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Chloroquine Enhances Replication of Semliki Forest Virus and Encephalomyocarditis Virus in Mice

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Chloroquine (CHL) has been suggested to play an important role in the development of Burkitt's lymphoma by enhancing Epstein-Barr virus expression. Herpes zoster virus incidence is markedly increased following malaria infection in children being treated with CHL. Recently, CHL has also been shown to dramatically increase the transactivation of Tat protein purified from human immunodeficiency virus. These previous studies indirectly suggest that CHL may be involved in the enhancement of virus replication. This study demonstrates for the first time that CHL indeed enhances Semliki Forest virus and encephalomyocarditis virus replication in mice. These results raise the possible connection between the increased spread of AIDS in endemic malaria areas and the wide use of CHL in those areas for the chemotherapy of malaria.

The spread of AIDS has been rapid (4, 8, 9) in tropical Africa, where there is a high incidence of malaria, and chloroquine (CHL) has been frequently used in the chemotherapy of malaria (13, 19). Epstein-Barr virus is thought to be involved in the etiology (14) of Burkitt's lymphoma. Most Burkitt's lymphoma patients have high titers of antibody against Epstein-Barr virus (10, 12). CHL has been shown to enhance Epstein-Barr virus expression (11). Herpes zoster virus is normally uncommon in younger children; however, its incidence was markedly increased (5) in immunosuppressed children who were being treated with CHL following *Plasmodium falciparum* and *Plasmodium vivax* malaria infection. Recently, it has been shown (6) that Tat protein purified from human immunodeficiency virus type 1, the causative agent of AIDS, transactivates the viral promoter and this transactivation is dramatically increased by CHL. So far, most of these studies indirectly suggest that CHL may be involved in the enhancement of virus replication. Studies reported here clearly demonstrate for the first time that CHL dramatically enhances Semliki Forest virus (SFV) and encephalomyocarditis virus (EMCV) replication in mice. These results suggest that the widespread use of CHL in endemic malaria areas may predispose the population to significant viral infections, including AIDS.

BALB/c male mice (Charles River Laboratories, Kingston, N.Y.) weighing 17 to 20 g were used in these studies. SFV was obtained from the American Type Culture Collection, Rockville, Md., and EMCV was originally obtained from C. Buckler (National Institute of Allergy and Infectious Diseases, Bethesda, Md.). Both SFV and EMCV pools were made by intracerebral inoculation of mice. Brains from mice showing specific paralytic symptoms were collected aseptically, and a 10% (wt/vol) brain homogenate was prepared in Eagle's minimal essential medium. The 50% lethal dose (LD_{50}) of the virus in mice was estimated by the Reed and Muench formula. The pool of EMCV was assayed by cytopathic effect in L_B cells, in which it had a titer of 1×10^9 mean tissue culture infective dose per ml; the pool of SFV was assayed by cytopathic effect in Vero cells, in which it had a titer of 5×10^8 mean tissue culture infective dose per ml. CHL was obtained from Winthrop Laboratories, New York, N.Y. Mouse alpha/beta interferon (IFN-α/β; specific activity, approximately 5×10^6 international reference units per mg of protein) was purchased as a frozen, dried powder from Lee Biomolecular Research, Inc., San Diego, Calif. IFN was assayed by inhibition of EMCV-induced cytopathic effect in mouse L_B cells against an international standard obtained from the National Institutes of Health, Bethesda, Md. Poly(I)-poly(C) was purchased from Calbiochem-Behring Corporation, La Jolla, Calif., in freeze-dried form. A stock solution (10 mg/ml) was made in dimethyl sulfoxide, and further dilutions were made in normal saline. The final concentration of dimethyl sulfoxide after dilution in normal saline did not appear to have any adverse effects in mice or in tissue culture.

Mice were treated with CHL (200 μg per mouse) intraperitoneally (i.p.) 24 h before infection, simultaneously with infection, or 24 h postinfection. All the animals were infected with SFV (10 LD_{50} per mouse) subcutaneously (s.c.) and were observed for 14 days for mortality preceded by specific paralytic symptoms. Data (Table 1) show that CHL treatment enhanced the mortality of SFV-infected mice compared with that of untreated control mice, as evident by shortening of the mean survival time (MST).

