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Effect of Chloroquine on the Growth of Animal Viruses

By

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With 6 Figures

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Summary

The effect of chloroquine diphosphate on the growth of polio type 1, influenza, Newcastle disease, Sendai, vesicular stomatitis and vaccinia viruses was studied. Host dependency of the antiviral effect of chloroquine on Newcastle disease and Sendai viruses was shown using HeLa S 3 cells and primary chick embryo fibroblast cells.

The antiviral effect of chloroquine was extensively studied in chick embryo cells infected with vesicular stomatitis virus. Chloroquine at a concentration of 12.5 μg per ml markedly reduced the virus yield. The drug did not affect the adsorption of vesicular stomatitis virus to chick embryo cells. No evidence for inhibition of the virus uncoating process by the drug was obtained in our present system. The addition of chloroquine at various times after the latent period induced immediate inhibition of the synthesis of progeny virus. Chloroquine inhibited selectively the synthesis of viral RNA without affecting that of cellular RNA or the synthesis of viral antigens.

1. Introduction

Chloroquine diphosphate, an antimalarial drug, is known to combine with purine moieties of DNA (20) and inhibit the reactions catalysed by DNA- and RNA polymerase *in vitro* (3). Stabilization of the lysosomal membranes by this drug has also been reported (18, 21).

Yielding (22) demonstrated that chloroquine inhibited the replication of $\varnothing \times 174$ phage in *E. coli* by suppressing selectively the synthesis of virus directed DNA under conditions where the host cell growth continued. Recently, Kohno *et al.* (17) reported that chloroquine blocked interferon production in chick embryo cells infected with ultraviolet light irradiated Newcastle disease virus, without affecting the cellular incorporation of leucine and uridine into the acid insoluble fraction.

Little has been known about the effect of chloroquine on the replication of animal viruses. MALLUCCI (18) has reported an inhibitory effect of chloroquine on the growth of mouse hepatitis virus in mouse peritoneal macrophages due to its inhibition of the uncoating process by stabilizing the lysosomal enzymes.

In this paper, the effects of chloroquine on the growth of animal viruses were studied.

Vesicular stomatitis virus was used to study the site of action of this drug.

2. Materials and Methods

2.1. Virus

The Indiana strain of vesicular stomatitis virus (VSV) was grown in a primary culture of chick embryo cells (CEC) and stored at $-20°C$ until used. The virus stock had a titer of $10^{8.3}$ plaque forming units (PFU) per ml on CEC monolayers. The viruses other than VSV will be described in the text.

2.2. Tissue Culture

CEC cultures were prepared by trypsinization of 11-day old chick embryos (11). The growth medium consisted of 0.5% lactalbumin hydrolysate in Hanks' solution with 5% bovine serum (LH). Small test tubes (13×100 mm) received 1 ml of the cell suspensions, and plaque bottles (50 ml volume), 7 ml. A complete monolayer was obtained usually on the second day after inoculation of the cells and the number of cells reached about 2×10^6 per test tube. For virus infection, LH was replaced by a medium which consisted of 0.5% lactalbumin hydrolysate in Earle's solution with 0.45% glucose without serum (LE). HeLa S 3 cells and L cells were serially passaged by trypsinization and propagated in a medium consisting of LE supplemented with 0.1% yeast extract and 10% bovine serum (YLE).

2.3. Infectivity Titration

The infectivity of VSV was determined by the plaque assay method on CEC (12). Samples were diluted in LE solution and inoculated onto CEC monolayers in a 0.2 ml volume. Three plaque bottles were used per dilution. After adsorption of the virus for 1 hour at 37°C, the cultures were overlaid with an agar medium which consisted of LE with 1% Noble agar and 1:20,000 neutral red. The plaque count was made on the 3rd day after virus inoculation.

2.4. RNA Synthesis

CEC monolayers in the small test tubes were incubated with ^{3}H-uridine according to the experimental schedule. The radioisotope incorporation was stopped by the addition of 1 ml of 10% cold perchloric acid (PCA) into the culture to make 5% PCA. The culture was allowed to stand for 10 minutes in an ice water bath, and washed twice successively with 5% and 1% PCA. The cells were dissolved in 1 ml of 1 N NH_4OH and an aliquot was taken into a vial containing Bray's scintillation fluid (2). Radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer.

