The Effect of Chloroquine on Herpesvirus Infection *in Vitro* and *in Vivo* (37166)

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Chloroquine, a compound widely used clinically for the prophylaxis and therapy of malaria, was recently found to inhibit the replication of herpes simplex virus (HSV) in HeLa cell cultures (1). This observation and the current lack of clinically effective drugs of low toxicity for the treatment of severe systemic human herpesvirus infections prompted the present study. We have investigated the therapeutic efficacy of chloroquine on lethal systemic infection produced by HSV-1 and HSV-2 in the newborn mouse. The lethal endpoint of this assay system (2) is unequivocal and highly reproducible. Additionally, manifestations suggestive of central nervous system involvement which resemble those observed in human herpesvirus encephalitis result from the intraperitoneal inoculation of the virus (2). These features and the observation that chloroquine inhibited HSV replication in mouse embryo (ME) cell cultures made assay of the antiviral activity of chloroquine in HSV-infected newborn mice seem appropriate and advantageous.

Materials and Methods. Viruses. Stock strains of herpesvirus type 1 (68–157) and herpesvirus type 2 (68–158), originally isolated in human embryo kidney cell cultures and passaged twice in rabbit kidney cell cultures were kindly provided by Dr. Fred Rapp. Virus pools for use in these experiments were prepared in cultures of BHK-21 cells (3). Cytopathic effects were complete 48 hr after inoculation, at which time the entire cultures were frozen (—70°) and thawed three times, centrifuged (15 min, 2000 rpm, 4°, International Centrifuge Model PR2), and the clarified supernates stored in screw-capped vials at —70° until use.

Cells and Media. HeLa cells and secondary mouse embryo (ME) cells were propagated in Eagle's medium, enriched with 10% tryptose phosphate broth and 5% fetal calf serum (FCS) (Hyland Laboratories), (ETC), containing penicillin 100 units/ml, streptomycin 100 μg/ml and mycostatin, 50 units/ml.

Chloroquine. Chloroquine was added to tissue culture media as chloroquine hydrochloride (Aralen R, Winthrop Laboratories, New York, New York). It was added to media at a final concentration of 36 μg of chloroquine base/ml, the amount present in 60 μg of chloroquine diphosphate (1).

In vitro *HSV growth curves and virus assays.* Confluent monolayers of HeLa or secondary ME cells in 30 ml plastic tissue culture flasks (Falcon Plastics No. 3012) were drained and infected with 0.2 ml of virus suspended in 0.025 *M* Tris (hydroxymethyl) aminomethane buffer (TBS) (pH 7.4), containing 3% FCS. The number of cells present at the time of infection was determined by cell counts performed on replicate cultures. When virus adsorption was complete (37°, 2 hr, intermittent manual rotation), the inoculum was removed with three washes of phosphate-buffered saline (PBS), and the cultures fed with 4 ml of ETC containing 5% FCS. At appropriate intervals after washing, replicate whole cultures were harvested by freezing and stored at —70° until thawed for virus plaque assays. They were then frozen and thawed twice more to disrupt cells, clarified by centrifugation (2000 rpm, 15 min, 4°) and the supernates diluted in TBS containing 3% FCS. Plaque assays were performed according to the method of Rapp (4) using confluent monolayer cultures of BHK-21 cells in 60 mm plastic petri dishes (Falcon No. 3002). The specimens to be assayed

were diluted in TBS containing 3% FCS and 0.2 ml of appropriate dilutions inoculated into 3 replicate BHK-21 monolayers. After adsorption at 37° for two hours with intermittent manual rotation, the monolayers were overlayed with 5 ml of ETC supplemented with 0.5% Methylcellulose (Matheson, Coleman and Bell, Inc., Cat. No. MX850), 3% FCS, and additional $NaHCO_3$ (0.23%) without prior removal of the inoculum. After four days of incubation (36°, 5% CO_2 in humidified air) the cultures were fixed with neutral formalin, stained with Jenner-Giemsa stain, and the resulting plaques counted macroscopically.

Inoculation of animals. Duplicate litters of Swiss Webster mice (Simonsen Laboratories, Gilroy, California), each consisting of approximately eight animals weighing 1–2 g each were used 24–48 hr after birth. Mice which died within the first 24 hr after inoculation were excluded from the data tabulations. Intraperitoneal injections consisted of 0.05 ml of TBS containing 3% FCS in which virus had been diluted. Chloroquine was diluted with normal saline and injected in the same manner. The dosages of HSV-1 and HSV-2 are expressed as plaque-forming units (PFU) assayed in BHK-21 cells (see above). All chloroquine dosages and concentrations, respectively, are expressed as micrograms or as micrograms per milliliter of chloroquine base.