In the next experiment, mice were infected with SFV (1 LD_{50} per mouse) s.c. or EMCV (1 LD_{50} per mouse) i.p. and simultaneously treated with various doses of CHL (0 to 200 μg per mouse); the mice were then observed for 14 days. CHL even at relatively low concentrations (10 to 50 μg per mouse) significantly enhanced the mortality caused by SFV and EMCV (Table 2). In addition, direct virus titers in brain were determined to demonstrate whether the enhanced mortality was correlated with the increased virus replication. Groups of mice were injected i.p. with CHL (200 μg per mouse), and control mice received saline in place of CHL. Animals were subsequently infected with SFV (10 LD_{50} per mouse) s.c. or with EMCV (10 LD_{50} per mouse) i.p. Three mice from each group were sacrificed daily on each of four days, and brains were collected, pooled, and homogenized (10% wt/vol) in Eagle's minimal essential medium and were then centrifuged to remove the cell debris. Supernatants were assayed for virus titers. An earlier onset of virus in brains of mice treated with CHL compared with untreated,

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TABLE 1. Effects of time of administration of CHL on SFV infection

Treatment[a]	Cumulative no. of mice dead postinfection on day:									MST[b]
	1	2	3	4	5	6	7	8	9	
Virus alone						1	1	4	5	7.8
CHL 24 h before virus					2	4	5	5	5	5.8
CHL with virus					2	5	5	5	5	5.6
CHL 24 h after virus				1	1	4	5	5	5	5.8

[a] Mice (five per treatment) were infected with SFV (10 LD_{50} per mouse) s.c.; CHL (200 μg per mouse, i.p.) was given at the times indicated.

[b] MST was calculated as follows. The total number of deaths occurring each day was multiplied by the number of days that each animal remained alive. The process of computation was continued until day 14, when all surviving animals were scored as having succumbed on that day; MST is expressed as a quotient obtained by dividing the total number of days mice were observed alive by the number of animals in the group.

infected animals was seen (Fig. 1). The magnitude of enhancement in virus multiplication in CHL-treated mice was highly significant. We have also determined the appearance of virus titer in various organs of mice infected with EMCV in the presence or absence of CHL. Three mice from each group were sacrificed after 2 days, and different organs were collected, pooled, and homogenized (10% wt/vol) in Eagle's minimal essential medium. Virus titers of supernatants were determined in L_B cells. Data (Fig. 2) show that CHL treatment enhances the virus replication in various organs. Maximum enhancement was obtained in brain and spleen (30- to 1,000-fold), and lower enhancement was obtained in liver, lung, and kidney (5- to 10-fold). Similar enhancement in virus replication was seen in various organs in mice infected with SFV and treated with CHL (data not presented).

It is possible that CHL may inhibit the endogenous production of IFN, which may be responsible for the enhancement of virus virulence. In order to explore this possibility, we treated virus-infected mice with IFN or poly(I)-poly(C) in the presence or absence of CHL. Mice were injected with IFN (40,000 U per mouse, intramuscularly [i.m.], for SFV and 50,000 U per mouse, i.m., for EMCV) or poly(I)-poly(C) (20 μg per mouse, i.p.) or left untreated. After 16 h, CHL (50 μg per mouse, i.p.) and either SFV (2 LD_{50} per mouse, s.c.) or EMCV (10 LD_{50} per mouse, i.p.) were administered. The second doses of IFN (10,000 U per mouse, i.m.) and CHL (200 μg per mouse, i.p.) were given 24 h after the first dose of IFN and/or CHL. Animals