2.5. Fluorescent Antibody Technique

Immunofluorescent staining was made by a direct method. Anti-VSV rabbit serum globulin was conjugated with fluorescein isothiocyanate by the procedure of MARSHALL *et al.* (19), and was further purified by chromatography with Sephadex G-50 and subsequently with a DEAE cellulose column. The cells were dried and fixed with carbon tetrachloride for 40 minutes at room temperature.

2.6. Reagents

Chloroquine diphosphate (Sumitomo Chemical Company, Japan) was dissolved in Hanks' solution, filtered through a Millipore filter type HA, and stored in the dark

at 4°C until used. Actinomycin D was supplied through the courtesy of Dr. BOHONOS, Lederle Laboratories. Uniformly labeled uridine with tritium (2.46 Ci per mM) was purchased from the Radiochemical Centre, Amersham, England.

3. Results

3.1. Effect of Chloroquine on the Growth of Animal Viruses

The effect of chloroquine was studied on the production of several viruses both in CEC and HeLa cells. CEC and HeLa cell tubes were pretreated with chloroquine for 1 hour at 37°C at concentrations of 25 and 50 μg per ml and then infected with appropriately diluted viruses. One hour after infection, the cells were washed twice with Hanks' solution and refed with fresh LE containing the original concentrations of chloroquine. At the end of incubation periods, the infected tubes were subjected to two cycles of freezing and thawing and the total virus was measured. Polio type 1 virus (Mahoney strain) was assayed by PFU count on HeLa cells, and Newcastle disease virus (NDV, California strain), influenza A virus (WSN strain), VSV and vaccinia virus (IHD strain) were titrated on CEC. Sendai virus (Fushimi strain) was assayed by egg titration. Hemagglutinin was measured by the Salk pattern method.

Table 1. *Antiviral Spectrum of Chloroquine*

Virus[1]	Host cell	Inoculum[2]	Infectivity[2]			Hemagglutinin		
			Drug concentration (μg/ml)					
			0	25	50	0	25	50
Polio 1	HeLa	3.0	8.5	8.1	7.2			
NDV	HeLa	7.0	7.3	7.0	7.2	32	32	32
	CEC	7.0	6.8	3.7	3.7	32	< 2	< 2
Sendai	HeLa	7.0	6.2	5.5	5.7	32	32	32
	CEC	7.0	7.0	4.5	4.5	32	< 4	< 4
WSN	HeLa	7.0	6.7	4.0	3.7	48	< 2	< 2
	CEC	7.0	7.2	4.2	4.3	64	< 2	< 2
VSV	HeLa	7.0	7.2	4.0	4.1			
	CEC	7.0	7.5	4.5	4.5			
Vaccinia	HeLa	3.0	5.0	4.5	4.5			
	CEC	3.0	4.8	4.2	4.3			

[1] Seed viruses were grown in the following systems. Polio: HeLa S 3 cell. NDV, Sendai and WSN: chorioallantoic cavity of fertile egg. VSV and vaccinia virus: primary chick embryo cell.

[2] Log $_{10}$PFU/ml, except for Sendai virus being expressed as EID_{50}/ml. Infectivity of polio type 1 and vaccinia viruses was measured at 48 hours after infection and the others at 12 hours. Detailed in the text.

As shown in Table 1, the growth of WSN and VSV in both CEC and HeLa cells was inhibited at either concentration of chloroquine. The growth inhibition of NDV and Sendai virus was demonstrated only in CEC but not in HeLa cells, showing host dependency of the chloroquine effect. The growth of polio 1 and vaccinia viruses was not affected. Recently, no inhibitory effect of chloroquine on the growth of NDV in chick embryo cells was reported (17), where the authors used a different strain (Miyadera strain) from ours. Because of the ease of plaque titration, the inhibitory effect of chloroquine on virus growth was studied in detail hereafter in a VSV and CEC system.