Chloroquine assays. At intervals after intraperitoneal inoculation of chloroquine to uninfected mice the animals were killed and their livers and brains individually collected. Tissues were weighed, homogenized in 2.8 ml of 0.5 *N* NaOH and 0.2 ml ethanol and extracted with 15 ml of heptane (5). Chloroquine levels were determined by means of gas chromatography and suitable standard and blank controls were run simultaneously. The results of chloroquine determinations are expressed as micrograms of chloroquine per gram of wet tissue weight.

Results. 1. Inhibition of in vitro *virus replication.* At a concentration of 36 μg/ml, chloroquine effectively inhibited the replication of both infectious HSV-1 and HSV-2 in ME as well as in HeLa cell cultures as shown in Table I. However, while small increases in the amount of HSV-1 occurred between 6 and 36 hr after the completion of virus adsorption, the titers of HSV-2 progressively declined. These results suggest a differential susceptibility of HSV-1 and HSV-2 replication to inhibition by chloroquine *in vitro*. Similar results were also obtained when HeLa cell cultures were used instead of ME cell cultures.

The appearance of the plaques produced in BHK-21 cell monolayers by HSV-1 and HSV-2 is shown in Fig. 1. HSV-2 consistently produced plaques which at 96 hr were much larger (5–6 mm) than HSV-1 plaques (2–3 mm) and tended to be irregular rather than round in shape. The observed irregularity appeared to result from focal retraction of the cell sheet along cell cleavage planes in the confluent BHK-21 fibroblast monolayer.

2. Infection of mice with HSV-1 and HSV-2. The intraperitoneal injection of 100 pfu

TABLE I. Effect of Chloroquine (36 μg/ml) on Replication of HSV-1 and HSV-2 in Mouse Embryo Cell Cultures.[a]

	HSV-1 (MOI = 2.5)[b]		HSV-2 (MOI = 0.5)	
Hours	Chloroquine	None	Chloroquine	None
0	7.07×10^{3}[c]	6.75×10^{3}	6.88×10^{2}	3.85×10^{2}
6	2.83×10^{4}	2.40×10^{4}	1.28×10^{2}	4.30×10^{1}
18	1.09×10^{3}	7.65×10^{5}	1.95×10^{1}	7.75×10^{2}
36	6.50×10^{3}	5.78×10^{5}	$<1.8 \times 10^{1}$	1.32×10^{3}

[a] Each number is the mean virus content, expressed as plaque-forming units per 10^6 cells, of duplicate cultures. Plaque assays were performed in BHK-21 cell cultures in triplicate (See Methods).

[b] MOI = multiplicity of infection, plaque-forming units (pfu) per cell.

[c] pfu/10^6 cells.

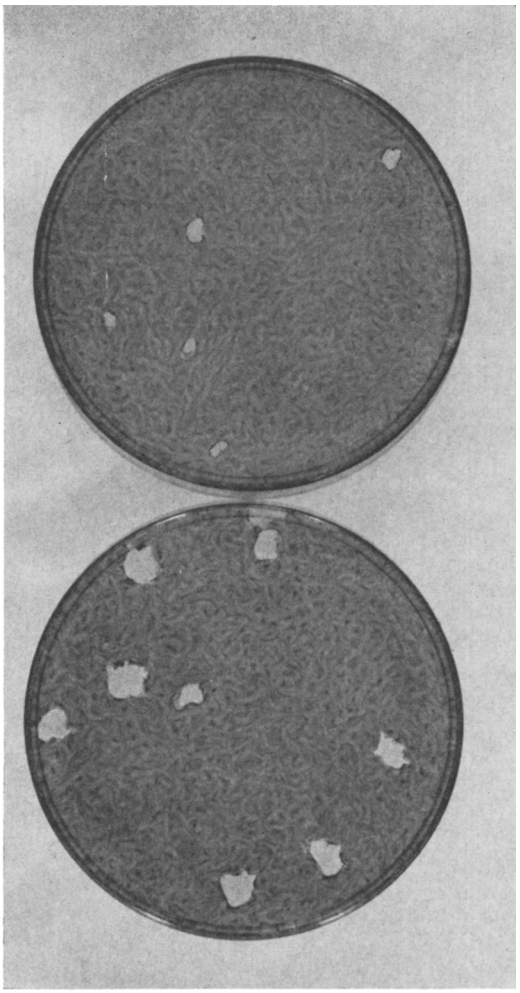

FIG. 1. Plaques (see Methods) produced in BHK-21 cell monolayers by HSV-1 (top) and HSV-2 (bottom) 96 hr after infection (Petri dish diameter = 60 mm).

