LATENT VIRAL INFECTION OF CELLS IN TISSUE CULTURE

I. Studies on Latent Infection of Chick Embryo Tissues with Psittacosis Virus*

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The problem of latent infection is of particular interest in the case of psittacosis virus since it has been shown that the virus persists for varying lengths of time in the tissues of several hosts including psittacine birds, man, mice, and chick embryos (1–4). In previous studies on the growth of psittacosis virus in cultures of chick embryos, it was demonstrated that cells maintained in a nutritionally deficient medium (inorganic salts and glucose) lose their ability to support the growth of psittacosis virus and this capacity could be restored by a variety of nutrient materials (5). Furthermore virus could be shown to remain in these starved cells for as long as 5 days without evidence of multiplication and then upon addition of embryo extracts or synthetic medium 199 of Parker (6) could be stimulated to active growth and virus production, suggesting that an *in vitro* latent infection of these cells had been induced (5).

The present investigation was designed to study the nature of the virus-host cell relationship in this *in vitro* model system of latent infection of tissues with psittacosis virus.

Materials and Methods

Virus.—The 6BC strain of psittacosis virus, originally obtained from Dr. K. F. Meyer, which had been repeatedly passed in eggs by the yolk sac route was used. A uniform source of virus was obtained by infecting 7-day-old embryonated eggs and harvesting the yolk sacs after 4 to 6 days of incubation at 35°C. These yolk sacs were pooled, homogenized, and diluted to give a 20 per cent suspension by weight in beef heart infusion broth and finally frozen in glass ampoules and stored at −40°C. Dilutions of this standard virus inoculum were made in Hanks's balanced salt solution (7) to give a final concentration of virus in the culture fluids used to infect the tissue cultures of $10^{2.0}$ to $10^{4.0}$ LD_{50} per ml.

Tissue cultures.—The tissue culture technic used was similar to that described previously (5). Nine- to 10-day-old chick embryos were minced with scissors and washed with four 10

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ml. changes of Hanks's balanced salt solution containing 0.0125 ml. of a 1.4 per cent sodium bicarbonate solution per ml. The washed tissue was planted with perforated cellophane discs in 10 ml. Erlenmeyer flasks to which was then added 1.8 ml. of Hanks's balanced salt solution containing 0.025 ml of 1.4 per cent sodium bicarbonate per ml., subsequently referred to as BSS. This initial fluid was completely removed after 24 hours' incubation at 36°C. and renewed with fresh BSS. Thereafter, the fluids were changed every 4 days unless otherwise noted.

These flask cultures were infected at various intervals as described and the amount of virus in the initial infecting fluids and the nutrient fluids removed from the cultures after 24 hours determined. Thereafter, the amount of virus in the fluid of each individual culture was determined by completely removing the nutrient medium from each culture at the interval stated and injecting it into 7-day-old embryonated eggs. The various substances used to stimulate virus growth were added to the fresh nutrient media introduced into the cultures as described. Three cultures were treated identically for each experimental group.

In certain experiments the tissues were examined for their content of virus. The culture fluids were removed and the cellophane discs and tissues washed for from 2 to 4 times with 2 ml. of BSS. The cellophane disc and tissue were then transferred to a mortar with a small amount of alundum and ground with a pestle in 5 ml. of nutrient broth. The washing fluids and the homogenized tissue were injected into twelve 7-day-old embryonated eggs as described below to determine their viral content. In the instances in which the eggs remained alive, they were sacrificed on the 10th day of incubation and the yolk sacs of each group pooled, ground in broth, and injected into twelve 7-day-old eggs to eliminate the possibility that virus was present in amounts too small to kill the eggs in the initial group.

