Factors Related to the Growth of Psittacosis Agent (Strain 6BC). VI. Host Cell Purine Nucleotide Pyrophosphorylases.* (27482)

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Recent investigations of the agents of the psittacosis group have shown that these organisms have synthetic capacities independent of the host cell(1,2,3,4) as well as many other properties which distinguish them from viruses(5). However, they are obligate intracellular parasites so the question arises as to the degree of their independence of the synthetic activities of the host cell. These viruses grow well in the mouse fibroblast cell line L of Earle(2) and since the purine analogs, 2, 6 diaminopurine and 8-azaguanine have been shown to inhibit intracellular growth of the agent(6), the question arises as to whether these analogs act directly on synthetic activities of the agent or indirectly by interfering with some metabolic processes of the host cell which are essential to the growth of the agent. Since cultures of L cells are readily derived which are resistant to the cell inhibitory action of these 2 analogs, the growth of psittacosis agent in resistant cells can be studied in presence and absence of the analogs. This cellular resistance has been related to the reduction or disappearance of the capacity of L cells and other cells(7,8,9, 10) to convert the analogs to their respective nucleotides, *e.g.*, resistance to 2, 6 diaminopurine (DP) is due to the selection of a mutant lacking adenosine 5'-phosphate pyrophosphorylase (AMP - pyrophosphorylase) and that to 8-azaguanine (AZA) by the development of mutants which lack guanosine 5'-phosphate pyrophosphorylase (GMP-pyrophosphorylase). In such analog resistant cells, the growth of the agent should be resistant to the action of the analog if the microorganism is dependent on the host cell for enzymatic conversion of the purine analogs to nucleotides or other complexes but if it possesses its own enzymatic mechanism, the agent should be inhibited by the respective analogs.

The growth of psittacosis agent in normal L cells and L cells resistant to the inhibitory effects of 2, 6 diaminopurine or of 8-azaguanine was studied to examine this question.

Materials and methods. The mouse fibroblast cell line L-929 was obtained from Dr. Harry Eagle, Nat. Inst. of Health, and was cultivated in plastic culture flasks (Falcon) using a modified Eagle(11) medium (MM) containing 4× the amino acids and glutamine originally described. Medium (GM) for growth of cells was prepared by adding 20% calf serum to the MM. All media contained 50 micrograms (γ) of streptomycin per ml. To obtain analog resistant cells, monolayer cultures were exposed to a toxic concentration of the analogs in GM until many of the cells had disintegrated. The analog was then removed and fresh GM added to permit the remaining cells to establish a new monolayer and the process repeated using a higher concentration of the analog.

The resistance of these cells was compared to that of normal cells by scratching a reference point on a plastic culture flask on which a cell inoculum had been deposited. A standard area was photographed with a microscope and camera at 20× and the cells counted. The same areas were photographed repeatedly in control flasks and in flasks to which GM containing various quantities of the analogs had been added and cells counted to determine the effect of purine analogs on cell growth.

Cells were inoculated into culture flasks in GM and when a monolayer formed were changed to MM and infected for studies of growth of the microorganism. The egg adapted strain (6BC) of the psittacosis agent was used(2) and the growth of the microorganism in a monolayer culture of cells in MM following inoculation of about 200 LD_{50} was

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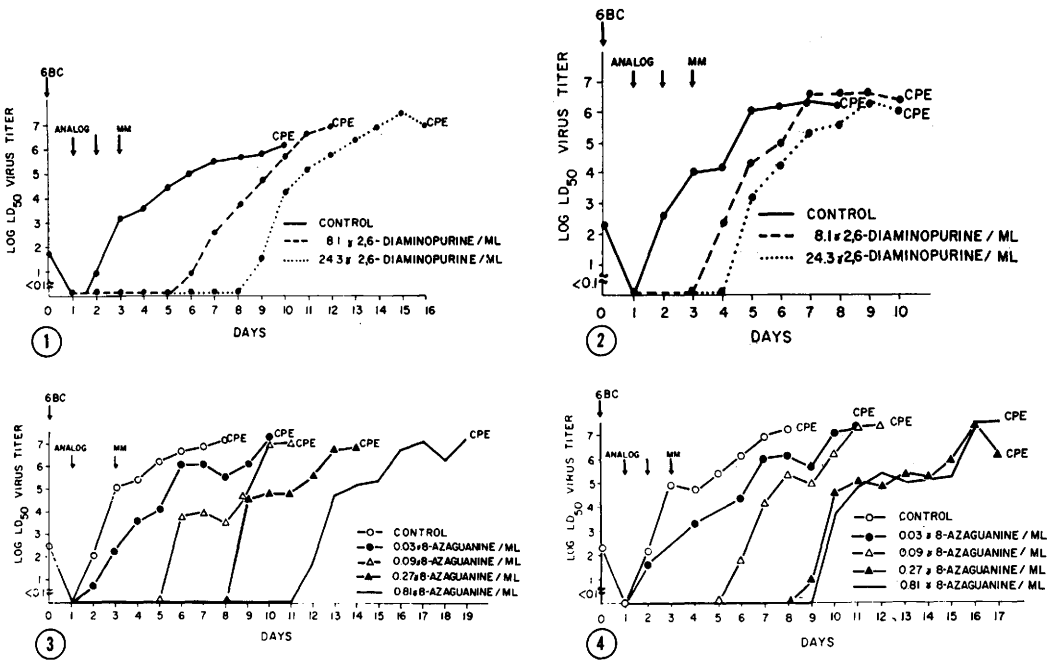