of either HSV-1 or HSV-2 into newborn mice resulted in an almost uniformly fatal infection characterized by the progressive development of lethargy, irritability, twitching, focal and generalized seizures, coma, and death. The patterns of illness produced by the two virus strains were grossly indistinguishable, except for the fact that the latent period following HSV-2 infection was approximately 24 hr longer. Less than a day generally elapsed between the onset of symptoms and death, and no animal exhibiting signs of illness recovered. No attempt was made to obtain serologic evidence of infection or to determine whether chronic HSV infection (6) developed in the small number of animals which remained asymptomatic and survived after receiving 10–100 pfu of either virus in preliminary *in vivo* virus pool titrations.

Despite the appearance of clinical manifestations which seemed consistent with and indicative of the presence of virus-induced lesions in the central nervous system (CNS), histopathologic evidence of encephalitis was observed in only one of six animals sacrificed and autopsied when severe symptoms suggestive of CNS damage were noted in all and when death appeared imminent. In contrast to this apparent sparing of the central nervous system, severe and extensive lesions characteristic of hepatitis and interstitial pneumonitis were present in every animal examined. Histopathologic lesions were not observed in other organs, and the lesions pro-

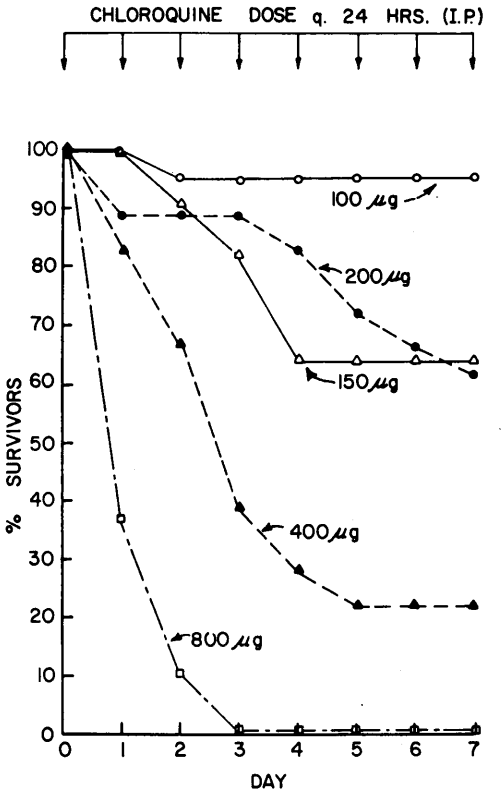

FIG. 2. Chloroquine toxicity in 1–2-day-old Swiss Webster mice. Duplicate litters each containing approximately 8 mice were injected intraperitoneally every 24 hr.

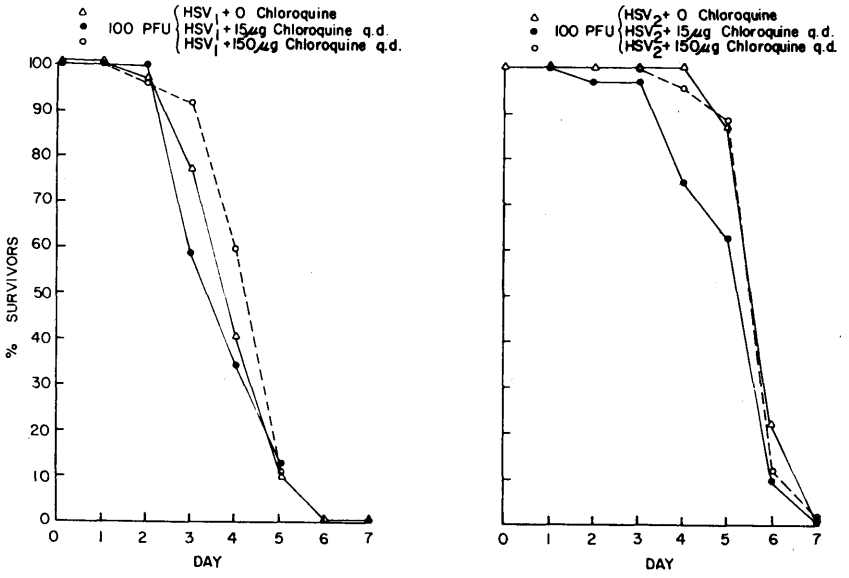

FIG. 3. Effect of chloroquine therapy on survival of mice infected with HSV-1 or HSV-2. Duplicate litters each consisting of approximately 8 mice were inoculated ip with 100 BHK-21 cell pfu of virus 6 hr after receiving chloroquine. Thereafter the drug was administered once daily.

