 PFU of BE-1. An equal amount of virus (0.1 ml) was added to one or two LAH control tubes containing the same volume of fluid as those tubes containing debris. After a 1 hr incubation at 37°, all samples were diluted to 2.1 ml by the addition of 1 ml of cold LAH and placed in an ice bath. One-tenth ml of each test and control sample was inoculated in triplicate assay bottles and the residual virus was determined by plaque counts. The percentage of surviving virus was calculated by comparing the mean plaque count of the bottles inoculated with the test sample with that obtained from the LAH control inoculated bottles.

Results. Determination of the consistency of the method used to assay virus receptor activity. Experiments to determine the degree of variation in plaque titers from similarly inoculated tubes of LAH were performed using 600 and 1000 PFU of BE-1 contained in 0.1 ml.

One-tenth ml of the appropriate concentration of virus was added to each of five replicate tubes containing 1.0 ml of LAH. After 1 hr of incubation at 37°, the tubes containing virus were placed in an ice bath and 1.0 ml of cold LAH was added to each of the samples. Table I presents the results of assays performed on CK for residual virus from these tubes using triplicate bottles for each virus sample.

Using the highest mean plaque count of virus from any one tube in a series, the

TABLE II. Percent Surviving BE-1[a] after 1 hr Incubation at 37° in Calf Kidney Cell Culture Debris.

Concentration of cell debris (%; v/v)	20	10	5	2.5	1.25	0.625	None
Surviving virus (%)	0.3	1.0	4.0	8.0	24.0	46.0	100

[a] All values are expressed as percentage of the mean plaque count recovered from a LAH control.

largest difference found between the highest and the lowest mean plaque titers was 18%. An application of the *F* test for analysis of variance, failed to show any significant differences at the 5% level between the various virus samples in any one test.

On this basis, a reduction of 30% or over between control and test samples was usually considered significant. However, in instances where the within-group variation was large, or the number of PFU per bottle was small, such arbitrary limits were not used and appropriate statistical tests (usually the *F* test) were applied.

Effect of calf kidney cell culture debris on the infectivity of BE-1. A 20% cell debris preparation was serially diluted twofold in LAH through a final 0.6% suspension. To 1.0 ml of each of the representative dilutions of cell debris was added 0.1 ml of BE-1, containing 1000–2000 PFU. After incubation for 1 hr at 37°, residual virus was assayed and compared to controls containing only LAH. The data in Table II show that all of the various concentrations of debris tested removed a significant proportion of BE-1 from the inoculum. Dilutions of the 20% CK debris through 8-fold, still resulted in greater than 90% inactivation of the infectivity of BE-1.

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Effect of heat on the BE-1 inactivating capacity of primary CK debris. Ten percent CK debris was heated for 1 hr at 60°, after which heated and unheated preparations were tested against BE-1. Unheated preparations inactivated 98% of the BE-1 inoculum, where heated material inactivated only 36%. Thus, inactivating material (receptor) in CK cell debris was heat-labile as was found for poliovirus receptor by Holland and McLaren (10), Kunin and Jordan (4), and Quersin-Thiry and Nihoul (9). The heat lability of the BE-1 receptor, thus provided a simple method for differentiating in later experiments, the inactivation of BE-1 by receptor from that due to residual antibody in calf kidney homogenates.

Propagation of BE-1 in calf kidney cell cultures. Bottles of CK containing 1.0 ml of maintenance medium were inoculated with 0.1 ml of BE-1 (4000–5000 PFU). Maintenance medium in bottles without cells similarly inoculated with the above virus, served as controls. Immediately following, and after 24 hr at 36°, bottles were removed from the incubator and frozen and stored at −60°. Virus assays were performed on CK and the amount of virus in the original sample was determined.

The BE-1, when inoculated into CK, replicated well within the first 24 hr following inoculation (Table III). Virus-inoculated cul-

TABLE III. Amount of BE-1 Recovered from Calf Kidney Cell Cultures 24 hr after Inoculation.

Sample	Time of harvest (hr) 0	24
Calf kidney cell culture	5.2×10^{2}[a]	2.3×10^{6}
Medium control	5.9×10^{2}	2.5×10^{2}

[a] PFU per 0.1 ml of harvest fluid.