At the close of representative experiments, the tissue in the culture was fixed by adding a mixture of equal parts of 95 per cent alcohol and ether to the flasks. The cellophane discs with the adhering cells were removed, stained by the Papanicolaou procedure, as modified by the Cytology Laboratory of Strong Memorial Hospital (8), and mounted on microscopic slides. By examining the stained discs, a rough estimate of the degree of cellular proliferation was made by observing the number of fibroblasts growing out from tissue fragments. The condition of the cells present on the discs was evaluated by observing the state of the nucleus and cytoplasm including the degree of vacuolation and granulation. The number of cells containing psittacosis viral inclusion bodies was also noted.

Virus Titration.—The single dilution method of Golub (9) was used for determining the amount of virus in culture fluids. The virus-containing fluids were injected undiluted or in a dilution of 10^{-1} in broth in 0.25 ml. amounts into the yolk sac of each of twelve 7-day-old embryonated eggs. The virus titers were expressed as the $\log_{10}$ of the LD_{50} for embryonated eggs. Titers of less than LD_{50} $10^{0.5}$ could not be determined with accuracy and so all are listed as <0.5 in the figures and charts unless there were no deaths of eggs injected and value is listed as <0.1.

Materials Used.—The embryo extract used throughout this work was prepared from muscle tissues of fresh beef embryos less than 40 cm. in length, which were homogenized in a Waring blendor and extracted with an equal volume of BSS without bicarbonate for 1 hour in the refrigerator. The mixture was then centrifuged at about 2000 R.P.M. for 10 minutes, the supernatant fluids removed and sealed in 2 ml. amounts in stoppered test tubes, quick frozen and stored at −40°C. This 50 per cent beef embryo extract (BEE) was used at a final concentration of 10 per cent in BSS.

EXPERIMENTAL

Stimulation of Viral Growth in Tissue Cultures Maintained in BSS for Varying Periods.—In the previous experiments (5), the tissues were regularly

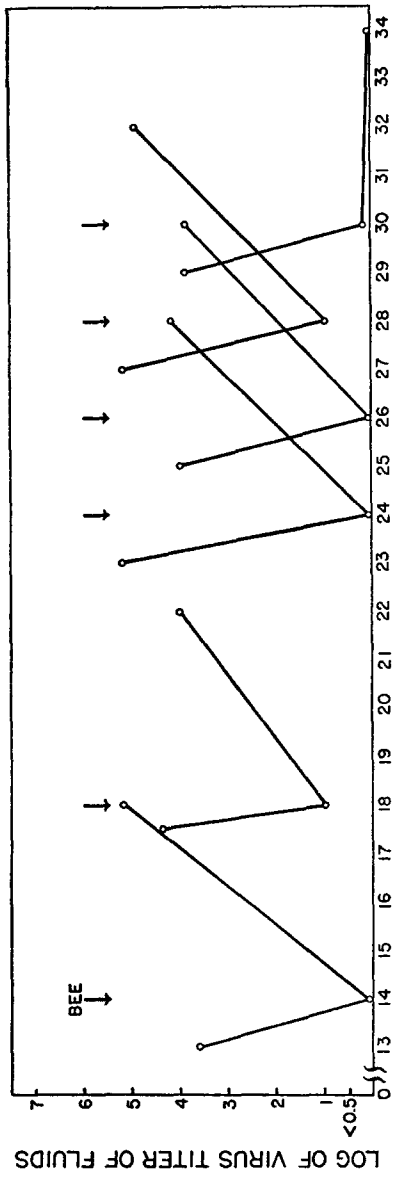

FIG. 1. Stimulation of growth of psittacosis virus in chick embryo tissues maintained in BSS with BEE 24 hours after infection.