3.2. Dose Effect of Chloroquine on the Inhibition of the Growth of VSV

The CEC culture tubes were infected with VSV at multiplicity of input (m.o.i.) of either 1 or 0.001 per cell and treated simultaneously with chloroquine at various concentrations ranging from 2.5 to 50 μg per ml. After adsorption for 1 hour, the tubes were washed twice with Hanks' solution and refed with fresh LE containing the original concentrations of chloroquine. Incubation was halted at 12 hours (m.o.i. 1) and at 18 hours (m.o.i. 0.001) and the total virus was measured as described.

As shown in Figure 1, chloroquine at 12.5 μg per ml reduced the virus yield to about 1:1,000 with both multiplicities. As the concentration increased, the inhibition was more pronounced with lower m.o.i. Chloroquine at 25 μg per ml

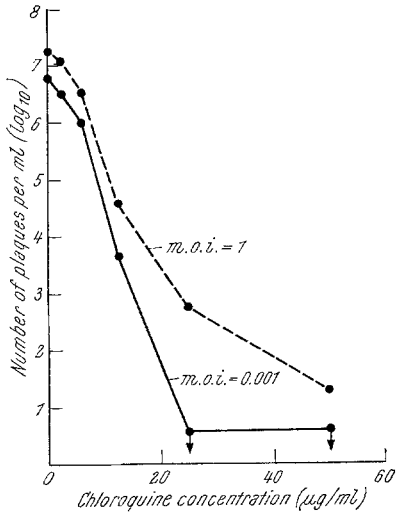

Fig. 1. Dose effect of chloroquine on the inhibition of the growth of VSV in CEC. (Detailed in the text.)

completely inhibited the cytopathic effect. In a parallel experiment, the effect of chloroquine on the viability of both CEC and HeLa cells was determined by the aid of neutral red staining and the counterstaining with trypan-blue (14). No difference in the sensitivity of CEC and HeLa cells to this drug was found. With the concentration of 50 μg per ml, cytoplasmic granulations occurred at 24 hours. At 48 hours, the loss of viability was about 10% at 50 μg per ml but none at 25 μg per ml. As a consequence of this result, the subtoxic dose of 25 μg per ml was used throughout the experiments hereafter.

3.3. Effect of Chloroquine on the Time Course of VSV Growth

The CEC tubes were infected with VSV at m.o.i. 4 in the presence of chloroquine. After adsorption for 1 hour, the cells were washed and refed with LE containing chloroquine. At indicated times, the extracellular and the cell-associated viruses were measured, respectively.

The results are illustrated in Figure 2, in which is included the control experiment. The cell-associated virus began to form from 3 hours and increased logarithmically up to 7 hours in the absence of the drug. The virus release occurred

abruptly from between 5 and 7 hours and continued beyond 9 hours. In the presence of the drug, the increase of the cell-associated virus was not found before 5 hours. The release of the virus seemed not to be affected, although the maximum yield was markedly reduced. The time course of VSV growth in

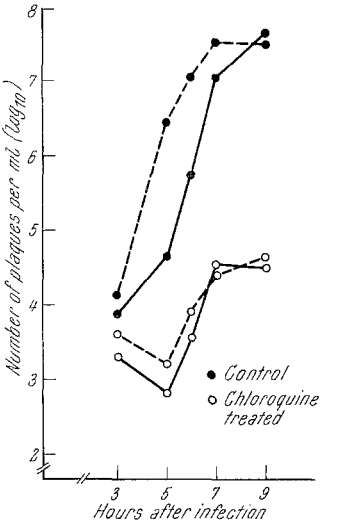

Fig. 2. Effect of chloroquine on the time course of VSV growth: solid line, extracellular virus; dashed line, cell-associated virus. (Detailed in the text.)

the present system is somewhat different from that obtained by FRANKLIN (13), but essentially no critical discrepancy is found between them; the way of the virus release is not of "burst type".

3.4. Irreversibility of the Chloroquine Effect

In order to inquire into reversibility of the chloroquine effect on the virus growth, the following pulse treatment was conducted. The CEC tubes were treated with chloroquine for 1 hour at various times starting from 2 hours before to 3 hours (at the end of the latent period) after virus infection at m.o.i. 4. After adsorption for 1 hour, the unadsorbed virus was washed out, the cells refed with fresh LE and the total virus yield was measured at 14 hours.

