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Inhibition of Human Immunodeficiency Virus Type 1 Replication by Hydroxychloroquine in T Cells and Monocytes

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ABSTRACT

Chloroquine and its analogue hydroxychloroquine (HCQ) have been shown to inhibit a variety of viral infections including influenza and adenovirus through blockade of viral entry via inhibition of endosomal acidification. We have extended these observations to human immunodeficiency virus type 1 (HIV-1) infection utilizing primary T cells and monocytes, a T cell line (CEM), and a monocytic cell line (U-937). HCQ inhibited HIV-1 replication (>75%), as measured by reverse transcriptase activity, in the primary T cells and monocytes as well as the T cell and monocytic cell lines. HCQ itself had no anti–reverse transcriptase activity and was not toxic to the cells at concentrations inhibitory to viral replication. Intracytoplasmic staining with an anti–p24 antibody, 24 h after infection, revealed the presence of intracytoplasmic virus, suggesting that the drug does not block viral entry. The production of steady-state HIV-1 mRNA was not affected by HCQ in that comparable levels of HIV-1 mRNA could be detected by Northern blot analysis and by in situ hybridization in both the HCQ-treated and untreated cells. However, HCQ does appear to affect production of infectious HIV-1 virions because viral isolates from HCQ-treated cells could not infect target CEM cells. These data suggest that HCQ may be useful adjunctive therapy in the treatment of HIV-1 infection.

INTRODUCTION

NUMEROUS AGENTS ARE PRESENTLY available that inhibit human immunodeficiency virus type 1 (HIV-1) replication in T cells and monocytes.[1,2] These compounds are limited in their usefulness, however, owing to significant toxicities and potential viral resistance associated with their long-term use.[3] Other agents, less toxic with a long safety record, would be of obvious importance in treating HIV-1 infection both as a primary modality or in combination with other therapies. The lysosomotropic agents, chloroquine and its analogue hydroxychloroquine (HCQ), have been shown to inhibit two retroviral infections (Rauscher and Moloney leukemia viruses) in murine model systems[4] and have been used safely for a number of years to treat both malaria and a variety of collagen vascular diseases.[5] In this study, we document the ability of HCQ to inhibit replication of various strains of HIV-1 in monocytic and T cell lines and primary T cells and monocytes. The mechanism of viral inhibition is distinct from HCQ's effect in other nonretroviral systems like influenza and adenovirus (blockade of entry).[5] HCQ appears to inhibit post-transcriptional production of HIV-1.

MATERIALS AND METHODS

Cell lines

The U-937 promonocytic cell line and the CEM T cell line were obtained from the American Type Culture Collection (Rockville, MD). All cells were grown in Iscove's modified Dulbecco's medium (GIBCO, Grand Island, NY), 10% fetal calf serum (FCS; GIBCO), 1% penicillin, and streptomycin (GIBCO), and 2 mM glutamine (GIBCO).

Isolation and culture of T cells and monocytes

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood of normal blood donors at the Mount Sinai

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Blood Bank. The cells were isolated by Ficoll-Hypaque (Pharmacia, Inc., Piscataway, NJ) density gradient centrifugation.[6] Isolated cells were washed in phosphate-buffered saline (PBS) and allowed to adhere onto plastic dishes for 45 min.[6] The nonadherent cells were removed, washed, resuspended, and adjusted to a concentration of 5.0×10^6 cells/ml in RPMI (GIBCO) supplemented with 10% FCS (GIBCO), 1% penicillin and streptomycin (GIBCO), and 2 mM glutamine (GIBCO) henceforth termed culture medium (CM). The adherent population was extensively washed with sterile PBS and cultured in CM. T cells were isolated by rosetting nonadherent cells with neuraminidase-treated sheep red blood cells (SRBC).[7] The cells were adjusted to a concentration of 0.5×10^6 cells, cultured in CM supplemented with 10% interleukin-2 (IL-2; Electronucleonics, Silver Springs, MD), and used for the infection protocol described below.

