AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 6, Number 4, 1990
Mary Ann Liebert, Inc., Publishers

Inhibition of Human Immunodeficiency Virus Infectivity by Chloroquine

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ABSTRACT

The effect of chloroquine, a drug known to affect intracellular exocytic pathways, was studied in two retroviral systems: human immunodeficiency virus (HIV-1) and avian reticuloendotheliosis virus (REV-A). With chloroquine treatment of virus-infected cells, significant size reduction of the cell- and virus-associated surface glycoproteins, gp90 of REV-A and gp120 of HIV-1, was observed. In the case of HIV-1, extracellular virions derived from treated cells contained very little gp120. Infectivity and reverse transcriptase assays of HIV-1 demonstrated that by chloroquine treatment the majority of the virions released was noninfectious and the total virus yield was also reduced. The data suggest that chloroquine inhibition of infectious virus production is most likely due to interference with terminal glycosylation in the *trans*-Golgi network.

INTRODUCTION

Weak bases such as chloroquine are known to cause vacuolation and swelling of intracellular acidic compartments and to raise the pH of these compartments.[1-6] This group of agents has been used to study cellular endocytic/exocytic pathways and posttranslational processing of glycoproteins.[3,4,5,7,8] They have been extensively used to study the entering events of animal viruses into cells.[9-18] In a recent report,[19] we described proteolytic processing and novel glycosylation pathways for retroviral envelope proteins and characterized the *env* precursor polyprotein of avian reticuloendotheliosis virus (REV-A) as being modified by unusually large sialic acid-rich complex-type carbohydrate moieties. These results prompted us to study in this viral system the effect of chloroquine, which has been previously shown to inhibit the proteolytic processing and terminal glycosylation of secretory proteins.[7,8] With REV-A, a type C retrovirus, we have found that chloroquine profoundly affected glycosylation of the surface glycoprotein. In light of these results, it was of special interest to us to examine the effect of chloroquine on human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS). Although previous studies of

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Stein et al.[18] showed that HIV entry into T cells by the CD4 receptor-mediated endocytosis is pH-independent and not inhibited by weak bases, the effect of chloroquine on the exocytic pathways leading to the maturation and release of infectious HIV was not studied. In this publication we report that chloroquine, a member of 4-aminoquinoline series and most commonly used antimalarial agent[20] strongly inhibits the infectivity of HIV-1 produced in chronically infected H9 cells.

MATERIALS AND METHODS

Cell lines and viruses

A chicken bone marrow cell line persistently producing REV-A (REV/cBMC)[21] was used to study intracellular and extracellular REV-A proteins. The H9 cell line that was persistently and productively infected with HTLV-IIIB[22,23] was used for the similar studies, and that infected with HIV-RFII,[22,23] for studies of chloroquine effects on HIV infectivity. The HIV-1s were obtained from R. Gallo, and were prepared and stocked as described previously.[24] The CEM-ss, a biological clone from the CEM cell line, was used for the microtiter syncytial-forming assay.[24,25] The cell lines, both infected and uninfected, were maintained in RPMI-1640 supplemented with heat-inactivated 10% fetal bovine serum (FBS), 1% L-glutamine and penicillin-streptomycin.

Antisera

The antisera raised in rabbits against various REV-A proteins were prepared and characterized as described.[19,26–28] The antisera to HIV p24 were obtained by immunizing rabbits with inactivated HTLV-IIIB, and those to gp120 was raised in rabbits by immunizing with HTLV-IIIB gp120 C-terminal synthetic peptides.

Metabolic labeling of cell cultures and viruses, radioimmunoprecipitation, SDS-PAGE, and autoradiography

The procedures were described in general previously[19] and are detailed in the legends to Figures 1 and 2.

Microtiter syncytial-forming assay

The assays performed as described by Nara et al.,[24,25] were used to determine the HIV infectivity, and the effects on the infectivity by chloroquine (Sigma).