Table 2. *Effect of Pulse Treatment of Chloroquine on Virus Production*

Chloroquine present (hours)	Virus titer at 14 hours (PFU/ml)	Virus yield (per cent)
None	2.6×10^7	100
$-2\sim-1$	1.8×10^5	0.7
$0\sim1$	1.3×10^5	0.5
$1\sim2$	1.8×10^5	0.7
$2\sim3$	2.9×10^5	1.1
$3\sim4$	2.1×10^5	0.8

As shown in Table 2, the yield of VSV was greatly reduced by the 1-hour pulse treatment with chloroquine. The addition of the drug even at 2 hours before

infection induced the maximum inhitition. These results might indicate that chloroquine inhibits the virus growth irreversibly through the action on the host cells. Moreover, the sensitive stages to chloroquine exist in the VSV infected CEC through the latent period of the virus growth.

3.5. Effect of Chloroquine on the Adsorption and Penetration of VSV

Since chloroquine at 50 μg per ml did not show any virucidal activity, it was thought from the foregoing results that chloroquine immediately exerted its action in the cells and the inhibitory effect of chloroquine could be expected even at later stages than virus uncoating. To support this idea the effect of chloroquine on the virus adsorption and penetration was studied. CEC tubes were infected with VSV at m.o.i. 0.3 either in the presence or absence of chloroquine. At various time intervals, the unadsorbed virus and the cell-associated virus were assayed. The thermal inactivation of VSV was also examined under the same conditions.

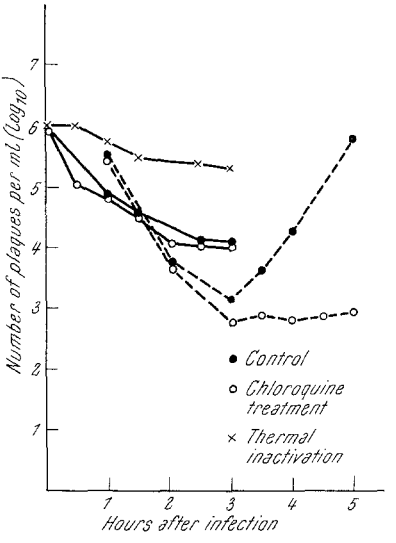

Fig. 3. Effect of chloroquine on the adsorption and penetration of VSV: solid line, extracellular virus; dashed line, cell-associated virus

The results are illustrated in Figure 3. The presence of the drug seemingly did not affect the virus adsorption process. The titer of the culture fluids was reduced to 1:100 of the original titer during the period of 2 hours incubation. The cell-associated virus in the presence or absence of chloroquine was reduced in titer to the order of 1:1,000 of the original at 3 hours after infection, showing that virtually 100% of the adsorbed virus was ultimately eclipsed. The progeny virus started to form at 3 hours in the absence of chloroquine while it did not by 5 hours in the presence of the drug. No significant thermal inactivation of VSV was obtained under the given conditions.

3.6. Effect of Chloroquine on the Various Stages of VSV Infection

The following experiment was carried out to see the effect of chloroquine on the virus yield when added at various times after infection. The CEC tubes were infected with VSV at m.o.i. 4. After adsorption for 1 hour, the cells were washed,

refed with fresh LE and then divided into 2 groups. One served as a control in which was followed the one step growth of VSV by measuring the total virus at hourly intervals. To the other was added the drug at the indicated times and the total virus yield each was measured at 12 hours after infection.

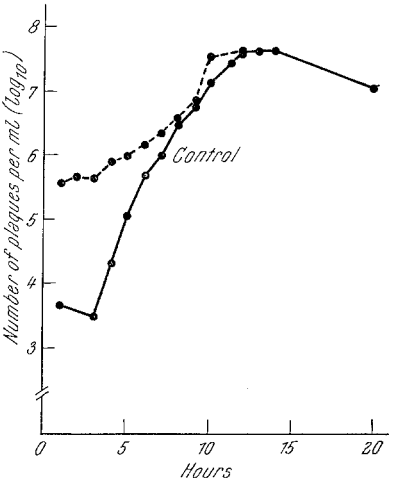

Fig. 4. Effect of chloroquine added at various stages of the virus growth on the production of VSV. The CEC tubes were infected with VSV at m.o.i.4. At hourly intervals, the total virus yield was assayed (solid line). At the indicated times, chloroquine was added to the infected cells. The total virus yield at 12 hours after infection was plotted against the time when the drug was added (dashed line)