HIV-1 strains and patient isolates

HIV-1_{III-B} and HIV-1_{BR-1} were obtained from the AIDS Research and Reference Laboratory and have been well characterized.[8,9] The patient isolates were obtained by mitogen stimulation of PBMC from HIV-1–infected patients.[10] Isolated T cells were adjusted to a concentration of 0.5×10^6/ml and cultured in CM containing PHA (1 μg/ml) (GIBCO) for 3 days after which time cell-free supernatants were harvested.[11] Viral concentrations of these supernatants were determined by reverse transcriptase activity (RT) and p24 assays (see below). For infection, all virus stocks were standardized to 80,000 cpm/ml in the RT assays.[11,12] This is equivalent to a $TCID_{50}$ of 8×10^2 for both HIV_{IIIB} and HIV_{BR-1}.

HIV-1 infection of primary T cells and monocytes and T cell and monocytic cell lines

Primary T cells and monocytes, the CEM T cell line and U-937 monocytic line were infected with various strains of HIV-1 including HIV-1_{III-B}, HIV-1_{BR-1} as well as several patient isolates as previously described.[11,12] One-half million cells were infected by cocultivation of an infectious supernatant, standardized to 80,000 cpm/ml, for 90 min at 37°C. After washing to remove residual virus, CEM and U-937 cells were cultured at a final concentration of 0.5×10^6/ml in CM and Iscove's, respectively, at 37°C. In some experiments, cells were incubated for 60 min with serial 10-fold dilutions of 1 mM HCQ or sterile PBS before infection.

RT assay and p24 antigen capture assay

RT activity and p24 antigen were measured in the culture supernatants 7 days after infection of the cell lines or 72 h after phytohemagglutinin (PHA) stimulation of the PBMC cultures. RT activity was detected in supernatants of infected cultures by a modification of the technique of Willey et al.[13] using [^{3}H]thymidine triphosphate incorporation onto a template primer poly (rA · oligo (dT) (Pharmacia, Inc.) and is displayed as the mean cpm/ml of culture supernatant. The p24 antigen values were determined using the HIV-1 p24 core profile ELISA kit (Dupont NEN Research Products, Wilmington, DE).[14] For both the RT and the p24 assays, CEM and U-937 HIV-1_{III-B} infected and uninfected cultures were used as both the positive and negative controls, respectively.

Intracytoplasmic staining and immunofluorescence

HIV-1 infection of HCQ-treated and untreated cells was further determined by intracytoplasmic staining of alcohol fixed cells using an anti-p24 monoclonal antibody (Dupont, NEN Research). Staining was determined by cytoflourographic analysis as previously described.[6]

Toxicity of HCQ for the CEM and U-937 cell lines

Serial 10-fold dilutions of 1 mM of HCQ were incubated with 0.5×10^6 of either CEM and U-937 cells for 1 h. The cells were maintained in culture for 3, 5, and 7 days. Eighteen hours before harvesting, 1 μCi of [^{3}H]thymidine (ICN, Irvine, CA) was added and the cells were harvested onto glass filters and incorporated radiolabel was measured by scintillation counting.

Detection of HIV-1 mRNA by Northern blot analysis and in situ hybridization

RNA probes were prepared from the cDNA clone pGM92 obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, and was the generous gift of Dr. John Rossi.[15] These were complementary to nucleotides 8475–9576 of the HIV-1 HXB2 GenBank Sequence. For Northern blot analysis, total cellular RNA was extracted using RNAzol solution (Linnai Biotechnology Laboratories, Inc., Dallas, TX). Twenty-microgram RNA samples were electrophoresed on a 1.1% agarose-formaldehyde gel, transferred onto nitrocellulose, and hybridized at 60°C overnight to the antisense ^{32}P-labeled probe generated from pGM92 by the methods outlined by the Promega Corp. (Milwaukee, WI). Filters were washed once each in 2 × PSSC, 1 × PSSC, 0.5 × PSSC, and finally 0.1 PSSC with 1% sodium dodecyl sulfate (SDS) and exposed to X-ray film with an intensifying screen at −70°C overnight.[16] The intensities of the respective HIV-1 RNA bands were measured by a scanning densitometer (GS 300, Hoefer Scientific Instruments, San Francisco, CA). Quantitative variations in either isolation or transfer of control and treated RNAs were accounted for by dehybridization and reprobing the same Northern blot with a cDNA probe for GAPDH,[17] which served as an internal control. For the in situ hybridization, ^{35}S-labeled sense and anti-sense RNA transcripts were generated with SP6 and T7 polymerases, respectively, after endonuclease linearization of pGM92. Insert orientation and specificity was confirmed by restriction analysis. HCQ-treated and untreated HIV-1_{III-B} infected CEM cells were probed for HIV-1 mRNA with 1.0×10^6 cpm/15 μl of riboprobe for 12–15 h at 51°C utilizing a commercially available in situ hybridization kit from Oncor Corp. (Gaithersburg, MD).