TABLE IV. Replication of BE-1 in Freshly Excised Calf Kidney Mince Inoculated after 0, 1, 2, 3, 4 Days Incubation at 37°.

Day of inoculation:	0		1		2		3		4	
Time of harvest (hr):	0	24	0	24	0	24	0	24	0	24
Calf kidney mince	350[a]	140	380	1000	390	1100	350	270,000	370	390,000
Medium control	390	140	400	100	420	100	360	120	350	130

[a] Total PFU per 0.1 ml of harvest fluid.

tures yielded titers approximately 4 logs greater than cell-free control fluids held for the same period at 37°.

Correlation between the propagation of virus and the development of a cell receptor-like response in untreated calf kidney mince. Kidneys were removed from freshly slaughtered calves within 0.5 hr after death. Portions of the cortex were finely minced with scissors. The tissue fragments thus obtained were washed three times in LAH, centrifuged at 250*g* for 10 min, resuspended in LAH medium at a final concentration of 5% (v/v) and distributed into screw cap tubes using 1.0 ml/tube.

After incubation of the tissue mince at 36° for 0, 1, 2, 3 ,and 4 days in the roller drum, duplicate mince and LAH medium control tubes were inoculated with 0.1 ml of stock BE-1/tube. Aliquots were taken at the time of inoculation, like samples pooled, and stored at —60°. All inoculated tubes were removed from the roller drum after 24-hr incubation at 36°, quick frozen in an alcohol-dry ice bath, and stored as before. Plaque assays were done as outlined previously and titers of virus were recorded as the total number of plaques per 0.1 ml of the harvest fluid.

The data presented in Table IV show that BE-1 replicated in calf kidney mince only if inoculated after 24-hr incubation *in vitro*. No virus multiplication could be demonstrated within the first 24 hr when fragments were inoculated shortly after removal from the host, whereas with extended times of incubation prior to virus inoculation (3–4 days), higher virus titers were routinely obtained from the 24-hr harvests.

For determinations of receptor activity present in incubated and nonincubated calf kidney fragments, 10–12 culture tubes of 5% mince (prepared at the same time as those used for virus inoculation) were removed from incubation at time intervals corresponding to the times of virus inoculation listed above (0, 1, 2, 3, and 4 days). Tubes of tissue mince from the same day were pooled, washed two times with LAH, centrifuged at 250*g* for 10 min, and resuspended to a 10% concentration (v/v) in LAH. The suspended fragments were homogenized with a motor driven Teflon homogenizer, the homogenate was distributed in 0.5 ml amounts in Pyrex tubes, sealed with a rubber stopper and subjected to three cycles of freezing and thawing. The final homogenate preparations were stored at —60° and representative samples from each day were assayed at the same time for receptor activity against BE-1 as outlined in "Materials and Methods" for cell culture debris.

The development of sensitivity to infection by BE-1 (Table IV) was directly correlated with the appearance of increased levels of BE-1 inactivating materials in the incubated calf kidney fragments (Table V). The

TABLE V. Percentage Surviving BE-1 after Incubation for 1 hr at 37° in 10% Homogenates from Calf Kidney Mince Incubated *in Vitro*.

	Days of incubation						0 day homog. heated 60° 1 hr	Medium control
	0	1	2	3	4	5		
Survivors (%)	69[a]	43	15	25	33	36	74	100

[a] Expressed as percentage of LAH control.

TABLE VI. Replication of BE-1 in Freshly Excised Calf Kidney Mince Incubated in 0.1 μg/ml of Colchicine and Inoculated after 0, 1, 2, 3 Days at 37°.