Results are shown in Figure 4. In the control experiment, there was an exponential increase of the infectivity from 3 to 9 hours and the maximum titer was obtained at 12 to 15 hours. The addition of chloroquine at the earlier stages of infection allowed some infectious processes to develop although the final virus yield was greatly reduced. Chloroquine when added at later than 7 hours immediately stopped the further increase of the infectivity. These results clearly indicated that in the VSV infected cells, the sensitive stages to chloroquine existed even at the logarithmic growth phase.

3.7. Synthesis of RNAs in VSV Infected Cells in the Presence of Chloroquine

In order to reveal the effect of chloroquine on the synthesis of virus specific RNA, the CEC tubes were infected with VSV at m.o.i. 4 and received chloroquine, 0.5 μg per ml of actinomycin D and 0.1 μCi of ^{3}H-uridine simultaneously. All the samples were taken out at hourly intervals and the radioactivity count in the acid insoluble fraction was made. Controls were included to see the synthesis of normal cellular RNA and the effect of chloroquine, actinomycin D and VSV each on the cellular RNA synthesis. The synthesis of virus specific RNA was disclosed by infecting the cells in the presence of actinomycin D. Infection of the cells in the presence of chloroquine revealed the effect of the drug on the virus induced inhibition of cellular RNA synthesis.

Figure 5 shows the results. Actinomycin D, either alone or with the combination of chloroquine, almost completely inhibited cellular RNA synthesis, while chloroquine alone did not. Infection of the cells with VSV caused a marked inhibition of the cellular RNA synthesis. In the presence of actinomycin D, the virus specific RNA synthesis could be detected at 2 hours after infection and a gradual increase

was observed up to 5 hours. In the presence of chloroquine, however, the synthesis of virus specific RNA was not detectable (VSV, chloroquine, Act.) and the virus-induced inhibition of cellular RNA synthesis did not occur to a significant extent (VSV, chloroquine).

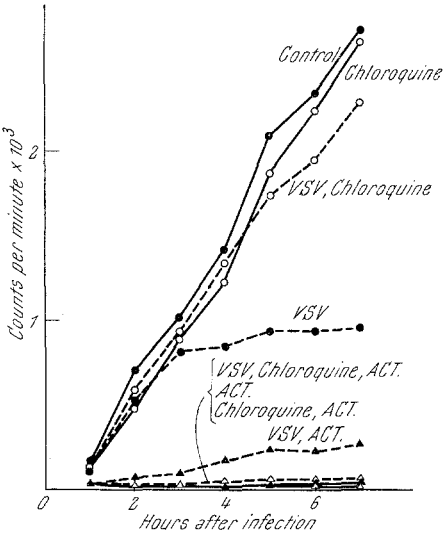

Fig. 5. Effect of chloroquine on the synthesis of RNA in the VSV infected CEC. The CEC tubes were divided into the following 8 groups where the m.o.i. used was 4, the concentration of actinomycin D, 0.5 μg per ml and ^{3}H-uridine, 0.1 μCi per tube. In all the experiments, the label was added at time 0. In the experiments specified, chloroquine and actinomycin D were also added at time 0. In each experiment, the label in the acid insoluble fraction was counted at hourly intervals. Each point represents the average of 3 determinations: (1) uninfected and treated with no drug, (2) uninfected and treated with actinomycin D, (3) uninfected and treated with chloroquine, (4) uninfected and treated with chloroquine and actinomycin D, (5) infected and treated with no drugs, (6) infected and treated with actinomycin D, (7) infected and treated with chloroquine, and (8) infected and treated with chloroquine and actinomycin D

3.8. Effect of Chloroquine on the Various Stages of Viral RNA Synthesis

In this experiment, chloroquine was added to infected cells at various times after infection and its effect on the synthesis of virus specific RNA was observed. The CEC tubes were pretreated with actinomycin D for 1 hour and infected with VSV at m.o.i. 4. At indicated times, 0.1 μCi ^{3}H-uridine with or without chloroquine was added to the cells. At 7 hours after infection, all the samples were taken out and the label in the acid insoluble fraction was counted.

