Chloroquine Mediated Alterations in Mammalian Cell Metabolism and Viral Replication[1] (35477)

GERALD J. LANCZ, LEROY C. MCLAREN, CHARLES G. JAMES AND JOSEPH V. SCALETTI

Department of Microbiology, University of New Mexico Medical School, Albuquerque, New Mexico 87106

Chloroquine is an effective antimalarial drug that has also been successfully employed clinically in treating lupus erythematosus (1), as well as rheumatoid arthritis (2). A number of studies performed to examine the biological effects of chloroquine have revealed a diversity of activities. Some of the drug effects included a stabilization of lysosomal membranes (3), an activation of *E. coli* tyrosine transfer ribonucleic acid (RNA) (4), binding to bacterial deoxyribonucleic acid (DNA) with an alteration of the polymerase reactions (5–8) and an inhibition of a number of metabolic enzymes such as alcohol dehydrogenase (9) and succinic dehydrogenase (10). There is a relative paucity of information describing the effects of chloroquine on mammalian cell metabolism and no information on the effects of this drug on the replication of animal viruses. Since chloroquine is employed clinically as well as for investigative purposes, this study sought to examine the effect of this drug on mammalian cell biosynthetic processes and to survey its effect on the replication of several unrelated viruses.

Materials and Methods. Cells. HeLa cells maintained in 16-oz prescription bottles as stationary monolayers were periodically dispersed by trypsinization as described (11) and distributed to 2-oz French square bottles (A. H. Thomas Co.) 18–24 hr prior to the onset of each experiment. All experiments to measure the rates of protein, RNA, and DNA synthesis and all virus growth studies were performed using these smaller monolayers, containing 2 to 5 $\times$ 10^6 cells/bottle. A continuous rat heart cell line, used to quantitate vaccinia and herpes virus, was similarly treated.

Medium. Growth medium consisted of Eagle's medium (12) in Hanks' balanced salt solution (BSS) (13), supplemented with 10% newborn calf serum (N. Am. Biological), 10% tryptose phosphate broth, Aureomycin (50 μg/ml), neomycin (75 μg/ml) and Mycostatin (25 μg/ml).

Extraction of nuclear RNA and DNA. HeLa cells were incubated with medium which contained or was free of chloroquine for 1 or 24 hr. The monolayers were then exposed to fresh medium containing 2.0 μCi of ^{3}H uridine or 2.0 μCi ^{3}H thymidine for 30 min. Following this period, the monolayers were washed twice with large volumes of cold physiological saline. The cells were then scraped into 1 ml of phosphate buffered saline (PBS) with a rubber policeman and the clumps of cells were dispersed. One ml of PBS containing 1% nonidet P40 (NP-40) was added to solubilize the plasma membrane (14) during a 15-min incubation on ice; and then the nuclei were removed by centrifugation at 500*g*. Nuclear RNA was extracted by the phenol–sodium dodecyl sulfate (SDS) procedure (15), precipitated with cold ethanol, and redissolved in saline twice. A sample of the final RNA solution was assayed for radioactivity in the Beckman L-S 100 scintillation counter. Cellular DNA was extracted by resuspending the nuclei in 1 ml of PBS and adding 0.1 ml of 5% SDS to solubilize the nuclei. Carrier DNA (1 mg) was added to each sample followed by an equal volume of 10% trichloroacetic acid. The precipitate was removed by centrifugation and redissolved in alkaline

[1] This investigation was supported by Research Grant Nos. AI-06450 and CA 10239 from the National Institutes of Health.

saline (0.85% NaCl; 0.05 *N* NaOH). A sample of the final solution was then assayed for radioactivity. The rates of nuclear RNA and DNA synthesis were expressed as counts per minute per milligram of cytoplasmic protein.

Extraction of cellular protein. HeLa cells were exposed to 1 ml of medium containing 0.25 μCi of ^{14}C amino acid mixture for 30 min. The monolayers were then washed twice with cold physiological saline and the protein was extracted as described (16). The rates of cellular protein synthesis were expressed as counts per minute per milligram of protein. All determinations of protein content were performed colorimetrically by the method of Oyama and Eagle (17) using crystalline bovine serum albumin as the protein standard.