Reverse transcriptase assay and cell viability

A standard reverse transcriptase (RT) assay procedure was performed as follows. To a 10 μl sample, Triton X-100 and dithiothreitol (DTT) solutions were added to final concentrations of 0.5% Triton X-100 and 0.015% DTT before the assays were performed. Ten microliters of the samples were then mixed with 30λ of magnesium cocktail and 10λ of [^{3}H]TTP (preparations of the solutions are described as below). The mixture was incubated at 37°C for 30 min. Samples were harvested onto DE81 ion-exchange paper (Whatman) and allowed to absorb for 15 min. The papers were soaked and washed in 5% Na_2HPO_4 buffer, followed by additional washings with distilled water. The papers were then dried and counted. The formula for magnesium cocktail: 1 M Tris-HCl pH 7.8 (2 vol), 3 M KCl (1 vol), 0.3% (w/v) DTT (5 vol), 0.1 M magnesium acetate (5 vol), poly(rA)-p(dT)$_{12–18}$ of 2 U/ml (Pharmacia) (10 vol), distilled water (6.5 vol), and 10% Triton X-100 (0.5 vol); the formula for ^{3}H[TTP] solution: [Methyl-^{3}H]thymidine 5′ triphosphate (ammonium salt) diluted in 1:5 with water before use. Each sample was run in triplicate and the results are shown in counts per milliliter (cpm) of each of the supernatants. Each of the relative values is also shown in parentheses using the 0 μM treatment as 100. Cell viability was determined by trypan blue dye exclusion.

