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Latent Viral Infection of Cells in Tissue Culture. VIII. Morphological Observations of Psittacosis Virus in L Cells.* (26245)

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With psittacosis virus, a latent infection can be established in cultures of L cells by maintaining them in a balanced salt solution (1) or a synthetic medium deficient in an amino acid(2) for 2 days prior to infection. When the virus infects these cells, it enters a latent phase which will persist in the cells for several days, during which time the addition of a medium consisting of water-soluble vitamins and amino acids will cause the virus to multiply. The objective of this study was to examine infected cells during this period of latency and early in the resumption of viral propagation in an attempt to determine whether any morphological structures occur which might be characteristic of this latent infection and how many cells are infected at various intervals of time after inoculation with virus.

Materials and methods. 1. *Media*: A lact-albumin-horse serum-yeast extract (LHYE) medium(1), Earle's balanced salt solution (BSS), and a synthetic medium (CM) consisting of salts, water-soluble vitamins and amino acids(2), and this synthetic medium minus its content of phenylalanine (PDM) were employed. 2. *Virus and viral titrations*: The 6BC 3cl strain of psittacosis virus, obtained from Dr. J. W. Moulder and maintained in the laboratory by allantoic fluid passage, was diluted 1/1000 in BSS or PDM and inoculated into monolayer cultures of L cells maintained in BSS or PDM. Fluids were changed daily and titrated by the single dilution method of Golub as modified by Dougherty *et al.*(3). On the appearance of a cytopathic effect, each cell layer was scraped off and the suspension was shaken with 0.5 g of 0.9 mm-diameter glass beads. Supernatant fluids were removed from each suspension and were dispensed in ampules and preserved at –70°C for inoculation of tissue cultures. All virus titrations were carried out by the modified titration method(3). 3. *Experimental protocol*: Petri dishes containing cover slips were seeded with L cells in LHYE medium and placed in a CO_2 incubator at 37°C. When a monolayer of cells had been established on the cover slips, the cell sheet was rinsed 3 times with warm BSS. L cells were maintained for 48 hours in BSS or PDM with daily fluid changes, the cultures were inoculated with a 1/1000 dilution of stock virus containing $10^{3.5}$ LD_{50} for infection, and culture fluids and lysed cells were tested for virus content as indicated. 4. *Light microscopy technics*: a) Methyl alcohol-fixed cells on cover slips were stained with May-

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Grunwald-Giemsa (MGG) stain as described by Jacobsen(4); b) Feulgen staining, as described by Chargaff and Davidson(5), was performed on methyl alcohol-fixed cells. Slides were counterstained with 0.1% light green to increase the contrast between Feulgen-negative and Feulgen-positive structures; c) Acridine orange staining, as described by Armstrong and Niven, was employed(6). A standard Zeiss microscope with a mercury lamp was used for observation. 5. *Electron microscopy*: Infected cells were sedimented by centrifugation and fixed at 0.5°C for 30 minutes in veronal acetate-buffered 1% osmium tetroxide containing 0.22 molar sucrose (7). Pellet fragments were dehydrated in ethyl alcohol and embedded in n-butyl-methacrylate. Sections were stained with lead hydroxide(8) and sandwiched with formvar (9). Electron micrographs were made with a Siemens Elmiskop 1 electron microscope.

Results. When L cells maintained for 2 days in each medium were exposed to the virus in each of the 3 different media, *i.e.*, LHYE, BSS, and PDM, for 8 hours, the virus was found to disappear from the culture fluids and to be detectable in small amounts in the cells after washing and lysis. However, tests of cells 24 hours after infection failed to reveal any infectious virus in the cells maintained in BSS or PDM, though virus reappeared within 24 hours after addition of the synthetic medium (CM) containing a complete complement of water-soluble vitamins and amino acids as has been previously reported(1,2). These results indicate that the virus infects cells maintained in BSS or PDM and enters a latent phase until cell nutrition is altered by supplying the missing vitamins and/or amino acids.

To determine the number of cells that were infected when exposed to virus, cover slips were removed from each preparation and cells were stained and examined for characteristic virus inclusions 24 hours after infection in LHYE and 24 hours after addition of the CM medium to those cells that had been infected in BSS or PDM. In all 3 cases, approximately 10% of the cells were found to contain characteristic virus inclusions.

An attempt was made to increase the number of cells infected (a) by reexposing the cells maintained in each of the media to fresh inocula of virus at 2-hour intervals during an 8-hour period at 37°C in which the cells were gently agitated, and (b) by reexposing the cells to fresh inocula of virus at 8-hour intervals for 24 hours at 4°C. With the LHYE medium, after 24 hours of incubation at 37°C and with BSS following the addition of the CM medium and 24 hours of incubation at 37°C, again only about 10% of the cells were found to be infected when examined in stained preparations. Thus agitation or repeated exposure of cells to the virus did not increase the proportion of cells susceptible to infection.

Morphological changes in infected cells were then studied, using the May-Grunwald-Giemsa and Feulgen stains as described. With the LHYE medium, inclusion bodies were first visible at 16 hours after infection and these became more intensely Feulgen-positive at 22 hours as the individual elementary bodies became visible. Such cells also revealed the characteristic collections of psittacosis elementary bodies when examined with the electron microscope. By 72 hours of incubation, 70% of the cells were infected, and when overt signs of cell destruction were visible at 96 hours, almost all cells contained inclusion bodies. When similar examinations of infected cells maintained in the BSS or PDM were made, no inclusion bodies were seen during the period of latent infection with light or electron microscopy. Within 22 hours after these cells were fed with the CM medium, inclusion bodies appeared within the cells and the infection spread to involve all the cells with destruction of the culture.