Infection of cells and assay of viruses. HeLa cell monolayers were infected with type 1 poliovirus at a multiplicity of 10 plaque-forming units (pfu)/cell ($m = 10$); vaccinia virus strain wr, ($m = 6$), vesicular stomatitis virus Indiana strain (vsv; $m = 10$) and herpes simplex virus, O'Connell strain ($m = 2.5$). The viruses were allowed to adsorb to the cells for 0.5, 2, 1, and 2 hr, respectively; and the monolayers were washed twice with large volumes of BSS. The monolayers then received 2 ml of medium $\pm$ 60 μg/ml of chloroquine. At various times after infection duplicate monolayers were frozen. The poliovirus infected monolayers were subsequently thawed, the cells were scraped into the medium and disrupted with the addition of an equal volume of PBS containing 1% NP-40. For the other viruses, cellular disruption was achieved by thawing followed by sonication for 15 sec with a Branson sonifier. Polio and VSV were quantitated by plaque formation on HeLa cells as described (18). Vaccinia and herpes simplex were quantitated by plaque formation on rat heart cells using an agar-free overlay.

Chemicals. ^{14}C chloroquine (1.71 mCi/m*M*; New England Nuclear Corp.) ^{3}H thymidine (2 Ci/m*M*); ^{3}H uridine (5 Ci/m*M*); and a ^{14}C amino acid mixture (protein hydrolysate, 52 mCi/mA; Amersham/Searle Corp.) were purchased. Chloroquine diphosphate was a gift of Sterling-Winthrop, New York. All references to drug concentration employed in experiments refer to the concentration of chloroquine diphosphate.

Results. Chloroquine mediated cytotoxicity was evaluated by exposing stationary phase HeLa cell monolayers to varying concentrations of drug and examining them microscopically at various times. A chloroquine level of 80 μg/ml or more was cytotoxic as determined by pycnosis and cell sloughing while 60 μg/ml or less did not produce a cytotoxic reaction in monolayer cultures in 48 hr (Fig. 1).

In addition, the effect of chloroquine on the replication of HeLa cells was studied. Culture bottles were seeded with approximately 10^6 cells and then incubated with medium alone or medium containing various concentrations of drug. At daily intervals duplicate control and chloroquine treated cultures were trypsinized and cell counts were performed. The data indicated a concentration of 5 μg/ml would retard cellular proliferation while 60 μg/ml effectively blocked HeLa cell division.

The kinetics of chloroquine uptake was examined by determining the level of radioactive chloroquine found intracellularly over time. As shown in Table I, chloroquine was readily taken up by HeLa cells as approximately 16% of the total radioactivity was found intracellularly in 1 hr. In addition, extending the incubation period to 24 hr resulted in only a 2-fold increase in the total

TABLE I. Uptake of ^{14}C Chloroquine by HeLa Cells.[a]

Length of exposure (hr)	Total intracellular radioactivity (cpm)
1	31,700
2	31,500
4	32,600
8	33,500
24	65,400

[a] HeLa cell monolayers were exposed to 1.0 ml of medium containing 0.1 μCi of ^{14}C chloroquine (19 μg) and a total of 60 μg/ml (41 μg of nonradioactive) chloroquine. At various times, duplicate monolayers were thoroughly washed with saline and the quantity of intracellular radioactivity was determined.

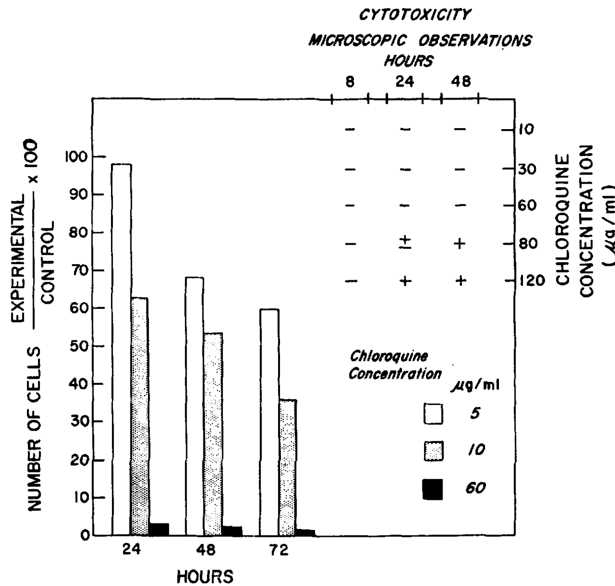

FIG. 1. Chloroquine cytotoxicity (upper right): HeLa cell monolayers were incubated with medium containing varying concentrations of chloroquine. Drug cytotoxicity (+) was determined microscopically. Effect on HeLa cell growth (lower left): Approximately 10^6 cells, seeded into culture bottles, were incubated with medium containing varying concentrations of chloroquine or medium alone (controls). Cell counts were performed daily as described in Methods.

intracellular concentration of ^{14}C chloroquine over the 1-hr level.

