Primaquine Diphosphate: Inhibition of Newcastle Disease Virus Replication

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The response of Newcastle disease virus replication to primaquine, an antimalarial drug, was examined in chicken embryo cells (CEC). Virus-induced hemadsorption was completely inhibited by 250 µg of primaquine per ml. At lower concentrations, hemadsorption inhibition was dose dependent. Primaquine retarded virus-induced redistribution of receptor sites on the host cell plasma membrane as shown by the failure of infected, drug-treated CEC to be agglutinated with concanavalin A. The production of infectious progeny virus was substantially inhibited by the addition of primaquine at various times postinfection. When the drug was added early in the virus replication cycle, viral ribonucleic acid (RNA) synthesis was inhibited; however, when the drug was added late in the cycle, stimulation of RNA synthesis was observed. Primaquine was also shown to retard the incorporation of [^{14}C]amino acids into proteins of virus-infected CEC. We suggest that the major role of primaquine is inhibition of protein synthesis; this results in changes in: hemadsorption, redistribution of lectin receptors, release of progeny, and virus-induced RNA synthesis.

The antimalarial aminoquinolines are divided into two subgroups: the 4-aminoquinolines, which appear to act as inhibitors of nucleic acid synthesis (17), and the 8-aminoquinolines, whose mechanism of antimalarial action has not as yet been clearly defined. The problem in defining the mode of action of the 8-aminoquinolines may be that their antimalarial action lies in the intermediates formed during metabolic transformations in the host (18).

Primaquine (Fig. 1) is an 8-aminoquinoline which was introduced as a curative antimalarial agent in 1950 (9); it is used extensively against the exoerythrocytic stage of malaria. Investigators have reported that primaquine binds to nucleic acids (23), inhibits protein synthesis (16), affects lipid synthesis (25), and interacts with biological membranes (4). Since these phenomena are also involved at various stages during the replication of enveloped vertebrate viruses, we studied the effects of primaquine on the replication cycle of Newcastle disease virus (NDV).

MATERIALS AND METHODS

NDV, strain Cal. RO, was obtained from the NDV repository of the University of Wisconsin. Procedures for the propagation, concentration and purification of stock virus, cultivation of primary chicken embryo cells (CEC), and plaque assay were as previously reported (6). A quantitative hemadsorption procedure (6) was modified by using fresh guinea pig erythrocytes that were aldehyde fixed before use (2).

For determining the agglutination of virus-infected CEC by concanavalin A (con A), the culture medium was first removed by aspiration and the monolayers were washed twice with calcium- and magnesium-free saline (CMF-S: 1.37 M NaCl, 50 mM KCl, 55 mM glucose, and 40 mM NaHCO$_3$). The cells were detached from the culture vessel with 0.2% disodium ethylendiaminetetraacetic acid (EDTA), washed with CMF-S, and resedimented. The pellet was suspended in CMF-S and filtered through two layers of gauze. The filtrate was then adjusted to a cell density of approximately 2.0×10^6 cells/ml and 0.5 ml was added to each tube (10-mm diameter). Con A was dissolved in CMF-S to a concentration of 1.0 mg/ml and 0.5 ml was added to each cell suspension. After incubation for 15 min at room temperature, cell agglutination was observed under a microscope by using a hanging-drop slide.
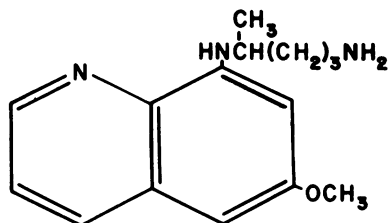
For measurement of ribonucleic acid (RNA) syn-

FIG. 1. *Structure of primaquine.*

thesis, cells were pulse-labeled by the addition of [5-³H]uridine (28 Ci/mmol; Schwarz Bioresearch Inc., Orangeburg, N.Y.) at a concentration of 0.5 μCi/ml for a period of 15 min. The medium was then decanted and 2.0 ml of cold 5% trichloroacetic acid was added to each culture and allowed to stand overnight at 4 C. After the acid-insoluble material was washed three times with cold 5% trichloroacetic acid, the precipitate was suspended in 2.0 ml of 5% trichloroacetic acid and heated for 1 h at 90 C. Samples of the hydrolysate were used to measure radioactivity. A 0.2-ml portion of the hydrolysate was diluted with 10 ml of scintillation fluid (cocktail D, Beckman; 100.0 g of napthalene, 5.0 g of 2,5-diphenyloxazole, brought to 1 liter with dioxane) and counted. RNA content was determined by the orcinol method of Borland and Mahy (3); yeast RNA was used as a standard.