Day of inoculation:	0		1		2		3	
Time of harvest (hr):	0	24	0	24	0	24	0	24
Untreated mince	250[a]	160	280	130,000	460	54,000	300	200,000
Colchicine-treated mince	290	40	340	78,000	440	21,000	350	28,000
Medium control	310	120	300	60	300	150	290	110

[a] Total PFU per 0.1 ml of harvest fluid.

amount of virus recovered from homogenates prepared from nonincubated calf kidney mince, inoculated with 1000 PFU of BE-1, was approximately 69% of the control, whereas the surviving virus in homogenates prepared from mince after 1- and 2-days incubation was 43 and 15% of the control level, respectively. The level of inactivation (31%), observed with homogenate from nonincubated mince, (0 day), was essentially unchanged (26%) when samples were heated at 60° for 1 hr, therefore it was assumed that this initial, antiviral activity was not due to specific cell receptors.

Correlation between the propagation of virus and the development of a cell receptor-like response in colchicine-treated calf kidney mince. Microscopic observation of incubated fragments of calf kidney tissue revealed small areas of cell outgrowth after 3 days. Therefor, it was possible that the acquisition of susceptibility to virus infection by these tissue fragments was due to the emergence of a new virus-sensitive population of cells. Marcus and Robbins (11) showed that cells arrested in metaphase by colchicine (0.05–0.1 μg/ml of medium) do not produce virus, but do possess receptor activity. Cells in the population, not in mitotic block, were not prevented from synthesizing virus in the presence of colchicine at these concentrations.

Fresh calf kidney was minced, washed, and resuspended to a 5% concentration as previously described. To one-half of the 5% mince suspensions was added colchicine, sufficient to give a final concentration of 0.1 μg/ml. Tubes of colchicine-treated and untreated mince were then subjected to the same experimental conditions as described before with untreated mince. The data presented in Table VI confirm the finding of the earlier experiment with untreated calf kidney mince, in that BE-1 does not replicate in fresh mince within the first 24 hr.

Inoculation with BE-1 of either colchicine-treated or untreated mince, following incubation of the mince for 24 hr prior to virus inoculation, resulted in replication of virus to approximately the same titer in both. The virus titer in the untreated mince was 13×10^4 PFU/0.1 ml and 7.8×10^4 PFU/0.1 ml in the colchicine-treated mince. Relatively similar virus titers were also found in treated and untreated mince which had been incubated for 2 days prior to inoculation. Cultures inoculated after 3 days of incubation yielded titers of 2.0×10^5 PFU/0.1 ml in untreated mince as contrasted to 2.8×10^4 PFU/0.1 ml in colchicine-treated mince. The probably significant difference in virus titers at this time was presumably due to a more virus-sensitive cell population in the untreated mince which resulted from slight replication of the cells at the periphery of the fragments.

Results of assays for BE-1 inactivating materials in treated and untreated mince are presented in Table VII. The data show that a detectable increase of viral receptor appeared in mince with *in vitro* incubation and that colchicine did not interfere with the development of these virus receptors.

Discussion. The experiments reported here show that although monolayer cultures of calf kidney cells yield high titers of BE-1 within 24 hr after virus inoculation, similarly inoculated, freshly removed, calf kidney tissue fragments fail to do so. In addition, 10% debris prepared from calf kidney cell cultures inactivated greater than 90% of a BE-1 inoculum, whereas homogenates prepared

TABLE VII. Percentage Surviving BE-1 after Incubation for 1 hr at 37° in 10% Homogenates Prepared from Calf Kidney Mince Incubated in Colchicine-Treated (0.1 μg/ml) and Untreated Medium.

Sample	Days of incubation at 37°				Medium control
	0	1	2	3	
Untreated mince	89*	57	62	47	100
Colchicine-treated mince	75	60	33	32	

* Expressed as percentage of LAH control.

from minced, freshly removed calf kidneys did not demonstrate a similar activity. An analogous situation has been described for the monkey kidney–poliovirus system by Holland (2), who showed that homogenates of monkey kidney tissue removed immediately after death did not display significant poliovirus receptor activity, whereas tissues known to replicate this virus bound significant percentages of infective virus.