Table 3. *Effect of Chloroquine on the Various Stages of the Synthesis of Viral RNA*

Chloroquine and ^{3}H-Uridine present (hours after infection)	Viral RNA synthesized (c.p.m.)[1] chloroquine absent	chloroquine present
0～7	242	6
1～7	230	n. d. [2]
2～7	158	18
3～7	104	1
5～7	21	2

[1] Values are the average of 3 determinations.
[2] Not done.

As presented in Table 3, the viral RNA synthesis was markedly inhibited in the cultures treated with chloroquine at later than 2 hours after infection as well as at the time of infection.

3.9. Synthesis of the VSV Antigens in the Presence of Chloroquine

The synthesis of the antigens specific for VSV in the chloroquine treated cells was examined by the direct fluorescent antibody technique. Since we had many difficulties in performing this kind of experiment in quantitative manner in the CEC, L cell monolayers were used instead. Although the kinetic experiments of chloroquine in L cells were not shown herein for the sake of brevity of description, essentially chloroquine acted equally in L cells as in CEC.

Coverslip cultures of L cells grown in Petri dishes (16) were treated with chloroquine at the time of infection with VSV at m.o.i. 0.8. The specimens were taken out at indicated time intervals and stained with fluorescent antibody as described. Briefly, the process of the viral antigen development in L cells can be divided into 3 stages. In the first stage *i.e.* 3 to 4 hours after infection, at the end of the latent period, fluorescent granules appear in some non-specified area of the cytoplasm. In the second stage *i.e.* 4 to 6 hours, at which the infectivity titer increase logarithmically, the fluorescent granules occupy nearly entire area of the cytoplasm. In the third stage *i.e.* 6 to 8 hours, at which virus growth is almost maximum, the antigen granules move toward the cell margin, the cytoplasm being

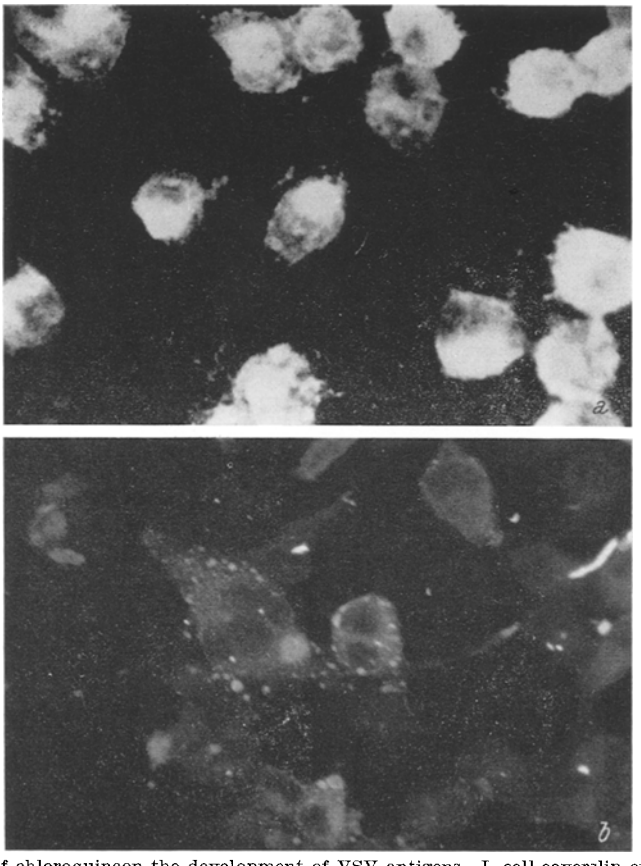

Fig. 6. Effect of chloroquineon the development of VSV antigens. L cell coverslip cultures were infected with VSV at m.o.i. 0.8 either in the absence (a) or the presence of 25 μg per ml of chloroquine (b). Immunofluorescent staining was made at 7 hours after virus infection

full of the antigen masses with intense immunofluorescence (Fig. 6a). In the cells treated with chloroquine, development of the virus antigens occurred in a limited number of the cells and most of the fluorescent cells remained in the stages 1 to 2 (Fig. 6b).