Passage of HIV-1 generated from HCQ-treated cells

Cell-free supernatants were isolated from the HCQ-treated and untreated HIV-1_{III-B}–infected CEM T cell line and the monocytic U-937 cell line infected with various HIV-1 isolates. In order to compare the infectivity of these isolates, these

Figure 1a.

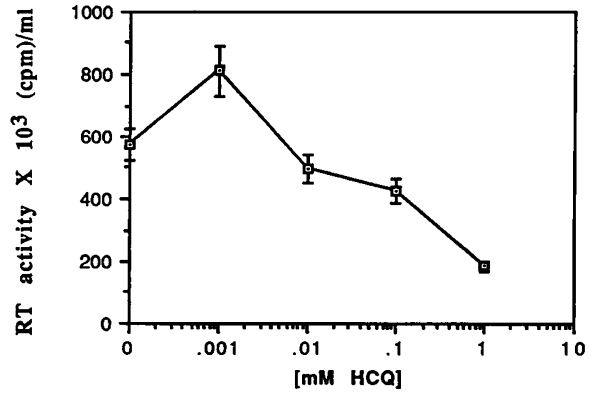

Figure 1b.

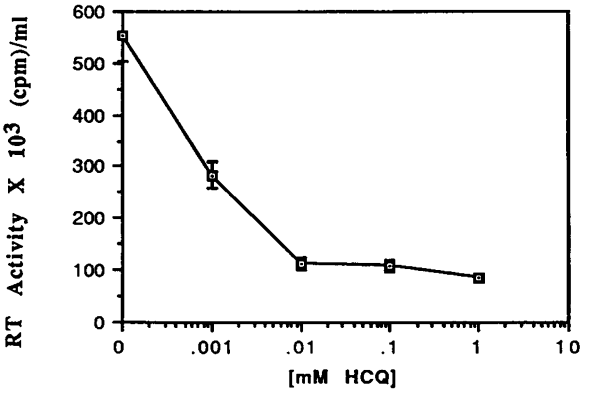

FIG. 1. RT production in CEM (a) and U-937 (b) cells infected with HIV_{III-B} is suppressed by HCQ. In a representative experiment repeated five times, cells were incubated with serial 10-fold dilutions of 1 mM HCQ or sterile PBS for 60 min before infection. After removal of excess virus, the cells were cultured for 7 days at 37°C and infection was measured by RT activity. Background = 2.3×10^3.

Figure 2a.

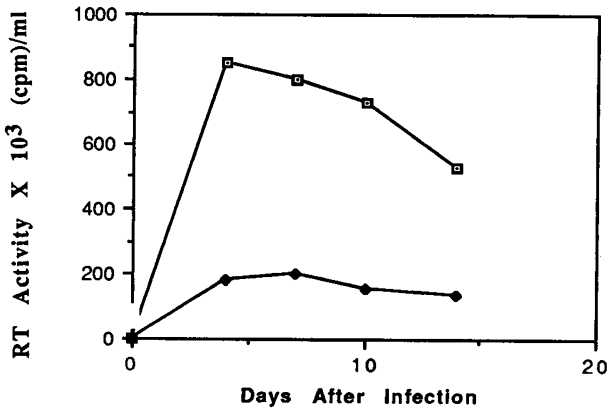

Figure 2b.

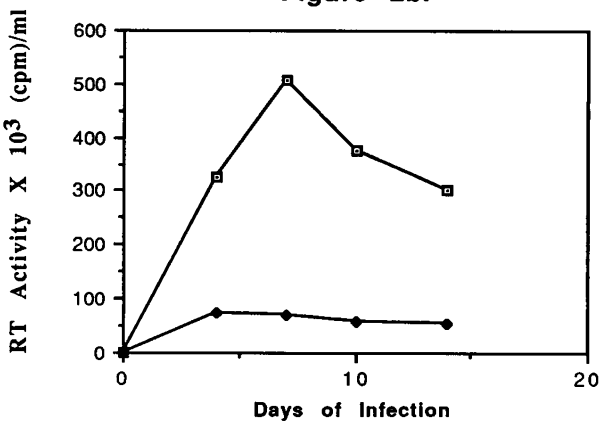

FIG. 2. HCQ suppresses HIV-1 replication in CEM and U-937 cells at different time points. The CEM T cell line (a) and the monocytic U-937 (b) cell line were pretreated with either 1 mM HCQ or PBS and infected with $HIV\text{-}1_{III-B}$. In a representative experiment, samples were taken at different time points after infection in both the HCQ-treated and PBS-treated cells and RT activity measured. (▫) Untreated; (◆) HCQ treated. Background = 2.3×10^3.

