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Induction of Prolonged Latency in Psittacosis Infected Cells by Aminopterin.* (27947)

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The phenomenon of virus latency was surveyed thoroughly in 1957(1). Viruses were not detectable when inoculated into newborn mice (lymphocytic choriomeningitis), or into clones of cells which survived the cytolysis of earlier viral infection (Newcastle disease virus), or into lines of cells which were visibly unaltered while producing virus (mumps). Mature psittacosis virus was not detected in cultures of nutritionally depleted "L" cells; however, infectious virus emerged from such cultures after treatment with amino acids and water soluble vitamins(2). Viral elements survived in such nutrient-depleted cultures for 8 days.

Psittacosis virus has been studied extensively by acridine orange fluorochrome staining of infected tissue cultures(3,4). This technic provided a visible cytochemical record of viral activity, as influenced by antimetabolites which modified the normal sequence of biosynthetic reactions associated with replication. Each virus particle maturated through a clearly defined sequence of cytochemical changes which reflected nucleic acid metabolism of the virus. The infective DNA virus particle entered the cytoplasm, became surrounded by a matrix of RNA, and from the resulting inclusion increased numbers of infectious DNA-staining virus particles emerged. The changes observed during this predictable virus maturation sequence reflected accurately the infectious status of the virus. During the period in which the virus inclusion was observed as a mass of RNA-staining material ("red ball"), it was not infectious and it represented an eclipse or latent stage of the virus.

Addition of antimetabolite drugs to the culture medium of infected cells interrupted the replication sequence of the virus; this effect was related to the stage of replication at

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which drugs were added. If fluorinated pyrimidines and virus were added simultaneously to tissue cells, no evidence of replication was seen; however, if addition of the drug was deferred for 18 hours after infection, visible accumulations of abnormal virus-related RNA and DNA appeared in the cells (5). The drugs had little direct effect on mature extracellular virus.

Psittacosis virus requires folic acid for replication, and the effect of antifolic drugs can be reversed by folinic acid (citrovorum factor)(6). Tissue cells infected with the virus contain increased amounts of folic acid or a related compound(7). The source of this increased factor, virus or cell, is uncertain. Colon has demonstrated folic acid-related agents in the virus(8). When aminopterin (AP) was added to the cells simultaneously with psittacosis or trachoma viruses, replication of virus material was stopped at the "red ball" stage(9). The AP-effect on cell and virus was reversed to some extent by folic acid; however, thymidine reversed the damage only to the host cell, not the virus. The sensitivity of virus to AP disappeared at 15 hours after onset of infection.

Continued studies of AP-psittacosis virus interaction have revealed: (a) that the virus produces or induces in the cell a factor (folinic acid-like) which reverses the inhibiting effect shown by AP on early stages of viral replication; and (b) that a prolonged state of viral latency can be induced in cells by AP, from which infectious virus can be recalled following addition of folinic acid to the culture medium.

Methods and materials. The methodology of the acridine orange fluorochrome procedure with psittacosis virus in tissue cell systems has been described(3,4). Psittacosis virus (TT strain), propagated in the chorioallantoic fluid of embryonated eggs, was pooled, ampouled and stored at −40°. A cell line (McCoy), derived from human synovial tissue was propagated as monolayers on coverslips in Leighton tubes. The medium (NF) consisted of Mixture 199 plus 10% heat-inactivated calf serum, 0.5% lactalbumin hydrolysate, and streptomycin 0.1 mg/ml. The stock virus was titrated on monolayers of McCoy cells by an adaptation(10) of the infected cell technic described by Weiss and Huang(11). Stock virus inoculum was diluted in NF so that at least 90% of the cells showed infection at 24 hours. Where indicated, AP† (25 μg/ml) and thymidine† (100 μg/ml) was added to the NF. All media were sterilized by filtration.

The following experiments were studied according to the general technics described above: A. NF containing AP was added to cell cultures at 0 or at 20 hours after onset of infection. Control cultures contained the NF described above. At 48 hours following onset of infection, the NF was replaced by drug-free NF, each culture was frozen twice, and titrated for virus particles in cultures of fresh McCoy cells(10).

B. TT virus and NF was added to cell cultures. Twenty hours later the cell cultures were suspended in NF by agitation with syringe and needle, and one-tenth of it was added to fresh cell monolayers with NF containing AP. At 12-hour intervals the cells on individual coverslips were examined microscopically and by counting infected cells per ml(9), extension of infection from the cell inoculum into the fresh cells was determined.

C. McCoy cells in a series of culture tubes were inoculated with TT virus, diluted to infect 5% of the cells. Twenty hours after onset of infection, new NF containing AP was added to the infected cultures. The cells in individual tubes were examined thereafter by the fluorochrome procedure to determine if the cells that were not infected by the initial inoculum were infected by repeated cycles of propagating virus, despite the presence of AP.

D. TT virus was added to cells at 0 time and supplemented with NF, AP, and thymidine. This medium was replenished at 4-day intervals, and sample cultures were examined microscopically and assayed for viable

† Aminopterin, C Grade and Thymidine, A Grade —Calif. Corp. for Biochem. Research, Los Angeles.

TABLE I.

Drugs added to nutrient medium at time 0*			
Aminopterin	Folinic acid	Microscopic appearance†	Assay virus particles/ml‡
0	0	mature	1.5×10^6
25	0	"red balls"	negative
25	.003	"	"
25	.01	mature	2.4×10^4
25	.03	"	5.5×10^4
25	.1	"	2.9×10^5
25	.3	"	8.4×10^5
25	1.0	"	2.2×10^6

* μg/ml.
† By acridine orange stain and fluorescence microscopy.
‡ At 48 hr after onset of infection.

virus. After 14 and 28 days, folinic acid‡ was added in graded doses to the NF of selected cell cultures; 48 hours later each culture was assayed for infectious virus according to the procedure described above.