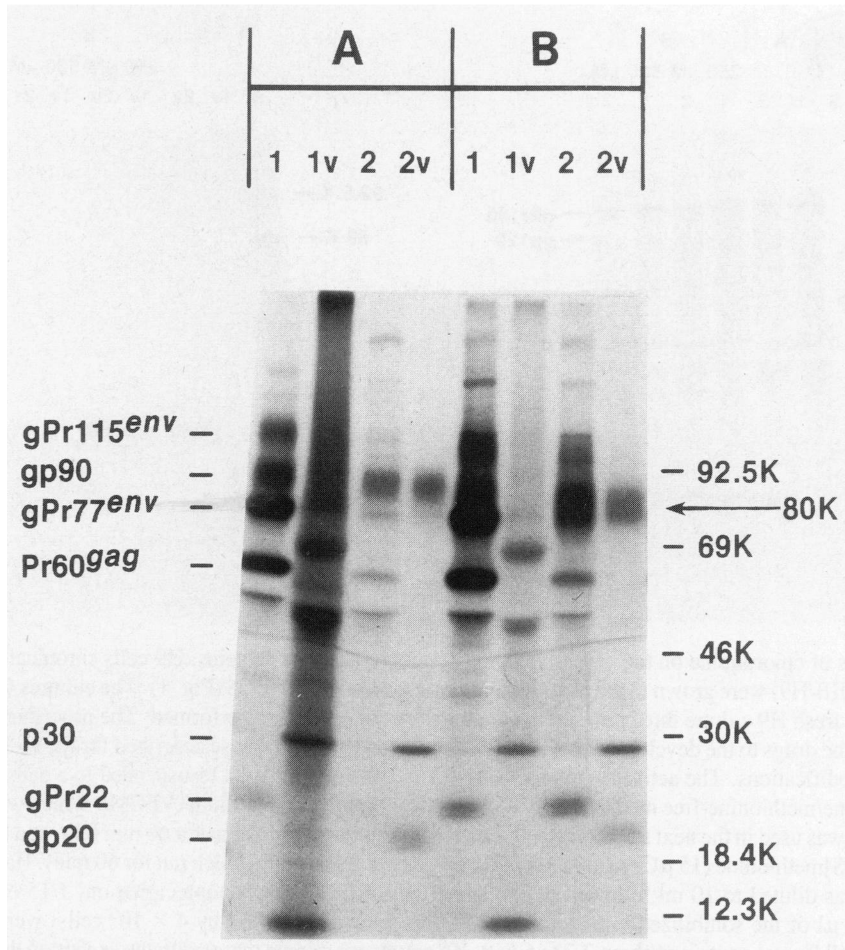

FIG. 1. Effects of chloroquine on the processing of REV-A *env* gene-encoded proteins. REV/cBMC was grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin. For drug treatments, the actively growing cells (~ 10^6 cells/ml) were clarified by centrifugation and resuspended at a density of 10^7 cells/ml in cysteine-free RPMI-1640 plus 10% dialyzed FBS, 1% L-glutamine, and antibiotics. A stock solution of the drug was added to a 10 ml cell suspension followed by labeling with [^{35}S]cysteine (30 μCi/ml) for 60 min of incubation in 5% CO_2 at 37°C. At the end of the incubation, the cell suspension was divided equally into two tubes and then clarified and washed by centrifugation at 500 to 1000 rpm for 5 min using an IEC HN-S centrifuge (Damon/IEC Division). After washing, one tube of the sample was then disrupted with 0.5 ml of lysing buffer [LB: 0.02 M Tris-HCl pH 7.5, 0.05 M NaCl, 0.5% sodium deoxycholate, 0.5% NP_4O, plus aprotinin (10 μg/ml) and phenylmethylsulfonyl fluoride (PMSF, 1 mM)]. Cell lysates were clarified at 15,000 rpm (Eppendorf microcentrifuge 5415) for 10–30 min. The virus was harvested by pelleting from clarified culture media using centrifugation at 40,000 rpm (Beckman L3-50 ultracentrifuge) for 90 min in nitrocellulose tubes that were cushioned at the bottom with 20% sucrose in TNE buffer (10 mM Tris-HCl pH 7.0, 0.1 M NaCl, 0.001 M EDTA). The virus pellets were then solubilized with 100 μl of LB. The other tube of the sample was resuspended in 10 ml of the complete medium and the incubation was resumed for an additional 150 min in the presence of the drug with the same concentration as for the labeling. At the end of the chase, cell lysates were obtained and viruses were harvested and solubilized as described above. The samples for the control were treated similarly without drugs. To perform immunoprecipitations, 10% of the samples was used. The samples were reacted with a mixture of antibodies to REV-A gp20, gp90 peptide and p30[19,26–28] in the presence of protein A Sepharose at 4°C overnight. The immunoprecipitates were washed and subjected to SDS-PAGE on a 7.5 to 18% gradient. The gels were dried and autoradiographs were developed from the gels. A stock solution of 0.1 M for chloroquine-diphosphate (Sigma) was prepared in double-distilled sterile water. The pH of the solutions that were diluted to the final concentrations with media were about 7.1 to 7.35. The autoradiograph made from an overnight exposure shows the following results: Panel A, control; Panel B, treatment with chloroquine-diphosphate (100 μM). Lanes 1 (cell lysates) and lv (viruses), pulse; lanes 2 (cell lysates) and 2v (viruses), chase.

