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Effect of Cyclic Nucleotide on Coronavirus Replication (40516)

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Although the human coronaviruses have been established as major pathogens of the respiratory tract, relatively little is known about characteristics of their activity in man or the total number of different strains that exist (1–4). This lack of information is directly a result of difficulty encountered in isolation and propagation of the agents. One of the viruses, 229E, is the only coronavirus which was originally isolated in cell culture and is serologically distinct from all others (5). Subsequent to the first description of 229E, isolation of related strains has been reported, often using cells not generally available. In contrast, none of the remaining coronavirus types has ever been isolated in cell culture. All were identified by virtue of their growth in tracheal organ culture, and only one of this group, OC (organ culture) 43 has been adapted to other systems, first to suckling mouse brain and then to cell culture (6–8). This virus can be considered as a prototype of the other organ culture viruses, since a number of them are related serologically to it and one, OC38, is identical to it (2). Thus its growth characteristics should resemble the other organ culture viruses. In an effort to identify methods for facilitation of coronavirus replication in cell culture, a number of different conditions of growth for OC43 virus with different passage histories was evaluated. In the present report, increased ease of adaptation of OC43 virus to cells in media containing dibutyryl cyclic AMP (dcAMP) is described.

Materials and methods. Viruses. Mouse adapted OC43 virus was originally obtained from Mr. H. S. Kaye, Center for Disease Control. It was passaged in the laboratory by intracerebral inoculation of suckling mice 24-hr old. For production of virus pools, mice were inoculated with a 1:20 suspension of infected mouse brain; animals were sacrificed 36 hours after inoculation, at a time when abnormal behavior was noticeable.

For adaptation of OC43 virus to cell cultures, a 1:20 suspension of mouse brain was inoculated into tube cultures of BS-C-1 cells. Tubes were incubated at 33° for 5 days and the material was harvested by freezing, after addition of gelatin, to a final concentration of 0.5%. Following six serial passages, the virus was considered to be adapted and an infectious pool made. The titer of this pool was $10^{5.5}$ $TCID_{50}$/ml.

Cell cultures used. WI-38 as well as the BS-C-1 cells were employed. The WI-38 cells were received as tube cultures (HEM Laboratories, Rockville, MD); the BS-C-1 cells were received as bottle cultures (Flow Laboratories, Rockville, MD). The cells were serially propagated in our laboratory and tube cultures made weekly. Growth medium was Eagle's Minimum Essential Medium (MEM) with 10% fetal bovine serum. Prior to use, the BS-C-1 cells were washed three times with Hanks' Balanced Salt Solution (BSS) and fed with serum free Eagle's MEM. WI-38 cells were fed with Eagle's MEM containing either 2% fetal bovine serum or 0.1% bovine serum albumin fraction V. Various additives were incorporated into the media used with both cell systems to test their effects in the growth of OC43 virus.

Growth and titration of OC43. Either a 1:20 suspension of infected mouse brain or a 1:20 dilution of the cell culture-adapted pool of OC43 virus was inoculated in 0.2 ml quantity into tubes of WI-38 or BS-C-1 cells. The number of tubes inoculated was sufficient so that two could be harvested on a regular basis on specific days during the week of inoculation. Additives were included in the media with both cell lines. Dibutyryl cyclic AMP (dcAMP) Sigma at a final concentration of 1.5 m*M* or 3 m*M* was incorporated into the media at day 0; the same quantity of dcAMP was added to the media again on days 2 and 4 of tubes still on test at that time. The infected WI-38 and BS-C-1 cells were incubated at 33° on a roller drum. Two of the appropriate tubes were harvested on indicated days and frozen at −70° until titration. To those tube cultures not containing serum

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or albumin, gelatin to a final concentration of 0.5% was added before freezing. All experiments were repeated a second time, separately.

All tubes from the harvests of a particular day from each of the replicate experiments were thawed, pooled and diluted in tenfold steps in Hanks' BSS. Each dilution was inoculated into 4 tubes of serum-free BS-C-1 cells. The tubes were incubated on a roller drum at 33° for 5 days. End points were read by hemadsorption using 0.2 ml of a 0.4% suspension of rat erythrocytes (9). The method of Reed and Muench was employed for determining 50% end points; the results from the replicate experiments were combined in this procedure (10).

Results. As a baseline, OC43 virus adapted to growth in BS-C-1 cells was evaluated in a homologous cell system. Serum is not required for maintenance of this cell line, so that none was used; dcAMP was included in two concentrations. Tubes were harvested at days 2, 4, and 6; titrations of the harvested material were carried out in BS-C-1 cells, which was the case in all experiments. As can be seen from the results in the left panel of Fig. 1, presence of dcAMP at either of the concentrations used did not increase the yield of virus; if anything, there was a reduction seen which was greater with the lower concentration of dcAMP. A different picture was seen when, instead of cell culture adapted virus, unadapted mouse brain material was used as the inoculum. As shown in the right panel of Fig. 1, there was better yield of virus when dcAMP was included in the medium, and again the lower concentration performed

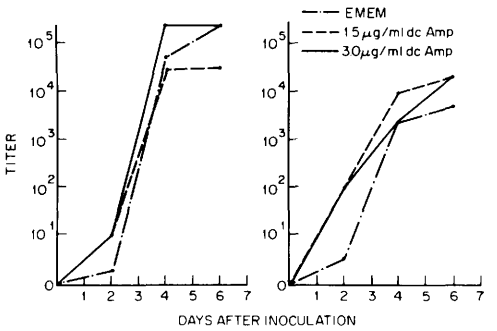

FIG. 1. Titers (Infectious $Dose_{50}$ per 0.2 ml) in BS-C-1 cells of OC43 virus grown in the same host system. Inocula used: left, cell culture adapted virus; right, mouse brain virus.