duced by HSV-1 and HSV-2 were indistinguishable from each other.

3. Effect of chloroquine on in vivo *HSV infection.* Although daily intraperitoneal doses of chloroquine greater than 100 μg produced significant mortality (Fig. 2), over 60% of injected mice appeared unaffected by dosages of up to 200 μg. Chloroquine was therefore tested for its potential antiviral effect *in vivo* both at potentially toxic (150 μg/day) and apparently nontoxic (15 μg/day) dosage levels (Fig. 3). Although virus was not administered until 6 hr after the first dose of chloroquine in an attempt to maximize the anticipated protective and therapeutic effects of the drug, no prolongation or enhancement of survival was noted with either virus even at the 150 μg/day dose level. No difference was noted between treated and untreated mice with respect to the onset, nature, or severity of the previously described clinical manifestations, or the duration of survival after onset of symptoms.

The data presented in Table II indicate that this result was probably not due to failure to achieve tissue levels of chloroquine comparable to those which inhibited HSV replication *in vitro*. Thus, intraperitoneal injection of 150 μg of chloroquine produced readily detectable hepatic tissue levels of chloroquine base within six hours and apparent cumulation of the drug in this organ thereafter. Levels achieved during the period corresponding to the incubation period of the infection were well in excess of protective levels *in vitro*. Chloroquine base was not detected in brain tissue at any time after chloroquine administration.

Discussion. Intraperitoneal inoculation of newborn mice with herpesvirus hominis results in viremia, infection of free peritoneal macrophages, visceral lesions, and hematogenous infection of the central nervous system

TABLE II. Tissue Levels of Chloroquine.[a]

	Chloroquine concentration (μg/gm)	
Time (hr)	Liver	Brain
6	7.0	<6.0
24	65.0	<6.0
30	98.1	<6.0

[a] One to two day old mice were injected with 150 μg of chloroquine. Livers and brains of two animals were harvested individually for chloroquine assay 6 and 24 hr later, and also 6 hr after a second 150 μg dose. Concentrations are expressed as micrograms chloroquine per gram of wet tissue weight.

(7). Our studies suggest that this sensitive and reproducible experimental model (1) may prove useful for the *in vivo* evaluation of the chemotherapeutic potential of substances which inhibit HSV replication *in vitro*.

The observed failure of chloroquine to enhance survival of HSV infected mice despite its ability to inhibit HSV replication in cell cultures of murine and human origin indicates that it is an unpromising candidate for the chemotherapy of human herpesvirus infections. Whether this compound might nevertheless alter the course of latent HSV infection (6) or of HSV-induced cell transformation (10) remains undetermined.

Our findings reemphasize the uncertainties inherent in extrapolating from *in vitro* data to the design of clinical chemotherapy. Lerner and Bailey (8) have recently advocated similar caution regarding utilization of data from *in vitro* assays of idoxuridine content of human body fluids as guides to the pharmacologic control of idoxuridine therapy (9) of patients with herpesvirus encephalitis.

The observed difference in the morphology of the plaques produced by HSV-1 and HSV-2 in BHK-21 cell monolayers resembles that noted by Lerner and Bailey (8) and recently also by McLaren (unpublished). This difference may prove useful for rapid presumptive identification of HSV strains of undetermined serotoype.

Summary. Despite *in vitro* inhibition by chloroquine of the replication of herpesvirus hominis types 1 and 2 in mouse embryo cell cultures, the course of *in vivo* infection of newborn mice was unaltered by chloroquine treatment. This discrepancy between *in vitro* and *in vivo* results suggest caution in the application of data derived from *in vitro* assays to the clinical therapy of herpesvirus infection. Plaques produced in BHK-21 cell cultures by HSV-1 and HSV-2 differed in appearance; this difference may prove useful for the presumptive identification of HSV isolates.

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