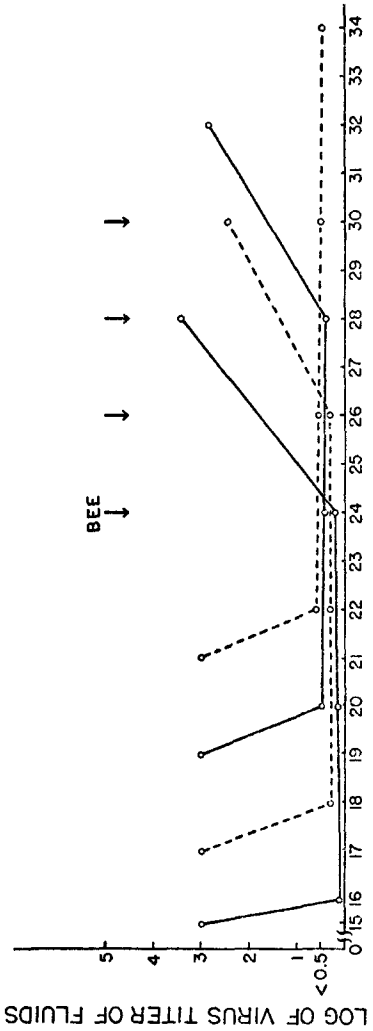

FIG. 2. Stimulation of growth of psittacosis virus in chick embryo tissues maintained in BSS with BEE 9 days after infection.

maintained in BSS for 13 days before infection with virus. It was of interest to determine whether or not this was a crucial time interval and of special importance to ascertain whether tissue lost its ability to respond to virus infection and subsequent stimulation of virus growth shortly after this time. In this experiment, the tissue was exhausted with BSS using changes at 4-day intervals for varying periods of time. The virus was then introduced and removed 24 hours later at which time BEE was added in fresh BSS with the results presented in Fig. 1. The data show that tissue maintained in BSS for as long as 27 days would still support virus growth if BEE was added on the 28th day. When the interval between virus infection and addition of BEE was increased to 9 days (Fig. 2) viral growth stimulation was observed in

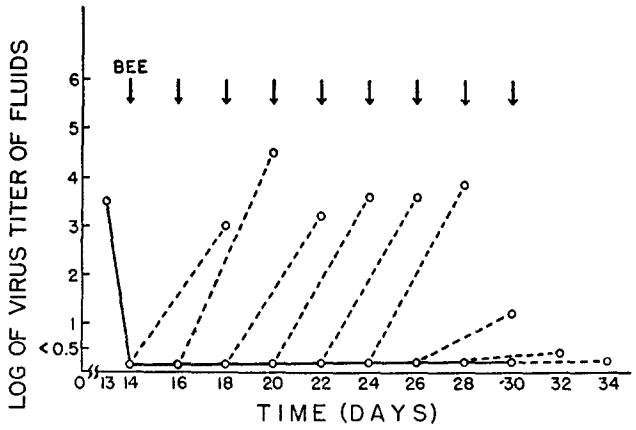

FIG. 3. Length of period of latent infection with psittacosis of chick embryo tissues maintained in BSS.

cultures infected on the 13th, 15th, 17th, and 19th day but not thereafter. It is of interest to note that again tissue maintained in BSS for more than 27 to 28 days would not support virus growth on the addition of BEE. Apparently the period of 28 days in BSS was the upper limit and, at this time, examination of the stained tissues showed very few recognizable cells. The period between 13 and 28 days of cultivation in BSS finds the tissue in a metabolic state that permits latent infection by psittacosis virus.

In another experiment, the BSS was changed every 2 days and, under these circumstances, when BEE was introduced 5 days after infection of tissues, virus growth was initiated in tissues infected on the 9th, 11th, and 13th day but not with tissues infected on the 15th day and stimulated with BEE on the 20th day. Thus the frequency of the changes of BSS was also a factor in the period of exhaustion of the tissues since more frequent changes apparently completely exhausted the tissues more rapidly.

Duration of the Latent Period of Viral Infection in Deficient Cells.—In the preceding experiments, the virus had been shown to remain dormant in these starved cells for as long as 9 days. It was decided to determine what the greatest period of latent viral infection was under these experimental conditions.