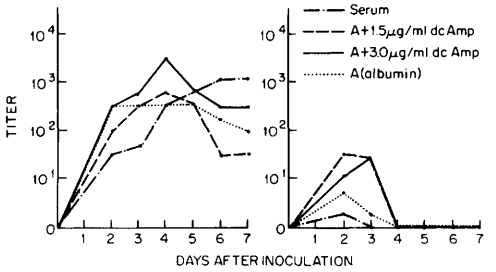

FIG. 2. Titers (Infectious $Dose_{50}$ per 0.2 ml) in BS-C-1 cells of OC43 virus grown in WI-38 cells. Inocula used: left, cell culture adapted virus; right, mouse brain virus.

better than the higher. As expected, yields in general were lower than that seen before with adapted virus. These results suggest the value of dcAMP in facilitating adaptation of the OC43 virus to growth in cells.

In the next study, virus was grown in WI-38 cells; the BS-C-1 cells were still used to determine yield achieved. WI-38 cells were chosen to represent a less permissive system for OC43 virus, one which would better serve as a model for the adaptation of other organ culture coronaviruses to cell culture. WI-38 cannot be maintained with serum-free Eagle's MEM, and 2% fetal bovine serum is usually used. This presented a problem, since dcAMP is inactivated in the presence of serum (11). Therefore, 0.1% bovine serum albumin was substituted, both by itself and in the presence of the two concentrations of dcAMP. In the left panel of Fig. 2 are shown the results when the inoculum consisted of the cell culture adapted OC43 virus. Until day 4, yield was lowest in those tubes containing the serum alone; but this increased gradually thereafter, suggesting gradual adaptation of the virus or alternately some early inhibitory effect of the serum. Inhibitory effect of serum on OC43 virus hemagglutination has, in fact, been demonstrated (12). In contrast, higher titer were observed on early days when albumin was used instead of serum. Still higher yields were observed when dcAMP was also incorporated in the media, and in contrast to the situation when BS-C-1 cells were used as the test system, the higher concentration produced better results.

The most extreme test was the inoculation of mouse brain grown virus into WI-38 cells. As shown in the right portion of Fig. 2, very little virus could be detected when bovine

serum was used. Some additional virus was produced when albumin was substituted for the serum, again suggesting an inhibitory effect of the serum. Best yields were achieved when dcAMP was added to the media. Thus, the effects of dcAMP were present only when less than optimal conditions were present, which indicates that the major use of the compound would be in adaptation of a virus to growth in cells; a similar result has been seen with other viruses, such as influenza (13). This interpretation was confirmed when it was found that dcAMP containing WI-38 tubes harvested at day 3 could be further passaged in dcAMP with increase in virus titer to 10^{-3}, while virus was lost in passage of WI-38 tubes in the presence of just albumin.

Discussion. High concentrations of certain cyclic nucleotides inhibit cellular synthesis of DNA, while lower concentrations promote it (14). In keeping with this observation, the effect of added cyclic AMP on virus infected cells varies; production of some DNA viruses may be depressed while that of RNA viruses may be increased (13, 15). The increase appears to be mediated through enhancement of virus-induced protein synthesis. Such enhancement has been demonstrated for influenza, but not for Sendai virus (13). With influenza, the effect was most apparent at low inocula of virus; this is analogous to the findings now described for OC43 virus in which increased yield of infectious virus did not result when the inoculum used was virus adapted to the cell system but was only observed when non-adapted strains were employed. This latter situation is somewhat similar to the use of a partially permissive cell system for primary isolation of an infectious agent.

Demonstration of the fact that dcAMP can aid in adaptation of coronaviruses to cell culture has several possible implications. Most of the known coronaviruses have never been grown in cell culture (2). Thus, use of compounds such as dcAMP may allow adaptation of such prototype stains to take place, which will permit further study of their past role in infections of population groups (16, 17). By the same mechanism dcAMP or substances of similar action could permit use of cell systems for primary isolation of a larger number of new coronaviruses which again would help in understanding the place of these agents in the etiology of respiratory infections of man.

Summary. The effects of the cyclic nucleotide, dibutyryl cyclic AMP on growth of the human coronavirus OC43 were evaluated. Systems used were the permissive cell line BS-C-1, and WI-38. There was no effect of the nucleotide when cell culture adapted virus was grown in BS-C-1. However, enhancement of growth in the presence of cyclic AMP was observed when the virus used was non-cell culture adapted and when WI-38 was employed. Such enhancement may be of value in attempts at growing non-adapted coronaviruses in cell cultures.

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