For measurement of protein synthesis, cells were pulse-labeled with a hydrolysate of ¹⁴C-labeled algal protein (0.2 μCi/ml; Schwarz Bioresearch Inc., Orangeburg, N.Y.) for 30 min. After 30 min, the medium was decanted and 2.0 ml of cold 6% trichloroacetic acid was added to each culture. The cells were suspended by scraping with a rubber policeman. The acid-insoluble material was first washed in 95% ethyl alcohol and then in ether. The residue was hydrolyzed overnight in 0.3 N KOH at room temperature. The protein content was determined by the method of Lowry (14); bovine serum albumin was used as a standard.

RESULTS

Primaquine was tested for cytotoxic effects on CEC. Cells were seeded into 12-oz (about 360 ml) prescription bottles containing various concentrations of the drug. At concentrations up to and including 100 μg of primaquine per ml, the cells attached and grew to confluency in 24 h. In other experiments, monolayered cells were exposed to various concentrations of primaquine for 8 h. The monolayers were then washed, fresh culture medium was added, and the cells were incubated for an additional 24 h. When the medium replacement technique was used, no signs of cell damage could be observed by microscope examination at primaquine levels of up to 250 μg/ml.

Hemadsorption (HAD). Viral HAD is the attachment of erythrocytes to the host cell plasma membrane. This attachment is mediated by virus hemagglutinin present either as intact virions and/or as virus-modified cell membrane. This rather simple assay was used to determine the effects of primaquine on the accumulation of virus hemagglutinin at the cell surface. Monolayered CEC were infected at an input multiplicity of 10 for 1 h at 39 C. Unattached virions were removed by washing with fresh culture medium. The cultures were then incubated for 1 h at 39 C to allow for virus

penetration before addition of the drug. There was an initial decrease in the percentage of hemadsorbing cells during the first hour postinfection (Fig. 2) presumably due to the uptake of the virus. A residual value of 45% at 1 h probably constitutes particles which have remained at the cell surface or those which have undergone fusion with the cell membrane. The increase at 2 h postinfection in the untreated cells may be a consequence of the resurfacing of intact virions and/or the resurfacing of virus membrane components from uncoated particles (8). Primaquine, when added at 1 h postinfection, had a bimodal effect on this resurfacing phenomenon. At a high concentration (250 μg/ml), the drug inhibited the resurfacing of parental virus components. The lowest concentration of primaquine tested (0.25 μg/ml) caused a consistent increase in the percentage of hemadsorbing cells at 2 h postinfection. Marked increases in HAD between 3 and 4 h postinfection, seen in the untreated cultures, parallel the time when virus maturation occurs. These increases were not seen in cultures treated with 250 μg of primaquine per ml. At the lower concentrations tested, inhibition was found to be dose dependent.

Production of infectious virus. The production of extracellular virus was partially inhibited when primaquine (50 μg/ml) was added at any time up to and including 7 h postinfection (Fig. 3). Inhibition of virus production ranged from 92% when the drug was added at 0 time to
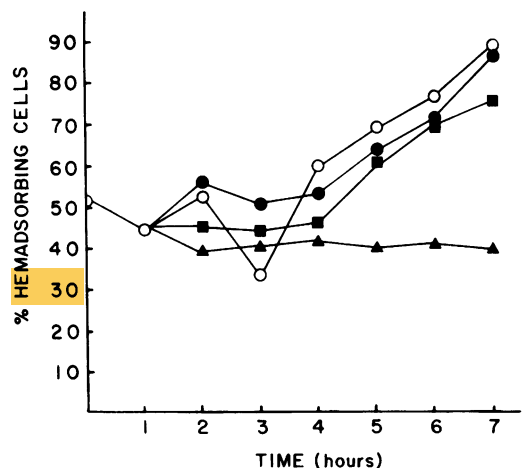

FIG. 2. *Effect of primaquine on viral hemadsorption. Primaquine was added to infected cultures of CEC 1 h postinfection at concentrations of 0.25 (●), 25.0 (■), or 250 μg/ml (▲); no primaquine (○). From a random count of 200 cells the percent exhibiting positive hemadsorption was recorded.*
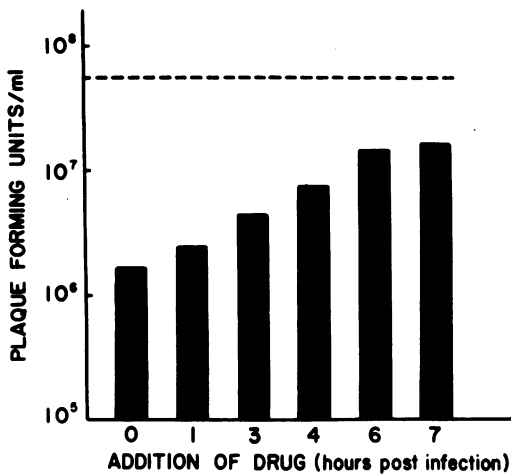
FIG. 3. *Effect of primaquine on the production of infectious virus. Primaquine was added to infected cultures of CEC at various times postinfection at a final concentration of 50 μg/ml. The cultures were assayed for plaque-forming units at 12 h postinfection. The dashed line represents the average plaque-forming units per milliliter found in cultures to which no primaquine was added.*

10% when it was added at 7 h postinfection. The results suggest that the production of extracellular virus was inhibited soon after the drug was added.