Exhausted cells were infected on the 13th day of cultivation in BSS with changes of medium every 4 days, the BEE was added at the intervals noted in Fig. 3 and viral growth determined 4 days later. Virus could remain in the tissues for as long as the 26th day, a period of 13 days as a latent infection. This was true even when the fluid changes after the 13th day were made only if the pH of the BSS dropped below 6.5 which meant less continuous exhaustion of the tissues than occurred with the regular schedule of BSS changes every 4 days. This period of latent infection could be extended to 15 days by using tissues exhausted before infection for only 11 days, though virus would

TABLE I

Stability of Psittacosis Virus at 36°C.

Time at 36°C.	Virus titer in BSS	Virus titer with heated tissue*
hrs.		
0	5.7*	5.9
24	<0.1	1.3
48	<0.1	<0.1
72	<0.1	<0.1

* Virus titers are expressed as log of the LD_{50} for embryonated eggs.

occasionally grow in these tissues during the initial 4-day period of observation. Under the conditions of these experiments, the latent virus infection of these cells was limited to the period from the 11th to the 26th day of exhaustion with BSS, since earlier, cells would support virus growth and after 26 to 28 days practically all the cells in the culture were dead.

Viral Invasion of Cells.—One of the possibilities that existed in the relationship of the virus to the exhausted cells was that virus was attached to the deficient cells and therefore was not washed away by the fluid changes, but had not entered the cells in the absence of factors present in BEE. This virus could again be released in the presence of BEE thus giving the appearance of growth. This possibility was investigated even though the fact that virus continued to grow over a period of days in the cells stimulated with BEE made it very unlikely. First, the stability of the virus was examined in BSS alone at 36°C. and in BSS in the presence of the same quantity of heat-killed tissues (60°C. for 2 hours) in flasks identical to those used in the experiments (Table I). It can be seen that in BSS alone or in the presence of dead cells, the virus rapidly loses its infectivity at 36°C. so that it must enter into some

close relationships with the living cells in the absence of BEE if it is not to become inactive on incubation.

To test the possibliity that BEE only enabled the virus to enter the cells following which it could proliferate freely, tissue cultures were exhausted for the standard 13-day period and BEE added to the medium at the time of infection (0 hours) and at 2 hours after infection and removed 2 hours later. Virus titrations on culture fluids were carried out at each of these periods and at 4 hours, and at 17 and 21 days, as indicated in Fig. 4. Three flasks were treated identically in each experimental group and a typical curve plotted for each group. It can be seen that exposure to BEE for 2-hour periods at the time of infection or 2 hours post infection did not cause the virus to multiply.

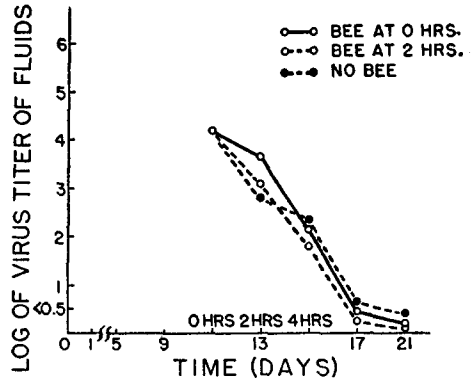

FIG. 4. Role of BEE in the entry of psittacosis virus into chick embryo cells maintained in BSS.

Therefore unless it required a period of longer than 2 hours to exert its effect, BEE did not cause viral growth as a result of enabling the virus to enter the exhausted cells which were refractory to infection in its absence. The earlier experiments (1) also presented evidence that psittacosis virus could infect the exhausted cells in the presence of BSS alone and the experiments of Heinmetz and Golub (10) indicated that psittacosis virus penetrates cells of the chorioallantoic membranes of embryonated eggs quite rapidly.