TABLE 2. Dose effects of CHL on SFV and EMCV infection

Treatment[a] (μg)	SFV		EMCV	
	No. of survivors (%)	MST	No. of survivors (%)	MST
Virus alone	4 (80)	12.2	2 (40)	8.2
Virus + CHL (200)	1 (20)	8.0	0 (0)	4.0
Virus + CHL (100)	1 (20)	7.8	0 (0)	4.0
Virus + CHL (50)	1 (20)	8.6	0 (0)	4.4
Virus + CHL (10)	3 (60)	11.2	0 (0)	4.6

[a] Mice (five per treatment) were infected with SFV (1 LD_{50} per mouse) s.c. or EMCV (1 LD_{50} per mouse) i.p. and were subsequently injected with various doses of CHL i.p. Animals were observed for 14 days, and then MSTs were calculated.

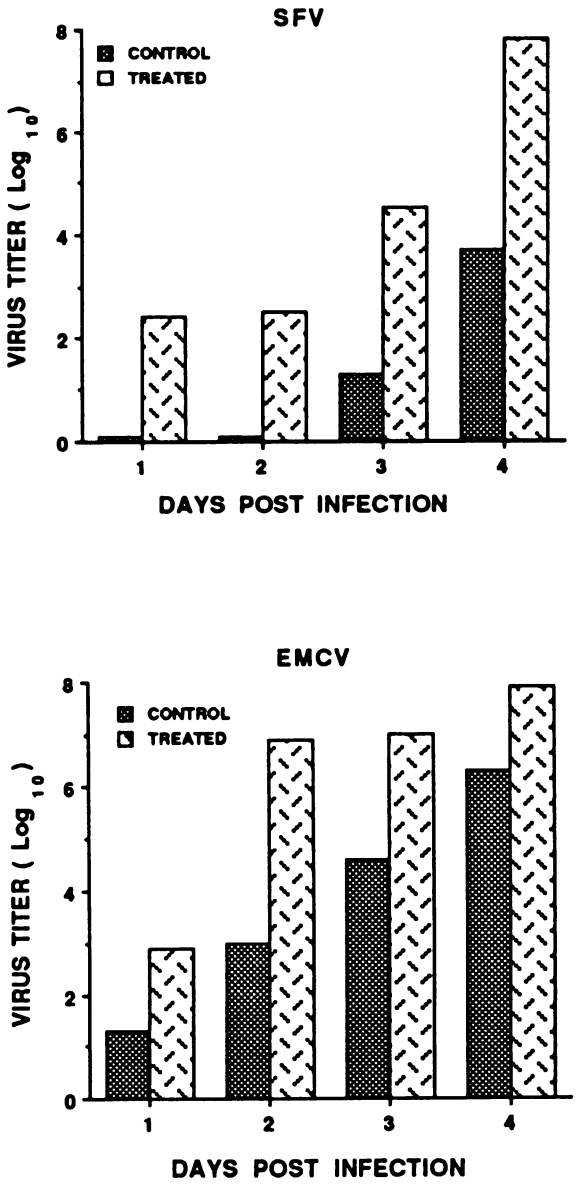

FIG. 1. Kinetics of the appearance of virus in brains of mice. Mice were infected with SFV (10 LD_{50} per mouse, s.c.) or EMCV (10 LD_{50} per mouse, i.p.) and injected simultaneously with CHL (200 μg per mouse, i.p.). Three mice from each group were sacrificed daily on each of four days, and brain homogenates (wt/vol) were prepared and pooled. The SFV titer was determined by cytopathic effect assay in Vero cells, and the EMCV titer was determined in L_B cells.

were observed over 14 days for paralytic symptoms and mortality. Mice infected with EMCV or SFV alone showed high mortality compared with normal, uninfected mice. Administration of IFN or poly(I)-poly(C) to virus-infected mice conferred significant protection. CHL itself did not protect mice against SFV or EMCV and in fact enhanced the severity of the disease, as evident by the shortening of MST compared with that in untreated controls. CHL administration significantly impaired the protective effects of IFN and poly(I)-poly(C) against SFV or EMCV (Table 3). These