Agglutination by con A. The agglutination of NDV-infected CEC by con A, a carbohydrate-binding protein, is due to the redistribution of con A receptor sites on the host membrane surface (19). The increased mobility of these sites is thought to be a consequence of cell membrane modification caused by the appearance of virus-specific glycoproteins at the membrane prior to maturation. To study this phenomenon in our system, monolayered CEC were infected as before. Fresh culture media containing various concentrations of the drug were added to the infected cells and the cultures were incubated at 39 C for 8 h. The results (Table 1) show that noninfected cells were not agglutinated by con A. Infected cells which were treated with either 100 or 125 μg of primaquine per ml appeared to be no different than the noninfected cells. At the lower concentrations of primaquine, the extent of inhibition of agglutination was dose dependent. Cells which were infected and not treated with primaquine showed substantial agglutination; aggregates consisting of 20 cells or more were readily apparent.

Effects of primaquine on macromolecular synthesis: RNA. Host cell deoxyribonucleic acid-directed RNA synthesis was severely in-

hibited (data not shown) by the addition of 1.0 μg of dactinomycin per ml (Merck & Co., Inc., West Point, Pa.). We therefore assumed that the incorporation of the labeled uridine under these conditons was primarily a consequence of virus-specific RNA synthesis. Cultures were infected as described previously. Primaquine was added to cultures in the presence of 1.0 μg of dactinomycin per ml at 1, 3, and 5 h postinfection, and RNA synthesis was determined at 7 h postinfection. The results (Table 2) show that the addition of primaquine at 1 h postinfection inhibited virus RNA synthesis at both concentrations tested. When the drug was added at 3 h postinfection, only a slight inhibitory effect was observed. The addition of 100 μg of primaquine per ml at 5 h postinfection inhibited the incorporation of the labeled uridine into RNA, whereas at a concentration of 50 μg/ml a consistent increase in virus RNA synthesis was observed.

Protein. The effects of primaquine on protein synthesis in infected and noninfected CEC are

TABLE 1. *Effect of primaquine on the ability of infected and noninfected CEC to be agglutinated with con A[a]*

Concn of primaquine (μg/ml)	Infected[b]	Noninfected
0	+ + + +	0
50	+ + +	0
75	+ +	0
100	0	0
125	0	0

[a] Primaquine was added to the culture medium at zero time, and agglutinibility was determined 8 h postinfection in the manner described in Materials and Methods.
[b] 0, No agglutination; + +, aggregates of less than 10 cells; + + +, aggregates of 10 to 20 cells; + + + +, aggregates of greater than 20 cells.

TABLE 2. *Effect of primaquine on the incorporation of [5-³H]uridine into the RNA of NDV-infected CEC[a]*

Concn of primaquine (μg/ml)	Incorporation (counts/min per μg of RNA) at:		
	1 h[b]	3 h	5 h
0	24.2	24.2	24.2
50	16.2	22.4	27.7
100	17.6	22.0	20.1

[a] Primaquine was added at various times postinfection, and RNA synthesis was measured at 7 h postinfection by pulse-labeling cells with [5-³H]uridine (28 Ci/mmol) at a final concentration of 0.5 μg/ml for a period of 15 min.
[b] Time postinfection.

shown in Table 3. Both infected and noninfected cells were untreated or were treated with 50 μg of primaquine per ml. In the cultures to which no primaquine was added, the specific activity (counts per minute per microgram of protein) of the noninfected cells was greater than that of the infected cultures. This effect is a consequence of NDV infection (24). The addition of primaquine inhibited the incorporation of the labeled amino acid into the acid-insoluble material of infected and noninfected cells by 75 and 84%, respectively.

DISCUSSION

During the replication cycle of NDV, viral glycoproteins are inserted into the host cell membrane. These membrane modifications are exemplified by viral HAD, the agglutination of infected cells by con A and syncytia formation.

The adsorption of erythrocytes to NDV-infected cells is a highly specific phenomenon requiring the presence of the virus envelope protein, hemagglutinin, at the cell surface. Primaquine affected both the early and late development of HAD activity (Fig. 2). At 2 h postinfection, those cells that had been treated with the low concentration of primaquine (0.25 μg/ml) exhibited an increase in percent hemadsorbing cells, whereas a decrease was recorded for those cells treated with 250 μg of primaquine per ml. We have reported this bimodal effect for another antimalarial compound, chloroquine (7). One might propose that the quinoline ring structure is in part responsible for this effect because the quinoline ring is common to both compounds.