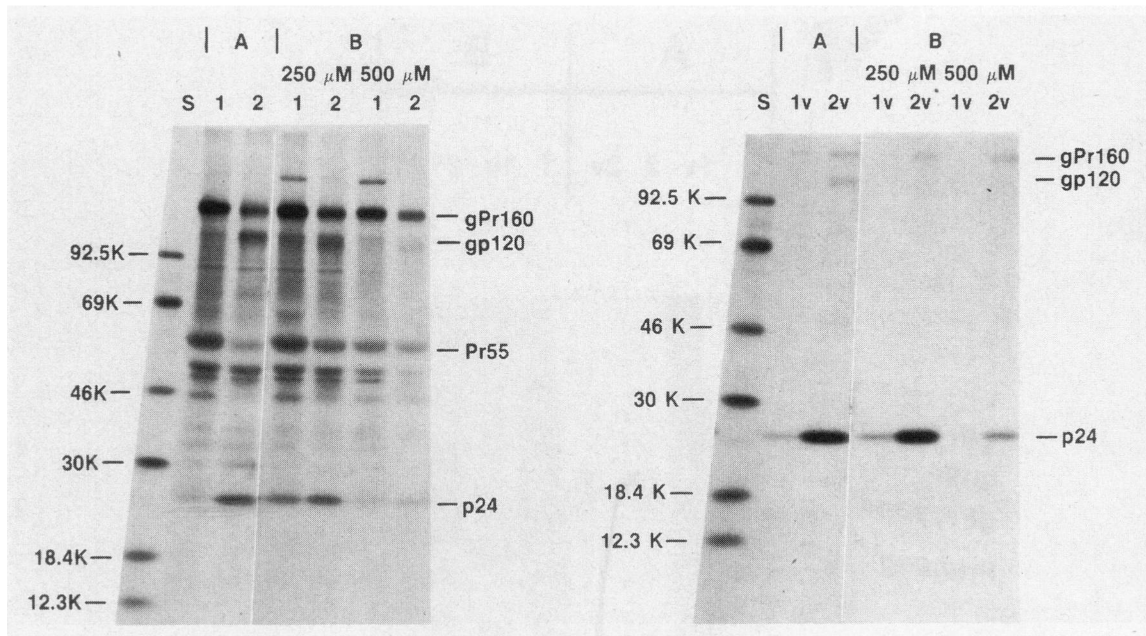

FIG. 2. Effects of chloroquine on the processing of HIV-1 gene-encoded proteins. H9 cells chronically infected with HIV IIIB (HIV-IIIB/H9) were grown in the medium that was used for REV/cBMC (Fig. 1). The cultures were diluted and replenished with fresh H9 culture three days before the drug experiments were performed. The procedures that involved the treatment of the drugs to the development of autoradiographs are similar to those described for REV/cBMC except for the following modifications. The actively growing cells at 2×10^5 cells/ml were resuspended to a density of 20×10^5 cells/ml in cysteine/methionine-free medium plus 10% virus-free conditioned medium (VFCM), which was also included in the media that was used in the next steps. The cells (10 ml) were treated with drugs for 60 min followed by labeling with [^{35}S]cysteine/[^{35}S]methionine (15 μCi each/ml) for 90 min (except the control, which ran for 60 min). Half of the labeled culture (5 ml) was diluted to 10 ml followed by the chase for 3.5 h. For immunoprecipitation, 1/15 of the cell lysate (100 μl) and 40 μl of the solubilized virus (equivalent to the amount produced by 4×10^5 cells) were used for each treatment. The cell lysates contained about 2.7 to 6.8×10^6 counts per minute (cpm) with the exception that B2 (500 μM) were in the range $0.3–1.5 \times 10^6$ cpm; the solubilized viruses contained 0.10 to 0.21×10^6 cpm. A mixture of two antisera was used: Antiserum to gp120, which was raised in rabbits by immunizing with HTLV-IIIB gp120 C-terminal synthetic peptide, and was shown to recognize gp120 and gp160; p24 antibodies, which was obtained by immunizing rabbits with inactivated HTLV-IIIB and was shown to precipitate p24 and Pr55. The results are shown in the autoradiographs: The left panel depicts cell lysates; the right panel, virus. Section A, control; Section B, treatment with chloroquine (250 and 500 μM). Lanes 1 (cell lysate) and lv (the virus), pulse; lanes 2 (cell lysate) and 2v (the virus), chase.

RESULTS AND DISCUSSION

First, we present our studies of the effect of chloroquine on the glycosylation pathways and the maturation of REV-A envelope glycoprotein. In a typical pulse-chase experiment, REV-A-producing chicken bone marrow cells (REV/cBMC)[21] grown in suspension culture were labeled with [^{35}S]cysteine in the presence and absence of chloroquine. Cells and extracellular virus were harvested after the pulse as well as the chase, and then lysed (see Fig. 1). Radiolabeled proteins were precipitated with a mixture of previously individually characterized monospecific antibodies to REV-A capsid protein (p30), transmembrane protein (gp20), and surface glycoprotein (gp90)[19,26–28] and analyzed by SDS gel electrophoresis followed by autoradiography. The results are shown Figure 1. The patterns from the culture grown in the absence of chloroquine (Panel A) are consistent with our previous findings[19] showing that gPr77^{env} is the high-mannose-type primary envelope precursor and gPr115^{env} is the complex-type secondary precursor, which is processed in the Golgi network into the mature gp90 (complex type) and gp22 (high-mannose type), the intermediate precursor to gp20 of the