Table 4 shows the development of the viral antigens which was monitored at 7 hours after infection. In the absence of chloroquine, the fluorescent cells were

Table 4. *Effect of Chloroquine on the Synthesis of Immunofluorescent Antigens*

Chloroquine treatment (hours)	Number of cells examined	Number of fluorescent positive cells	Per cent of fluorescent positive cells	Distribution of fluorescent positive cells at the stages[1]	
				I~II	III
−1~7	2050	137	6.7	113 (83)[2]	24 (17)
None	1468	451	30.7	0 (0)	451 (100)

[1] Detailed in the text.
[2] Parentheses show the ratio of the number of fluorescent cells at the indicated stage to the total number of fluorescent positive cells (%).

30.7%, all of which were at the third stage. On the contrary, in the presence of the drug, the number of the fluorescent cells was reduced to 6.7%, of which only 17% of the cells were at the third stage and the rest remained at the first or the second stage.

4. Discussion

There are evidences that animal viruses are taken up into phagosomes of the infected cells (4—7, 9) and exposed to the lysosomal enzymes (8, 10). There seems little doubt that lysosomal enzymes uncoat viruses (1). MALLUCCI (18) and WEISSMANN (21) have reported the stabilizing effect of chloroquine on lysosomes and this has also been confirmed in our present system (unpublished data) by histochemical study for acid phosphates activity (15). Therefore it seems reasonable that lysosomes stabilizers such as chloroquine inhibit virus multiplication by blocking the uncoating process.

MALLUCCI (18) has observed that mouse peritoneal macrophages pretreated with chloroquine for 6 hours produced less mouse hepatitis virus than the untreated cells. This inhibition of the virus growth was considered to be caused by prevention of virus uncoating through lysosomal stabilization of the macrophages by this drug. However, as he pointed out, because of the system used, he could not follow a single cycle of the virus growth and study the effect of chloroquine on the replicative phase of the virus growth.

In our present experiment, the effect of chloroquine was studied on a single cycle event of the VSV growth in CEC. In accord with MALLUCCI's result, chloroquine did not affect the adsorption of VSV to CEC but none of the evidence for the prevention of the uncoating by this drug was obtained. As shown in Table 2, a 1-hour pulse treatment of chloroquine even at the very end of the latent period decreased the virus yield. The processes responsible for the loss of infectivity after adsorption were not affected by chloroquine treatment (Fig. 3). Although we did not measure the freed viral RNA after adsorption, this process may be thought as a reflection of the virus uncoating. Thus the inhibition of the virus

growth by chloroquine in our system can not be explained by the inhibition of the uncoating process, but the inhibitory effect on the process(es) after uncoating must be considered. In fact, this was observed in the result given in Figure 4, from which it is evident that chloroquine when added during the logarithmic growth phase immediately stops the further increase of the progeny virus. Therefore, in the infected cell, the sensitive stages to chloroquine exist through the replicative phase of the virus growth after uncoating. However, whether the mechanism of the virus inhibition by chloroquine observed in the latent period is different from that in the replicative phase of the virus growth is unknown. Chloroquine inhibited selectively the synthesis of the viral RNA without affecting that of the cellular RNA (Fig. 5), and this inhibition could be observed when the drug was added even at the end of the latent period (Table 3). Chloroquine also inhibited both the synthesis of viral antigens and their development when added at the time of infection. Since the uncoating process proceeded normally in the presence of chloroquine, this might be due to inhibition of the expression of viral genome after it has been freed from the virion or due to limited synthesis of viral RNA.

Although the present data are not enough to understand the inhibitory mechanism of chloroquine for the growth of animal viruses, since the drug effect was shown to be host-dependent and pretreatment of the cells by the drug was effective for the induction of the inhibition of virus growth, it might be possible that chloroquine acts primarily on the host cells so that the synthesis of viral RNA and viral antigens is depressed. We did not made direct efforts to accomodate the conflict between MALLUCCI's results and ours but it seems not unlikely that chloroquine acts on macrophages in a different way from other animal cells.