Results. TT virus completed a replication cycle in McCoy cells within 48 hours, during which single RNA-staining "red ball" inclusions increased in number and maturated to DNA-staining infectious virus material. Addition of AP to the cell-virus combination at time 0 interrupted the development of the virus inclusion at the single RNA-staining ("red ball") stage. Addition of AP later than 20 hours after onset of infection resulted in damage to the host cell, but not to the virus.

The transfer of suspended cells containing 20-hour replicating virus to fresh cell cultures with AP in the medium was accompanied by a factor which enabled virus to complete maturation. Through repeated cycles of replication, the mature virus infected all the cells in the culture. Cell cultures, with 5% of the cells infected, were treated at 20 hours with AP; and during the next 3 days mature virus was produced in all of the remaining cells in the culture.

Folinic acid, in doses as small as 0.01 μg reversed the inhibitory effect of 25 μg AP on virus infected cells (Table I). In other experiments, a constant level of 0.05 μg folinic acid reversed the effect of up to 100 μg AP (Table II). AP-inactivated TT virus was observed as single "red ball" inclusions in the cytoplasm of cells. The inclusions were difficult to detect by day 12 of induced latency and were indistinct on day 14. Thymidine (100 μg) spared the cells the toxicity associated with AP; they appeared "healthy," and mitotic figures were observed. While the AP-treated cultures were not infectious when assayed on days 2, 4, 8, 12, and 16, addition of folinic acid (0.15 μg) and fresh NF on day 14 resulted in rapid resumption of virus propagation and maturation within 48 hours (Table III). Virus was recalled by folinic acid at 28 days after onset of latency. The effect of either leucovorin or folinic acid was the same.

Discussion. Aminopterin inhibits dihydrofolic reductase, thereby preventing conversion of dihydrofolic acid to tetrahydrofolic

‡ Folinic acid, calcium salt—Sigma Chemical Co., St. Louis, Mo.

TABLE II.

Drugs added to nutrient medium at time 0*			
Aminopterin	Folinic acid	Microscopic appearance†	Assay virus particles/ml‡
0	0	mature	2.8×10^6
25	0	"red balls"	negative
25	.05	mature	1.5×10^5
50	.05	"	7.8×10^4
100	.05	"	1.9×10^4
200	.05	"red balls"	negative

* μg/ml.
† By acridine orange stain and fluorescence microscopy.
‡ At 48 hr after onset of infection.

TABLE III. Revival of Aminopterin-Treated Psittacosis Virus in McCoy Tissue Cells.

Days after treatment*	Microscopic examination for inclusions†	Virus assay for virus/ml	Assay after FOA‡/ml
Control§	mature	1.3×10^6	
2	"red balls"	negative	
4	"	"	
8	"	"	
12	indistinct	"	
14	"	"	7.4×10^5

* Nutrient medium plus aminopterin, 25 μg/ml, and thymidine, 100 μg/ml.
† By acridine orange stain and fluorescence microscopy.
‡ At 48 hr after addition of folinic acid, 1 μg/ml. Exemplifies 4 trials with the above protocol.
§ No treatment.

acid(12). Cytotoxicity is attributed to the resulting deficiency. As described here and previously(4,7), TT virus is sensitive to AP at early stages of replication and indifferent to it at the later stages. Either a specific enzyme (viral dihydrofolic reductase) has been formed or induced by the 20-hour replicating virus, or by that time information needed for that organizational level of virus has already been transferred to the cell by the virus. In either case only minimal amounts of this maturation factor are needed for production of relatively large amounts of virus. The enzyme nature of this factor is now being examined. The benefit from folinic acid is apparent in virus and cell; whereas, the factor associated with 20-hour replicating virus benefits the virus primarily. TT virus propagates in the presence of cytotoxic amounts of AP, thereby indicating that the metabolic pathway for viral thymidine and DNA differs from that of the cell. The virus cannot utilize preformed thymidine, but the cell can.

Latency of psittacosis virus has been demonstrated by Morgan *et al.*(2,13,14) in nutritionally depleted chick embryo explants and in "L" cells. The virus survived at a declining level in "L" cells for 8 days. In the latency described by us survival was prolonged and was induced by a specific agent, AP, in cells which were otherwise normal in appearance. The interrupted replication involved an accessory factor, and was not the result of a lethal biosynthetic process.

The genetic code of TT virus survived the AP-induced immobilization, and rested eventually as an indistinct morphological entity, a provirus, for at least 4 weeks. Following addition of folinic acid to the culture medium, the virus resumed its maturation from the point at which it had been stopped. On the basis of the visible condition of the cells and the flourishing virus evoked by folinic acid, the state of viral latency could have been prolonged beyond the 4-week test period. The progeny of AP-interrupted virus was unchanged in its sensitivity to AP.

Summary. Psittacosis virus was sensitive to aminopterin during the early stages of replication in tissue cultured cells. After hour 20 of virus infection, a factor appeared in the infected cells which reversed the effect of aminopterin on virus. Folinic acid reversed the inhibitory effect of aminopterin on virus and cells. Psittacosis virus, inhibited in tissue culture cells by medium containing aminopterin, remained latent for at least 4 weeks, and resumed replication after addition of folinic acid to the culture medium.

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