mature virion. Treatment of cells with 100 μM chloroquine (Panel B) resulted in a substantial reduction of the molecular size of the sialic acid-rich complex-type glycoproteins, both the precursor, gPr115^{env} and gp90. This is clearly seen after the chase (lanes B2 and B2v). The observed ~ 10 kD size reduction (see band labeled 80K in lane B2v) is nearly equivalent to the total size of the three terminal sialoligosaccharide chains (~4 kD each) found in the three N-linked complex carbohydrate moieties of gp90.[19]

To determine whether chloroquine exerts any effect on HIV-1 glycoproteins, immunoprecipitation experiments were performed using H9 cells chronically infected with the HTLV-IIIB strain[22,23] of HIV-1 and a mixture of antibodies specifically recognizing gp120 and p24. The results are shown in Figure 2 (left panel for cell lysates and right panel for the virus). In the presence of 250 μM chloroquine, the cellular gp120 after the 3.5 h chase appears to be only slightly reduced in size (left panel, lane B2, compared with lane B1 of the 90 min pulse or with lanes A1 and A2 of the control), but its incorporation into the extracellular virion was apparently retarded. Note the presence of gp120 in lane A2V, left panel (control) and its nearly complete absence in lane B2v, right panel. As suggested from the REV-A study and to be discussed further later, the size reduction of HIV-1 gp120 may also be due to the effect of chloroquine on terminal sialylation. The minor size reduction of gp120 relative to the much greater effect on REV-A gp90 is consistent with recent findings from biochemical studies[29,30] showing that terminally sialylated complex-type oligosaccharides of gp120 contain only one or two neuraminic acid residues per chain in contrast to REV-A gp90 containing unusually large terminal polysialic acid chains.[19] When the cells were treated with chloroquine at a higher concentration (500 μM), the effects were similar to those with 250 μM, however, at the higher concentration less incorporation of the label into proteins was observed, probably due to cell toxicity and inhibition of protein synthesis. The effects of primaquine, a member of the 8-aminoquinoline series[20] were similar to those found with chloroquine (data not shown).

Next we studied the effect of chloroquine on the infectivity of HIV-1. H9 cells infected with HIV-1 RF11[22,23] were first treated with chloroquine at various concentrations for 45 to 60 min and then washed. Treatment with the drug in fresh medium was resumed for 4 h. The cell-free supernatants were then collected to determine the virus titers by infectivity assays. A microtiter syncytial-forming assay with cloned CEM cells (CEM-ss) as indicator cells was used to measure syncytial-forming units (SFU) as described previously.[24,25] The supernatants were also tested for reverse transcriptase (RT) activity. Cell viability was determined by trypan blue exclusion. The results of two independent experiments are shown in Table 1. For virus produced in the presence of 100 μM chloroquine, the number of SFUs was reduced to about 50% of the untreated. Increasing the drug concentration in the medium resulted in substantially greater inhibition. At the highest concentration of chloroquine used (300 μM), virus infectivity was inhibited by 84% in the first and 76% in the second experiment. The reductions were not due to cell death, since cell viability was apparently not affected at these concentrations (Table 1). Nor were the reductions due to the effect of the drug on the indicator cells during the virus adsorption period. Titration of supernatants collected from the control cells grown without chloroquine to which the drug was added (300 μM) before serial dilutions did not show significant reduction of SFUs compared with the control and shown by data of Table 1 [see line 0 μM (+)].