Nature of the Latent Viral Infection.—Since psittacosis virus could infect these exhausted cells and remain latent for as long as 2 weeks at which time it could be stimulated to grow by addition of BEE, experiments were designed to determine the state of the virus in the latently infected cells. Cells were exhausted with BSS for the standard period of 13 days in groups of four cultures each. At this time, the four cultures in each group were infected with virus. On the 14th day and at later intervals noted in Fig. 5, the BSS was removed and tested for virus and then BSS containing BEE added to 2 of

the 4 cultures and virus growth determined 4 days later. The tissues in the other two cultures were washed, ground, and tested for the presence of virus. As seen from the data, virus was present as a latent infection in these tissues as revealed by multiplication of virus on addition of BEE, but it could not be detected in the cells before addition of BEE. This was true as early as 24 hours after infection though virus stimulation occurred up to the 26th day. Infectious virus if present must have existed in minute amounts, since not only was no virus demonstrable in the eggs injected with the homogenized tissues, but on passage the yolk sacs of all but one set of the titration test eggs revealed no virus. Thus the virus existed in these tissues for as long as 13 days in a form which could not readily infect 7-day-old embryos but could

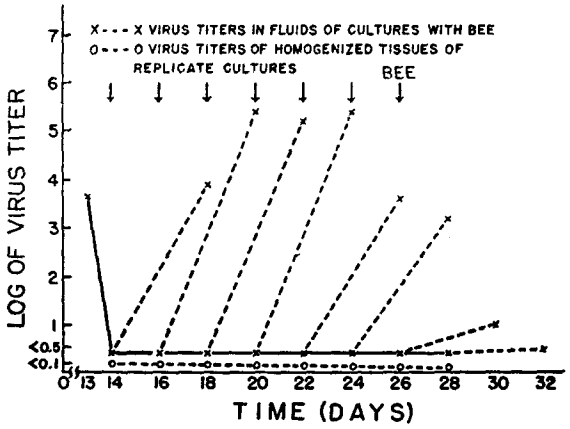

FIG. 5. Viral content of chick embryo tissues maintained in BSS and infected with psittacosis virus in a latent state.

be stimulated to multiply. When released from the cell, the virus would infect an identical series of chick embryos. It seems unlikely that this failure to infect was due to any inhibitor in the ground tissues, since on passage of the injected yolk sacs no virus was detected except in the one instance mentioned.

This virus thus existed in a non-infectious form by 24 hours after it came in contact with the cells and it was decided to investigate the state of the virus within the cells at earlier periods.

Tissues exhausted for 16 days in BSS were infected with $10^{3.5}$ LD_{50} of virus and after 4 hours the culture fluids were removed and tissues washed twice with an equal volume of BSS. At 4, 6, 8, 12, and 24 hours, the tissues of two cultures were homogenized and tested for presence of virus with secondary passage of yolk sacs harvested from the titration embryos. Small amounts of virus, *e.g.* $10^{0.2}$ to $10^{1.0}$ LD_{50}, could be detected in the tissues at 4 hours but none thereafter, though stimulation of the cultures with addition of BEE

would result in the growth of virus in the cells. This also indicated that virus was entering the cells in the absence of BEE and quite rapidly becoming non-infectious.

One question that could be raised was whether the homogenization of the tissues with alundum with a mortar and pestle might have denatured the virus, thus rendering it inactive. To investigate this possibility, one set of two cultures each of these experiments was disrupted by freezing and thawing and no virus was recovered.

In addition, an experiment was carried out to determine the lability of the virus under the process of homogenization and the results are presented in Table II. Virus inoculum ground with alundum or frozen and thawed three times showed no significant loss in titer. The small increase in the frozen and thawed yolk sac suspension may represent better dispersion of the virus as a

TABLE II

Stability of Psittacosis Virus to Homogenization

Treatment	Virus titer*
Original inoculum	2.9
Ground with alundum	2.9
Frozen and thawed	3.2

* Virus titers are expressed as log of the LD_{50} for embryonated eggs.

result of this treatment or the margin of error of the method used for virus titration.