With the acridine orange stain, no changes were visible in infected cells until 24 hours after infection at which time discrete, round or oval orange-red masses appeared within the cytoplasm. These masses were the same as the inclusion bodies seen in the MGG stain, for when the acridine orange stain was removed and the cells were restained with MGG, the orange-red bodies appeared as typical viral inclusions. When the infected cells were treated with ribonuclease, the inclusion bodies underwent a striking change,

appearing as deep green homogeneous masses, whereas treatment with desoxyribonuclease resulted in an intensification of their red fluoresence. These results suggest the presence of DNA and RNA in the virus inclusions as indicated by the studies of Armstrong and Niven(6).

Discussion. Infection of L cells with psittacosis virus in a variety of media, including LHYE, CM or BSS, reveals that virus penetrates the cells in BSS alone and thus apparently only salts and glucose are essential for this step and the subsequent step of development of a latent phase of virus infection. No viral structure characteristic of this latent infection could be visualized by light or electronmicroscopy in the cells examined, but only 10% of these were shown to be infected, and examination of much larger numbers of cells might have revealed some virus structures such as the early virus forms described by Litwin in infections with feline pneumonitis virus(10). Subsequent virus multiplication will not occur in these cells in absence of water-soluble vitamins and certain amino acids(2). However, under the conditions of these experiments, only 10% of the cells could be infected at any one time with virus grown in the same cells, though with continued incubation and release of virus from the infected cells, infection spread to all cells and the culture was destroyed. This occurred equally well in a synthetic medium (CM) containing only salts, glucose, glutamine, water-soluble vitamins and amino acids or LHYE. Since all the cells eventually became susceptible to infection over a period of 96 hours, a cyclical change in cell susceptibility is indicated. It might be thought that this would be related to the mitotic cycle, but the CM medium does not support cell multiplication so that this possibility is eliminated. Some physiological state of the cell appears most likely to be the determining factor of its susceptibility to infection, and over a period of 96 hours the virus infects and destroys all cells as conditions compatible with infection develop in segments of the cell population. A similar situation was observed by Officer(11) in infection of Chang's strain of human liver cells with the 6BC strain of psittacosis virus where only 2% of the cells could be infected at any one time.

There is a possibility that an asynchronous infection might have been established in these cultures in which only part of the infected cells were at a stage, at 24 hours following addition of virus, in which visible viral inclusions were present, but morphological studies on growth of this and related viruses have provided no evidence for asynchronous infection of cells(10,12).

The histochemical studies indicate that the intracellular inclusion bodies of psittacosis virus contain RNA and DNA in confirmation of other observations(13,14).

Summary. With the 6BC strain of psittacosis virus only 10% of L cells could be infected *in vitro* in several culture media, even with repeated exposure to virus over a period of 8 hours at 37°C or 24 hours at 4°C. However, all cells became susceptible to infection during a period of 96 hours at 37°C, suggesting a sequential change in susceptibility. Using the balanced salt solution of Earle or a synthetic medium deficient in phenylalanine, the virus infected the cells and entered a latent phase as well as it did with a complex horse serum-lactalbumin hydrolysate-yeast extract medium, but virus growth did not occur until the missing amino acid or acids were supplied. No structures characteristic of virus were visible in these cells during the phase of latent infection when examined with the light or electron microscope.† Histochemical studies of the growth of virus in L cells revealed that the virus inclusion body contained RNA and DNA.

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Relative Effectiveness of Phenothiazine Tranquilizing Drugs Causing Release of MSH.*† (26246)

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The ataraxic drug meprobamate (Miltown, Equanil) has been found to cause darkening of the light-adapted frog. This reaction does not occur in the hypophysectomized animal (1). Chlorpromazine (Thorazine), methoxypromazine (Tentone), and reserpine also have been found to cause melanophore dispersion in the normal frog and in the sand dab, *Lophosetta maculata.* On the other hand, azacyclonol (Frenquel) and oxanamide (Quiactin) have been found to be ineffective (2).

This paper is a report of the influence of 10 phenothiazine derivatives now used in medical practice on the pituitary melanocyte system of the frog, *Rana pipiens.* Marked differences in milligram potency of the drugs causing release of melanocyte stimulating hormone (MSH) from the hypophysis were observed.

Methods. Three hundred and thirty-one medium-sized *Rana pipiens* of both sexes were used in determination of the minimal effective doses (MED) of the drugs. The effects on 10 hypophysectomized frogs were also observed. Frogs were weighed and placed singly in 1000 ml beakers containing 200 ml of tap water. To provide a white background, beakers were wrapped with white cloth. Beakers containing the frogs were placed in white enamel pans under continual illumination from a pair of 4 foot, 40 watt fluorescent tubes mounted in a fixture 20 inches above the pans. Frogs were permitted to light-adapt overnight prior to injection. Equal mg% doses (mg of drug per 100 g of frog) of a drug were injected (0.1-1.3 ml) intraperitoneally into a series of 3 to 5 light-adapted frogs. Doses ranged from 0.01 to 8.0 mg%. The extent of melanophore dispersion was recorded from microscopic observation of the web of the foot at 2-5 hour intervals for 36 hours following injection. The melanophore index of Hogben and Slome(3) was used. Twenty-seven control frogs injected with carrier solutions and 5 uninjected controls were also run.

The mean melanophore response to each dose was calculated by averaging the single maximal responses of all frogs(3-11) injected with that dose. The MED was defined as the minimal dose eliciting a mean melanophore response of 3.0 on the Hogben and Slome index and was obtained by interpolating from a dose-response curve.

Hypophysectomy was performed according to the procedure of Hogben(4) with additional use of an ice bath to reduce blood flow. Complete removal of the hypophysis

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