In a recent comparable study, McClure et al.[16] showed that treatment of the HIV-infected T cells with NH_4Cl (30 mM) for 18 h resulted in 95% reduction in the production of infectious virus and 10 to 15% cell death, which is similar to the reduction of infectivity by chloroquine in our study. With chloroquine treatments, the RT activity was reduced in the two separate experiments to about 90–94%, 54–75%, and 41–55%, at 100, 200, and 300 μM, respectively (Table 1). The drug did not affect the RT assay itself (see footnotes to Table 1). The reduced RT activity is likely due to the decrease in the number of virions produced. However, the extent of reduction of RT was apparently less than that of infectivity (Table 1).

N-linked carbohydrate structures of HIV gp120 were found by biochemical studies to be of unique diversity containing high-mannose type, hybrid-type, and four categories of complex-type chains, with or without *N*-acetyl-lactosamine repeats and a core region fucose residue.[29,30] The functional roles of carbohydrates in studied by various approaches including the use of glycosidases[31–34] and lectins.[35,36] The effects of glycosylation inhibitors that block trimming enzymes functioning the early stages of the glycosylation in pathways were also studied.[31,36–42] Chloroquine is known to raise the acidic milieu of the Golgi apparatus,[1,3–5] and to affect the proteolytic processing of secretory proteins that occur in these compartments.[7,8] Furthermore, as shown in this study with REV-A, chloroquine appears to inhibit sialylation

TABLE 1. EFFECTS ON THE SYNCYTIAL FORMATION AND REVERSE TRANSCRIPTASE ACTIVITY OF HUMAN IMMUNODEFICIENCY VIRUSES PRODUCED BY INFECTED CELLS TREATED WITH CHLOROQUINE[a]

	Experiment 1			Experiment 2		
Chloroquine	*SFU/ml*	*RT (cpm × 10³/ml*	*Cell viability %*	*SFU/ml*	*RT (cpm × 10³/ml)*	*Cell viability %*
0 μM	72,540 (100)	404 (100)	90.3	76,230 (100%)	806 (100)	91.2
100 μM	33,656 (46.4)	303 (77.2)	89.8	41,220 (54.1)	762 (94.4)	89.2
200 μM	19,420 (26.8)	223 (53.5)	90.5	38,430 (50.4)	607 (75.1)	95.7
300 μM	11,400 (15.7)	158 (41.1)	86.3	20,460 (23.8)	447 (55.3)	91.4
0 μM(+)	68,940 (95.0)	334 (84.8)	—	69,660 (91.4)	992 (122.8)	—

[a]Quantitative microtiter syncytial-forming assay (SFA) developed by Nara et al.[(24,25)] was used to measure syncytial-forming units (SFU). Briefly, the infected cells (RFII/H9) growing in active phase in complete RPMI-1640 were washed and then treated with the drugs of various concentrations for 45-60 min. The treated cells were washed again and re-treated with the drugs in the complete media, respectively, in the same concentrations as for the pretreatments for an additional 4 h. The cells were then incubated at 37°C, 5% CO_2. For each treatment, 2 ml of cells (about 5 to 10 × 10^5 cells/ml) were used. The cells without the drugs served as the controls. After incubation, 1 ml of cell suspension from each of the treatments was centrifuged and cell-free supernatants were used for SFA and the RT assay. The remaining 1 ml of cell suspension was used to examine the cells for viability. The supernatants, undiluted and diluted at 1:3, 1:9, and 1:18, were used for SFA. For the controls, the supernatants were divided into two portions. One portion was diluted as above and indicated as the 0 μM treatments to serve as the controls without the drugs. To the other portion, the drugs were added to a concentration of 300 μM followed by dilutions as above, and the samples were indicated as the 0 μM(+) treatments to serve as the control for the drug effect on the CEM-ss cells during the virus adsorption period. Each dilution was run in duplicate in complete media and 50 μl sample from each was mixed with an equal volume (50 × 10^3 cells) of CEM-ss for SFA. The mixtures were then placed in 96-well tissue culture plates pretreated with poly-L-lysine and incubated at 37°C, 5% CO_2, for 60 min. The supernatants were then removed and 210 μl of complete media was replaced in each well. After a 3 day incubation, the SFUs were counted. The means of SFUs of the duplicate samples from each dilution in the range of 10–250 SFUs per well were taken to estimate SFU/ml of supernatants, and the means from two different dilutions were used to estimate the mean of SFU/ml for each of treatments as shown in the Table. Each relative value of SFU/ml is shown in parentheses as percentage on the basis of the 0 μM treatments as 100. In a separate experiment to determine the effect of chloroquine on the RT assay itself, the culture supernatants of virus-producing untreated RF/H9 cells were incubated with increasing concentrations of chloroquine for 4 h at 37°C before RT assays. The relative RT activities were 100%, 99%, 90%, and 96% at 0, 100, 200, and 300 μM chloroquine, respectively.