FIG. 1. Effect of DP on growth of psittacosis agent (strain 6BC) in analog sensitive L cells.
FIG. 2. Effect of DP on growth of psittacosis agent in analog resistant L cells.
FIG. 3. Effect of AZA on growth of psittacosis agent in analog sensitive cells.
FIG. 4. Effect of AZA on growth of psittacosis agent in analog resistant cells.

determined by removing an aliquot of the supernatant medium daily and estimating its content of agent by the embryonated egg titration method of Golub as described previously(6). Media containing various amounts of analogs were added to the cultures at intervals and then removed and replaced with MM to test the effect of the analogs on multiplication of psittacosis agent in comparison with cultures containing no analog.

Results. Development of resistant cell mutants. By repeatedly exposing L cells to 10 γ per ml and then to 100 γ per ml of 2,6 diaminopurine (DP), cells were obtained whose growth was not affected by presence of 72 γ per ml of DP, a concentration which completely inhibited the growth of sensitive cells. This represented at least a 10× increase in tolerance of the cells to DP. With 8-azaguanine (AZA), repeated exposure of the culture to 2 γ and then 5 γ per ml resulted in the selection of mutants which grew in 0.87 γ per ml of AZA. This prevented any growth of sensitive cells. These resistant cells showed 10× the tolerance of sensitive cells to AZA.

Effect of analogs on multiplication of psittacosis agent in analog resistant and sensitive cells. Cultures of DP sensitive and resistant cells were infected with the agent and fresh analog-containing medium placed on the cultures at 1 and 2 days following which it was replaced with MM, and the agent present in the extracellular fluids measured as shown in Fig. 1 and 2. At levels of 8.1 and 24.3 γ per ml the DP markedly delayed the appearance of virus in the extracellular fluids (4 and 7 days respectively) and the time at which a visible cytopathic effect (CPE) appeared. With resistant cells, only a slight delay in release of virus from the cells (2 and 3 days) occurred indicating that the DP was much less effective for inhibition of psittacosis agent growth in a population of DP resistant cells. Even with consecutive replacement of the DP (8.1 γ per ml) containing medium, daily for 5 days, the agent still appeared in the extracellular fluids by the 5th day thus

confirming the growth of fully infectious psittacosis agent in resistant cells at concentrations of DP which completely suppressed growth in sensitive cells as long as it was present in the medium (Fig. 1). Furthermore, if the agent was allowed to grow for 4 days in the resistant cells, repeated (3×) applications of media containing 8.1 γ DP per ml had no significant effect on the subsequent growth pattern or production of CPE.

With AZA as little as 0.09 γ per ml would produce a significant suppression of growth of psittacosis agent when applied to the cultures of either analog sensitive or resistant cells at 1 and 2 days after infection with complete changes of medium (Fig. 3, 4). No difference between the effect of the analog on the agent in sensitive and resistant cells was seen until 0.81 γ per ml was used (Fig. 4). Daily examination of the cultures of cells with this dose of analog revealed that most of the sensitive cells showed toxic effects and many disintegrated so that fewer cells were available to support growth of the agent. Thus the observed difference in effectiveness of growth inhibition by 0.81 γ AZA per ml in susceptible and resistant cells was an artifact due to the smaller number of cells available for infection in the cultures of sensitive cells.