The dose–response relationship of chloroquine level and cellular macromolecular biosynthetic potential was examined. Monolayer cultures were exposed to medium containing varying drug concentrations and after 1 or 24 hr, these cultures were exposed to medium containing chloroquine and ^3H uridine, ^3H thymidine, or ^{14}C amino acids. Controls were similarly treated with media without chloroquine. Following a 30-min incubation period, the amount of acid-insoluble radioactivity was determined as described in the methods. The data indicated that exposure to 10 and 30 μg/ml for 1 hr did not appreciably inhibit cellular biosynthesis of protein, RNA, or DNA (Fig. 2). However, a drug level of 60 μg/ml did produce a decrement in the biosynthesis of these macromolecules. In addition, extending the exposure time (60 μg/ml) from 1 to 24 hr resulted in only slightly greater levels of inhibition compared with the rates of incorporation at 1 hr.

An evaluation of the effectiveness of chloroquine as an antiviral agent was performed by comparing the growth curves of four unrelated viruses in HeLa cells in the presence and absence of the drug. The data indicated the

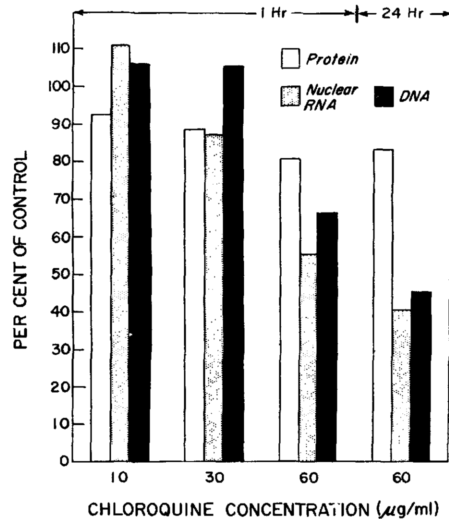

FIG. 2. HeLa cell monolayers were exposed to varying concentrations of chloroquine for 1 or 24 hr: The rates of cellular protein, nuclear RNA, and DNA synthesis were then determined as described in the methods and expressed as a percentage of controls.

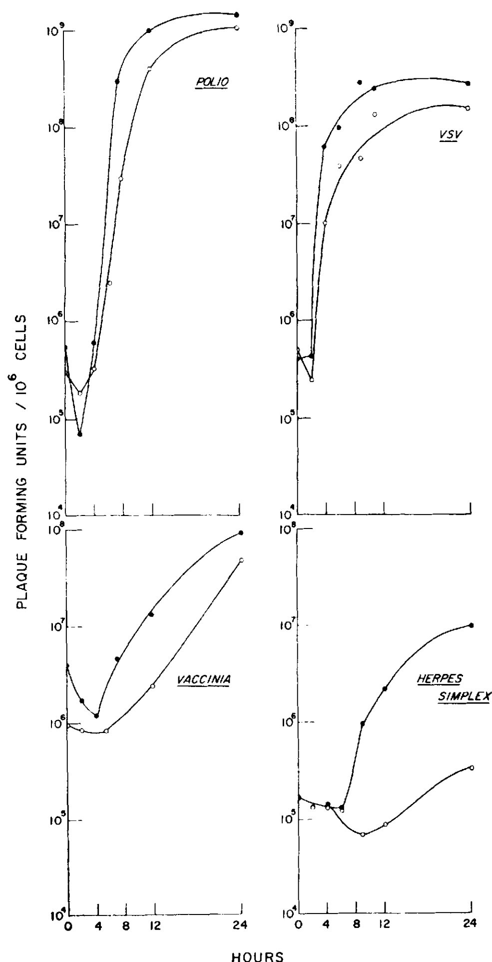

FIG. 3. The effect of 60 μg/ml of chloroquine on the replication of four viruses was determined: (●) control; (○) chloroquine treated.

replication of VSV, polio, and vaccinia virus were somewhat delayed but the ultimate production of progeny virus particles was virtually unaffected by chloroquine (Fig. 3). However, herpes simplex replication was found to be sensitive to this drug as the level of progeny virus production was depressed by 95% in 24 hr.