of REV-A gp90 that likely occurs in the *trans*-Golgi networks[19] with which sialyltransferases are associated in certain cell types.[4,43] Studies with monension showed that this drug reduced HIV infectivity and syncytial formation,[44,45] likely by blocking sialylation and partially affecting the addition of galactose and fucose.[44] Treatment of HIV with neuraminidase reduced its infectivity as well as the size of gp120.[34] These results support that terminal glycosylation of gpl20 may play a crucial role for HIV infectivity. Chloroquine may also inhibit cleavage of gp160 (see Fig. 1). Another weak amine, NH_4Cl, was shown to have such inhibiting effects.[46] However, the exact mechanisms by which chloroquine inhibits HIV infectivity has yet to be determined by direct analysis of HIV gp120/160 from chloroquine-treated cells. From our studies, chloroquine appeared to be an effective inhibitor of HIV-1 by reducing both the yield and infectivity of the virus produced in chronically infected cells. This effect of chloroquine is consistent with the reported inhibition of HIV infectivity by NH_4/Cl.[16] However, 100% inhibition was not obtained even at the highest concentration of chloroquine used. A likely explanation may be the relatively short exposure (45–60 min) to chloroquine prior to the start of collecting extracellular virus. Thus, normal infectious particles formed prior to treatment may have been present at low levels in the virus stocks we assayed.

During the preparation of our manuscript, Frankel and Pabo[47] reported that chloroquine (100 μM) had dramatically enhanced transactivation of HIV-1 promoter by exogenous *tat* protein, which was supplied to cells in the culture medium by inhibiting lysosomal degradation of *tat*. Although chloroquine had no effect on *tat* protein produced intracellularly, the authors made a connection between AIDS and malaria. They raised

the question whether chloroquine, a drug that has been frequently used for treatment of malaria, could enhance *tat* activity and thereby increase the chance of activating latent HIV. Although this question cannot be answered directly from our studies with chloroquine, it is clear, based on the results we presented, that chloroquine treatment of HIV-infected cells results in a strong inhibition of infectious virus production. Further studies are required to determine the effect of chloroquine on HIV-1 and its relatives produced by other cell types, for example, monocytes-macrophages and to evaluate whether chloroquine and its existing analogs[20] or newly synthesized related weak bases could be useful either alone or in combination with other drugs to attack various stages of the virus lifecycle in an infected individual.

ACKNOWLEDGMENTS

We thank Sally Lockhart for performing SDS-gel electrophoresis analysis, William Hatch for providing HIV-IIIB/H9 cell cultures, James P. Dobbs for the RT assay, Nancy Dunlop for invaluable advice on the microtiter infectivity assay, Dr. John R. Ortaldo for providing the BL-3 laboratory, Dr. Alan Rein for valuable discussion, and Cheri Rhoderick for preparation of the manuscript. We are also indebted to Terry Copeland for antiserum to HTLV-IIIB gp120 and to Julian Bess and Larry Arthur for antiserum to HTLV-IIIB p24.

This research was sponsored in part by the National Cancer Institute, DHHS under contract No. N01-CO-74101 with BRI, Basic Research Program. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

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