Furthermore, repeated changes with media containing 0.09 γ per ml on resistant cells for 5 successive days followed by washing and MM caused complete inhibition of agent growth for 9 days. It is apparent that the AZA resistant cell is not providing effective protection against the inhibitory action of the AZA on growth of psittacosis agent in contrast to the observations with the DP resistant cells. This failure was again demonstrated by the fact that if cells were infected and multiplication of the agent allowed to proceed for 2 days before change to MM containing 0.81 γ per ml of AZA, growth of the agent was inhibited and it did not appear again in the culture fluids until 6 days after the analog was removed and 2 daily changes of fresh MM were used to remove traces of AZA. Thus, the agent remains sensitive to the growth inhibitory action of AZA in cells with a 10× increase in tolerance to the analog.

Discussion. The differences in the action of DP and AZA on the growth of psittacosis agent in strains of L cells resistant to these 2 analogs are striking and knowledge concerning the mechanism of the host cell resistance to the analog has interesting implications regarding the metabolic activities of this agent. Since the agent whose growth is inhibited by DP in L cells sensitive to this analog was able to grow in an almost normal pattern in analog resistant cells in the presence of DP, some change must have taken place in the intracellular environment which makes such growth possible. The resistant cell is known to lack the AMP-pyrophosphorylase which renders it incapable of incorporating adenine or DP, and thus the analog cannot be converted to some toxic anabolite (*e.g.*, nucleotide) which exerts an inhibitory effect on growth of the cell and growth of the psittacosis agent in the cell(7). The effect of DP on growth of the agent is therefore related to the level of host-cell AMP-pyrophosphorylase, and the microorganism obviously cannot carry out independent anabolism of DP or the toxic effect of this analog would still be observed in the analog resistant cell. Thus, the psittacosis agent appears host-cell dependent for metabolic processes involving DP which are related to AMP-pyrophosphorylase.

In sharp contrast, the continuing sensitivity of the psittacosis agent to the growth inhibitory effect of AZA in analog resistant cells indicates that the agent is not dependent on the cellular metabolic activities that relate to the toxic effects of AZA. Since the cellular resistance has been identified as being a result of lack of GMP-pyrophosphorylase, it is apparent that the psittacosis agent appears to be independent of the host-cell metabolic activities involving AZA which are related to this enzyme. In fact, preliminary observations on the AZA resistant L cells infected with the agent support this concept since infected cells showed early toxic changes in presence of 0.81 γ per ml of AZA before any CPE was produced by growth of the agent whereas uninfected resistant cells showed no toxic changes in the presence of analog. This suggests that the presence of the psittacosis agent provided an enzymatic bypass for the

metabolic block to the toxic action of AZA on the resistant cells which is due to their lack of GMP-pyrophosphorylase(7,8,10). It is conceivable therefore that the psittacosis agent is capable of carrying out independently the conversion of AZA to a toxic component, *e.g.*, a toxic nucleotide(7,10). Recent experiments(12) demonstrating the development of resistance to 5-fluorouracil by a strain of psittacosis agent support the concept that this microorganism possesses specific enzyme systems relating to the biosynthesis of its nucleic acids which are, at least in part, different from those of the host cell itself.

Thus, the psittacosis agent appears to exhibit a host-cell dependence for metabolic functions related to the action of DP and AMP-pyrophosphorylase but not for those related to AZA and GMP-pyrophosphorylase. In the first instance in which it exhibits dependence on host-cell metabolism, this agent resembles a virus but in the second where it shows independence it does not.

This study demonstrates the value of the use of cells with known deficiencies in enzymatic functions in elucidation of the role of cellular enzymes in the growth of intracellular parasites and should prove to have wide applications in investigations of such agents including viruses.

Summary. The 6BC strain of psittacosis agent is sensitive to the growth inhibitory effects of 2, 6 diaminopurine (DP) and 8-azaguanine (AZA) when infecting normal L cells. However, when L-cell mutants resistant to the analogs are derived and are infected with the psittacosis agent, its growth is not effectively inhibited by addition of DP to infected cultures of DP resistant cells. In contrast, addition of AZA to infected cultures of AZA resistant cells is as effective in inhibition of growth of psittacosis agent as it is when placed on infected, normal L-cells. The implications regarding the dependence of this agent on the metabolic functions of the host cell are discussed and the usefulness of this technic for investigation of the specific cellular enzymatic dependence of intracellular parasites is considered.

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