cell-free supernatants were cocultured with either U-937 or CEM as described above and p24 antigen and RT activity were determined 7 days after infection.

RESULTS

Pretreatment of U-937 and CEM with HCQ cells suppresses HIV-1 replication

CEM and U-937 cells were pretreated with serial dilutions of HCQ (1–100 μM) for 1 h prior to infection with $HIV\text{-}1_{III-B}$. HCQ pretreatment resulted in the suppression of RT activity (Fig. 1, a and b) in a dose-dependent manner. The EC_{50} for HCQ inhibition of $HIV\text{-}1_{III-B}$ replication in the CEM cells was 10 μM and for inhibition of HIV_{BR-1} in the U-937 cells was 1 μM. The mean effective dose (ED_{50}) for HCQ was 10 μM in the CEM cells and 1 μM in the U-937 cells compared with 0.05 μM for 3′-azido-3′-deoxythymidine.[18] Similar data were obtained with $HIV\text{-}1_{BR-1}$ infection of U-937 and five patient isolates infecting CEM and U-937 (data not shown). $HIV\text{-}1_{BR-1}$ infection of CEM cells was not successful, consistent with the previously described restricted monocytotropic nature of this isolate.[9] To exclude the possibility that HCQ caused a shift in the kinetics of expression of HIV-1 protein in the infected CEM and U-937 cells, viral replication was measured by RT at multiple time points (Fig. 2, a and b). In these studies both CEM and U-937 were pretreated either with 1 mM of HCQ or sterile PBS for 1 h and subjected to the HIV-1 infection protocol described in Materials and Methods. RT activity was inhibited at all time points tested (as far out as 14 days) in both CEM and U-937

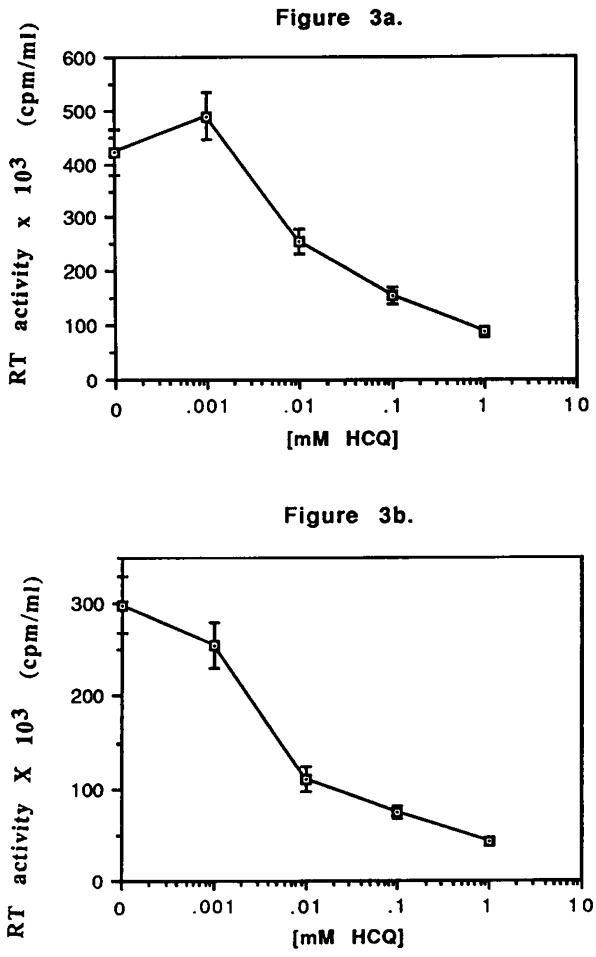

FIG. 3. HCQ suppresses HIV-1 replication in primary T cells (a) and monocytes (b). In a representative experiment repeated five times, primary T cells and monocytes were treated with serial 10-fold dilutions of 1 mM HCQ or sterile PBS before infection. HIV-1_{III-B} was used to infect the T cells while HIV-1_{BR-1} was used to infect the monocytes. After removal of excess virions, the cells were maintained in culture for 7 days and infection was determined by RT activity. Background = 2.3×10^3.