DISCUSSION

Chick embryo tissues maintained in BSS with changes of medium every 4 days can be infected with psittacosis virus on the 13th day, but the virus does not multiply until BEE is added. The tissue will continue to show this property until the 28th day of cultivation in BSS after which time the cells all die. During this period of time the virus exists as a latent infection in the cells and it can remain in such a state for as long as 15 days. Not only does the virus not multiply, but it cannot readily be isolated from these tissues while in this latent state. It would appear that the virus invades these deficient cells and then enters upon a non-infectious phase in which state it remains until the BEE is added. A similar non-infectious phase has been described in the growth of the closely related meningopneumonitis virus in tissue culture (11).

This latent infection of nutritionally deficient chick embryo cells in tissue culture by psittacosis virus is an *in vitro* model for study of the problem of

latent infections with this agent which are a characteristic of its parasitic relationships with several hosts including the chick embryo, psittacine birds, mice, and man (1–4). Latent infections with recurrence of active disease in the host are usually attributed to an alteration in the immune response of the host which allows the infection to become active. The data presented in this study indicate that a change in the nutritional status of the cell may also be a factor in the transformation of a latent to an active infection in the case of psittacosis virus.

The finding that psittacosis virus exists in this latent infection in what seems to be a non-infectious phase suggests that the virus infecting such nutritionally deficient cells enters upon this phase, but is unable to proceed further in the absence of some factor not present in these starved cells. Furthermore, though it remains viable within the deficient cells for at least 15 days, in this state it cannot infect new susceptible cells such as those of the yolk sac of the chick embryo. This may indicate that certain viruses may exist in this non-infectious state in cells of their respective hosts for considerable periods of time and thus not be detectable until there is some alteration of the host cell permitting them to complete their cycle of multiplication. This possible mechanism raises many interesting speculations with reference to host-virus relationships in which silent infections are prominent, such as those characteristic of swine influenza virus in the earthworm (12, 13) and certain virus-induced tumors, as discussed in detail in a recent review (14).

The fact that psittacosis virus may enter these cells and remain in a non-infectious phase until the cells are altered suggests that other viruses may enter cells that are non-susceptible (*i.e.* do not permit the virus to complete its developmental cycle and thus be recognized upon release from the cell) and remain in them in the non-infectious phase. Such cells would be classified as completely resistant to infection by the virus even though it had invaded them.

These studies on the growth of psittacosis virus in chick embryo tissues cultivated with BSS are an unusual departure in investigations of the growth of viruses in tissue culture, which usually employ enriched media providing for maximal multiplication of the cells. Viral growth in rapidly growing cells is not comparable to the growth of viruses in most tissues of a host in which cellular multiplication is very slow. The infected cells *in vivo* are in a far different metabolic state than those maintained *in vitro*. It may be that the use of relatively deficient media for the cultivation of tissues provides *in vitro* a cell population much more like that existing in a susceptible host, and thus observations on viral growth in such cells may be more directly applicable to the problems of viral multiplication in the host.

The observation that the synthetic medium 199 of Parker (6) will induce

virus multiplication in these starved cells (1) has made it possible to elucidate the important nutritional factors in studies which will be reported subsequently.

SUMMARY

Chick embryo tissues maintained for from 11 to 28 days in Hanks's balanced salt solution lost their capacity to support the multiplication of psittacosis virus. The virus however infected such cells, as active multiplication of the virus occurred on the addition of beef embryo extract to this nutritionally poor medium at any period up to 28 days of cultivation in balanced salt solution. The virus remained in a state of latency for as long as 15 days in these starved cells in a non-infectious phase.

These results obtained in this *in vitro* model system for the investigation of latent infections of cells with psittacosis virus suggest that cell nutrition as well as an alteration in the immunological defenses of the host may prove to be an important factor in the activation of latent viral infections.

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