References

1. ALLISON, A.: Lysosomes in virus-infected cells. In: "Perspectives in Virology" **5,** pp. 29—61, New York and London: Academic Press, 1967.
2. BRAY, G. A.: A simple efficient liquid scinzillator for counting aqueous solutions in a liquid scintillation counter. Ann. Biochem. **1,** 279—285 (1960).
3. COHEN, S. N., and K. L. YIELDING: Inhibition of DNA and RNA polymerase reactions by chloroquine. Proc. nat. Acad. Sci. (Wash.) **54,** 521—527 (1955).
4. DALES, S.: An electron microscope study of the early association between two mammalian viruses and their hosts. J. Cell Biol. **13,** 303—322 (1962).
5. DALES, S.: The uptake and development of vaccinia virus in strain L cells followed with labelled viral deoxyribonucleic acid. J. Cell Biol. **18,** 51—72 (1963).
6. DALES, S., and P. W. CHOPPIN: Attachment and penetration of influenza virus. Virology **18,** 489—493 (1962).
7. DALES, S., P. J. GOMATOS, and K. C. HSU: The uptake and development of reovirus in strain L cells followed with labelled viral ribonucleic acid and ferritin-antibody conjugates. Virology **25,** 193—211 (1965).
8. DALES, S., and R. KAJIOKA: The cycle of multiplication of vaccinia virus in Earle's strain L cells. 1. Uptake and penetration. Virology **24,** 278—294 (1964).
9. DALES, S., and L. SIMINOVITCH: The development of vaccinia virus in Earle's L strain cells as examined by electron microscopy. J. biophys. biochem. Cytol. **10,** 475—503 (1961).
10. DAVID-FERREIRA, J. F., and R. A. MANAKER: An electron microscope study of the development of a mouse hepatitis virus in tissue culture cells. J. Cell. Biol. **24,** 57—78 (1965).

11. DULBECCO, R.: Production of plaques in monolayer tissue cultures by single particles of an animal virus. Proc. nat. Acad. Sci. (Wash.) **38,** 747—752 (1952).
12. DULBECCO, R., and M. VOGT: Plaque formation and isolation of pure lines with poliomyelitis viruses. J. exp. Med. **99,** 167—182 (1954).
13. FRANKLIN, R. M.: Studies on the growth of vesicular stomatitis virus in tissue culture. Virology **5,** 408—424 (1958).
14. GIRARDI, A. J., H. MCMICHAEL JR., and W. HENLE: The use of HeLa cells in suspension for the quantitative study of virus propagation. Virology **2,** 532—544 (1956).
15. GREEN, M. H., and E. L. VERNEY: A simple method for the study of intracellular acid phosphatase in HeLa cells. J. Histochem. Cytochem. **4,** 106—109 (1956).
16. KASHIWAZAKI, H., and N. ISHIDA: Inhibition of Sendai virus by N^1-Isobutylbiguanide hydrochloride as studied by immunofluorescent staining. Proc. Soc. exp. Biol. (N.Y.) **120,** 139—143 (1965).
17. KOHNO, S., M. KOHASE, and M. SUGANUMA: Inhibition of interferon production by chloroquine diphosphate. Jap. J. med. Sci. Biol. **21,** 239—248 (1968).
18. MALLUCCI, L.: Effect of chloroquine on lysosomes and on growth of mouse hepatitus virus (MHV-3). Virology **28,** 355—362 (1966).
19. MARSHALL, J. D., W. C. EVELAND, and C. W. SMITH: Superiority of fluorescein isothiocyanate (Riggs) for fluorescent antibody technic with a modification of its application. Proc. Soc. exp. Biol. (N.Y.) **98,** 898—900 (1958).
20. O'BRIEN, R. L., J. G. OLENICK, and F. E. HAHN: Reactions of quinine, chloroquine, and quinacrine with DNA and their effects on the DNA and RNA polymerase reactions. Proc. nat. Acad. Sci. (Wash.) **55,** 1511—1517 (1966).
21. WEISSMANN, G.: Labilization and stabilization of lysosomes. Fed. Proc. **23,** 1038—1044 (1964).
22. YIELDING, K. L.: Inhibition of the replication of a bacterial DNA virus by chloroquine and other 4-aminoquinoline drugs. Proc. Soc. exp. Biol. (N.Y.) **125,** 780—783 (1967).

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