Discussion. The relationship between drug concentration and microscopic cytotoxicity indicated 60 μg/ml of chloroquine did not appreciably affect HeLa cell monolayers. Consequently, the observation that 60 μg/ml of chloroquine totally inhibited HeLa cell replication was surprising despite the previously reported similar effects of hydroxychloroquine on L cells (19). These data indicated a lack of microscopic cytotoxicity does not preclude biologic activity. In addition, these observations also suggest that the apparent degree of biologic activity of chloroquine in other systems may be closely associated with the cultural and physiological state of the cells employed.

The data on the kinetics of chloroquine uptake indicated the drug attained near maximum intracellular concentrations in 1 hr. Comparing these data with the dose–response effects of chloroquine on cellular macromolecular biosynthesis indicated a drug level of 60 μg/ml resulted in a decrement in the rates of cellular RNA, DNA, and protein synthesis in 1 hr. The extension of both of these studies to a 24-hr period did not result in significant alterations of the 1-hr observations. These data indicate that the chloroquine-mediated alterations in cellular metabolism are rapid, occurring shortly following drug uptake. Similar conclusions have been reported from studies employing bacterial systems (20, 21).

Chloroquine was found not to effect the synthesis of polio and VSV (RNA viruses) or vaccinia (DNA virus) which replicate in the cytoplasm of the cell. In contrast, herpes simplex (DNA virus) replication was strongly inhibited by chloroquine in 24 hr. This differential inhibitory effect may be a consequence of the preferential nuclear localization of chloroquine at the site of herpes simplex maturation. The present data are consistent with the DNA drug binding mechanism proposed as the mode of chloroquine action (5, 7). In addition, evidence is presented which suggests chloroquine may be an effective chemotherapeutic agent against herpes simplex virus.

1. Merwin, C., and Winkelman, R., Mayo Clin. Proc. **37**, 253 (1962).

2. Scull, E., Arthritis Rheum. **5**, 30 (1962).

3. Weissmann, G., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **23**, 1038 (1964).

4. Muench, K., Cold Spring Harbor Symp. Quant. Biol. **31**, 539 (1966).

5. O'Brien, R., Olenick, J., and Hahn, F., Proc. Nat. Acad. Sci. U.S.A. **55**, 1511 (1966).

6. Cohen, S., and Yielding, K., Arthritis Rheum. 7, 302 (1964).

7. Cohen, S., and Yielding, K., J. Biol. Chem. **240**,

3123 (1965).
8. Cohen, S., and Yielding, K., Proc. Nat. Acad. Sci. U.S.A. **54**, 521 (1965).
9. Fiddick, R., and Heath, H., Nature (London) **213**, 628 (1967).
10. Sanabria, A., and Carbonell, L., J. Histochem. Cytochem. **7**, 391 (1959).
11. McLaren, L., Holland, J., and Syverton, J., J. Exp. Med. **109**, 475 (1959).
12. Eagle, H., Science **130**, 432 (1959).
13. Hanks, J., and Wallace, R., Proc. Soc. Exp. Biol. Med. **71**, 196 (1949).
14. Borun, T., Scharff, M., and Robbins, E., Biochim. Biophys. Acta **149**, 302 (1967).
15. Johnson, T., J. Neurochem. **14**, 1075 (1967).
16. Johnson, T., Lerner, M., and Lancz, G., J. Cell Biol. **36**, 617 (1968).
17. Oyama, V., and Eagle, H., Proc. Soc. Exp. Biol. Med. **91**, 305 (1956).
18. Holland, J., and McLaren, L., J. Bacteriol. **78**, 596 (1959).
19. Gabourel, J., J. Pharmacol. Exp. Ther. **141**, 122 (1963).
20. Ciak, J., and Hahn, F., Science **151**, 347 (1966).
21. Yielding, K., Proc. Soc. Exp. Biol. Med. **125**, 780 (1967).

Received Nov. 4, 1970. P.S.E.B.M., 1971, Vol. 136.