The inhibition of oxidative phosphorylation results in a rapid inhibition of HAD, suggesting that the development of HAD activity depends not only on de novo synthesis of viral glycoproteins but also upon energy generating systems for the transport of the proteins to the surface membrane (15). Primaquine has been shown to inhibit oxidative phosphorylation (5),

which could explain why the marked increases in HAD seen in the controls (no primaquine) were not seen in the cultures treated with 250 μg of primaquine per ml.

During the replication cycle of many of the enveloped RNA viruses, including NDV, both the physical and chemical properties of the host membrane are altered. One consequence of these alterations is that carbohydrate-binding proteins such as plant lectins will cause the agglutination of infected cells (1). At concentrations of 100 and 125 μg/ml, primaquine inhibited the agglutination of NDV-infected CEC by con A; however, cells which were not treated with the drug showed substantial agglutination (Table 1). These results, taken in conjunction with those obtained from the HAD experiment, suggest that primaquine suppresses the synthesis of viral glycoproteins and/or their transport or incorporation at the membrane surface.

Virus maturation takes place at the cell surface. Viral glycoproteins are concentrated in areas of the plasma membrane from which budding takes place (20). If primaquine inhibited membrane modification, then the release of progeny virus would also be affected. When primaquine was added to infected CEC at various times postinfection, the numbers of infectious virus found in the culture media were less than the numbers contained in cultures to which no primaquine was added (Fig. 3). Release of progeny virus is thought to be facilitated by the membrane glycoprotein, neuraminidase (12). The drug inhibited the appearance of viral hemagglutinin at the membrane surface (Fig. 2). Therefore, the appearance of neuraminidase might also be inhibited because these two glycoproteins are believed to be associated with each other (13).

The possibility exists that the effects of primaquine on virus-induced membrane modifications are consequences of the drug's direct interaction with the plasma membrane of the cell. Membrane components move laterally within the "fluid" matrix of the membrane (22). Primaquine may act to stabilize the plasma membrane thereby retarding the movement of viral glycoproteins within the membrane. This would result in changes in the development of HAD and could inhibit virus maturation. A decreased fluid state would also affect the redistribution of lectin receptors, which appears to be necessary for the agglutination of virus-infected cells by con A.

Primaquine was shown to inhibit viral RNA synthesis when it was added up to 3 h postinfection (Table 2). The addition of 100 μg of primaquine per ml at 5 h postinfection also sup-

TABLE 3. *Effect of primaquine on protein synthesis in infected and noninfected CEC[a]*

Treatment	Protein synthesis (counts/min per μg of RNA)	
	Infected	Noninfected
None	15.03[b]	20.65
Primaquine	4.12	3.37

[a] Primaquine was added at zero time postinfection at a final concentration of 50 μg/ml. Cultures were pulse-labeled with a [14C]protein hydrolysate for a period of 30 min at 7 h postinfection.

pressed viral RNA synthesis; however, the addition of 50 µg of primaquine per ml at 5 h postinfection caused an increase in RNA synthesis. Late in the infection cycle, the progeny RNA (50S) is being synthesized and, once made, it is incorporated into the nucleocapsid (10). Scholtissek and Rott (21) suggested that there is a competition for the progeny RNA between the proteins of the nucleocapsid and the RNA polymerase. Kaverin and Varich (11) reported that when cycloheximide, a known inhibitor of protein synthesis, was added at an early stage of NDV infection, the accumulation of virus-specific RNA was inhibited. When added at a later stage, cycloheximide enhanced the synthesis of small virus-specific RNA; at the same time, the synthesis of progeny RNA was suppressed. In this study, primaquine was shown to substantially inhibit protein synthesis in NDV-infected CEC (Table 3). If primaquine suppresses protein synthesis, the proteins of the nucleocapsid would not be present to encapsidate the progeny genome. Consequently, the RNA polymerase could attach to the progeny RNA, resulting in the synthesis of new RNA. This would account for the increase in RNA synthesis when primaquine (50 µg/ml) was added at 5 h postinfection.

Primaquine has been shown to inhibit the uptake of labeled amino acids (4) as well as interact directly with the protein synthesizing machinery of cells (16). Direct inhibition of protein synthesis in virus-infected cells would result in a decrease in the production of both structural and nonstructural viral proteins. This inhibition would directly affect the development of HAD activity, production of progeny virus, virus-induced membrane modification, and viral RNA synthesis. We therefore propose that primaquine's modification of the replication of NDV in CEC were consequences of the drug's inhibition of protein synthesis.

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