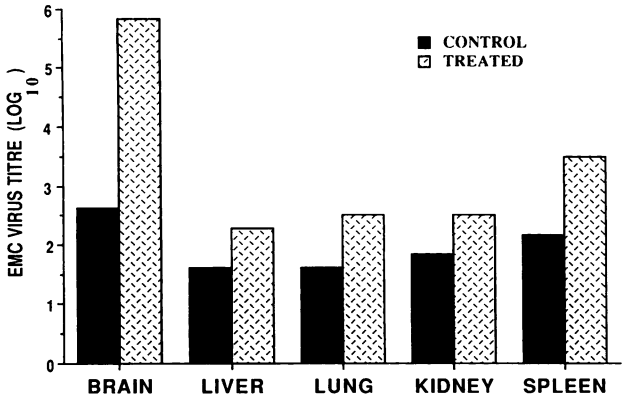

FIG. 2. Effects of CHL on the appearance of EMCV in various organs of mice. Mice were infected with 10 LD_{50} of EMCV, i.p. One group of mice was left untreated and the other group received CHL (200 μg per mouse, i.p.). After 2 days, three animals in each group were sacrificed and various organs (viz., brain, liver, lung, kidney, and spleen) were collected and a 10% homogenate was prepared. Samples were centrifuged at 4,000 × *g* for 20 min to remove cell debris, and virus titers of supernatants were determined in L_B cells.

results may have important clinical implications, especially in the use of IFN against viral infections in endemic malaria areas where CHL is one of the most widely used antimalarial drugs.

Studies reported in this paper clearly demonstrate for the first time that CHL enhances virus replication in mice. However, it remains unclear how CHL specifically enhances virus multiplication in vivo. Since the effects of CHL appear quite early, it is likely that CHL may affect certain cell populations responsible for maintaining the natural immunity of the body, such as natural killer cells or macrophages; the function of these cells is augmented by IFN, and these cells may be involved in the control of viral infections (1, 7). Any perturbation or inhibition of these cells by CHL may lead to the increase of viral multiplication (3, 17, 18). In fact, various antimalarial drugs (viz., CHL, mefloquine, quinine, pyrimethamine, and sulfadoxine) have been shown to suppress natural killer cell activity in vitro (15, 16). Preliminary results show that CHL inhibited the natural killer cell activity in vivo. Moreover, the tissue concentrations of CHL are well below those needed to elevate endosomal pH above the pH threshold for SFV fusion and entry. These results suggested that natural killer cells may play an important role in natural resistance against some tumors and viral, fungal, and bacterial infections; it is possible that people who receive treatment with antimalarials are at high risk of developing certain infections, including AIDS, in endemic malaria areas (5, 11, 14, 15).

TABLE 3. Effects of IFN, poly (I)-poly(C), and/or CHL on virus infection in mice

Treatment[a]	No. of survivors (%)	MST
EMCV alone	0 (0)	4.0
EMCV + CHL	0 (0)	3.0
EMCV + IFN	3 (60)	11.2
EMCV + IFN + CHL	0 (0)	5.2
EMCV + poly(I:C)	3 (60)	11.6
EMCV + poly(I:C) + CHL	0 (0)	6.2
SFV alone	1 (20)	7.4
SFV + CHL	0 (0)	5.0
SFV + IFN	3 (60)	12.5
SFV + IFN + CHL	0 (0)	7.6
SFV + poly(I:C)	4 (80)	13.2
SFV + poly(I:C) + CHL	1 (20)	8.8

[a] Mice (five per treatment) were injected with IFN (40,000 U per mouse i.m. for SFV and 50,000 U per mouse i.m. for EMCV) or poly(I)-poly(C) (20 μg per mouse); CHL (200 μg per mouse, i.p.) was injected after 16 h, and animals were subsequently infected with SFV (2 LD_{50}, s.c.) or EMCV (10 LD_{50}, i.p.). The second dose of IFN (10,000 U per mouse, i.m.) and/or CHL (200 μg per mouse, i.p.) was given 24 h after the first dose of either drug. Animals were observed for 14 days, and MSTs were then calculated.

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