The acquisition of susceptibility by calf kidney fragments to BE-1 infection after 24 hr incubation, suggested that the *in vitro* incubation of mince may have resulted in the development of virus receptors in cells at the mince surface. The demonstration of increased antiviral activity in homogenates of calf kidney mince, after 1–2 days incubation, was correlated with virus replication in calf kidney fragments at these times. Increased amounts of virus were obtained from BE-1 inoculated fragments which had been incubated for a total of 3 or 4 days prior to virus inoculation. However, homogenates prepared from these minces did not show a corresponding increase in antiviral activity over that prepared from mince incubated for 2 days. Therefore, it appears that cellular functions other than receptor development may be stimulated during *in vitro* maintenance which play an important role in determining the degree to which virus will replicate.

The data discussed above, which show that freshly removed calf kidney tissues do not support the synthesis of BE-1 within the first 24 hr, confirm the findings of Holland (2) with poliovirus and human kidney. Holland (2) showed that if freshly trypsinized cells from fetal human kidney were inoculated with poliovirus at time of seeding into tubes, that no replication of virus could be detected within the first 24 hr. After this period, virus multiplication was demonstrated and maximal titers were obtained by 48–72 hr postinfection. These data are in disagreement with the findings of Kunin (5), who showed replication of poliovirus as early as 8 hr in "noncultured" trypsinized monkey and fetal human kidney cells. However, as was found with BE-1 in calf kidney mince, Kunin (5) was unable to demonstrate any poliovirus replication in freshly excised, nontrypsinized mince fragments of various rhesus monkey tissues.

Holland (2) also has reported that human amnion, freshly obtained after delivery, did not support the synthesis of poliovirus and had no receptor activity. If trypsin dispersed amnion cells were allowed to reattach to the underlying collagen membrane, from which they were removed, they did not support the replication of, or adsorb poliovirus, whereas poliovirus multiplied in, and adsorbed to, similar cells allowed to attach to glass and incubated for the same time period.

The data contained in this report using calf kidney mince, are in agreement with the proposal by Holland (2), "that contact inhibitions imposed by the immediate environment of differentiated cells repress synthesis of receptor protein, and this repression is relieved by release of the cells from their normal contact relationships during *in vitro* culture." The demonstration of receptor activity in monkey kidney homografts (3) and the replication of poliovirus in newly forming scar tissue in monkey skin (12) seem also to support this concept.

The increase in BE-1 receptor activity in calf kidney mince did not appear to be related to the emergence of a new population of sensitive cells, since virus susceptibility

and receptor activity developed when division of cells was inhibited through the use of colchicine. Cells in metaphase arrest were apparently not the source of the virus increase, since Marcus and Robbins (11) found that although cells arrested in mitosis had virus receptor activity, virus replication did not proceed in those cells. Kunin (5) has reported similar findings with poliovirus-susceptible human fetal tissues. Treatment of freshly trypsinized human fetal kidney or lung cells with colchicine, mitomycin C and 5-fluor-2-deoxyuridine to inhibit cell division, did not affect the replication in these cells of type 1 poliovirus or Coxsackie B1 virus.

The mechanism, whereby virus receptor material develops with incubation outside the normal host environment, has not been resolved. Receptor activity *in vitro* results either from the synthesis of a new cellular component or from the release of receptor already present but in a bound form. Holland and Hoyer (13) attempted to prevent new protein synthesis in freshly prepared cell cultures by using puromycin and *p*-fluorophenylalanine, but the effect on receptor appearance was negated due to the death of the cultures from the added inhibitors.

The failure to find receptor activity in isolated subcellular fractions from insusceptible tissues, and the failure of insusceptible tissue homogenates to influence receptor activity in susceptible homogenates, negates the probability that receptor exists in cells *in vivo* in a "masked" form (2).

Summary. Bovine enterovirus (BE-1) replicates well in primary cell cultures of calf kidney within 24 hr after inoculation. Debris (2.5–20%) prepared from such cell cultures inactivates greater than 90% of the infectivity of a BE-1 inoculum. Freshly excised calf kidney will support the replication of BE-1 within 24 hr only if incubated *in vitro* for 24 hr or longer prior to virus inoculation. Correlated with the development of sensitivity to virus infection, is the appearance of virus inactivating materials (receptors) in homogenates prepared from the incubated mince. The development of virus sensitivity and receptor material presumably was not due to the growth of new cells, since similar events occurred when cell division was inhibited by the incorporation of colchicine into the maintenance medium.

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