infected with HIV-1_{III-B} when compared to the HCQ-untreated infected control cells. HCQ pretreatment had a similar effect on HIV-1 replication in primary T cells and monocytes (Fig. 3, a and b). RT activity was suppressed in a dose-dependent manner comparable to the observations in the cell lines. The background counts (uninfected cells) ranged from 2.3 to 2.9×10^3 cpm/ml depending on the experiment.

HCQ does not block HIV-1 entry into cells

To determine the inhibition of RT activity by HCQ related to HCQ blockade of HIV-1 entry into cells, HCQ-treated and untreated CEM cells were infected with HIV-1_{III-B} and analyzed by intracytoplasmic staining for the presence of *intracytoplasmic* p24 antigen 24 h after infection. If HCQ blocks viral entry into T cells and monocytes, then no detectable p24 should be observed after infection. As seen in Fig. 4, p24 antigen was detected intracytoplasmically in the HCQ-treated cells after infection although less p24 was detected relative to the control cells. These data suggest that, in contrast to its effect in other viral systems (e.g., influenza virus),[5] HCQ does not block HIV-1 entry into the cell.

HCQ does not inhibit HIV-1 mRNA transcription

Since HCQ at high concentrations has been reported to inhibit both DNA and RNA synthesis,[5] it is conceivable that such inhibition could be a mechanism of action for HCQ's effect on HIV-1 replication. To delineate the site of action of HCQ's inhibitory effect on HIV-1 replication, we assessed the steady-state levels of HIV-1 mRNA in the presence or absence of HCQ pretreatment in CEM cells infected with HIV-1_{III-B}. Northern blot analysis (Fig. 5) demonstrated comparable amounts of HIV-1–specific mRNA in the HCQ-treated and untreated cells. Two 4.3- and 1.8-kilobase bands, consistent with the known size of HIV-1 mRNA,[19] of comparable intensity were detected from the RNAs isolated from both the untreated (lane 1) and the HCQ-treated (lane 2) CEM cells. Similar bands were not found in the uninfected CEM cells (data not shown). In situ hybridization was performed to determine if equivalent numbers of cells expressed HIV-1 transcripts using the anti-sense RNA probe derived from pGM92. Fig. 6 demonstrates that virtually 100% of the CEM cells contained HIV-1 mRNA in both HCQ treated (panel D) and untreated (panel B) cells indicating that HCQ does not prevent cell infection or HIV-1 mRNA production. Panels A and C are the representative controls for the HCQ-untreated and treated cells, respectively, using the sense RNA probe.

HCQ is not toxic for T cell or monocytic cell lines and has no anti–RT activity

To ensure that HCQ has a direct antiviral effect and is not toxic to the T cell or monocytic cell lines, serial dilutions of HCQ (from 100 mM to 0.0001 mM) were incubated with both CEM cells and U-937, and proliferation was measured by thymidine incorporation. No inhibition of proliferation of either cell line was seen at concentrations between 1000 and 0.1 μM HCQ where antiviral activity was observed (data not shown). A 100% cell death in both the CEM and U-937 cells (determined by an absence of cellular proliferation and uptake of trypan blue) was observed at an HCQ concentration of 100 mM. The IC_{50} of HCQ for both the CEM and U-937 cells was 50 mM.

Lastly, the inhibition of viral replication seen might relate to a direct inhibitory effect of HCQ on RT activity. To assess this possibility, HCQ was added directly to standardized HIV-1 stocks and RT activity was measured. No difference in RT activity was detected in any culture (data not shown).

HIV-1 produced from HCQ-treated T cell and monocytic cell lines poorly infects other cells

HIV-1_{III-B} and HIV-1_{BR-1} produced from HCQ-treated and untreated CEM cells or U-937 were used to infect other CEM and U-937 cells. Supernatants from pretreated cells (HCQ or PBS control) obtained 1 wk after HIV-1 infection and standardized to 80,000 cpm/ml were utilized in these studies. Comparing

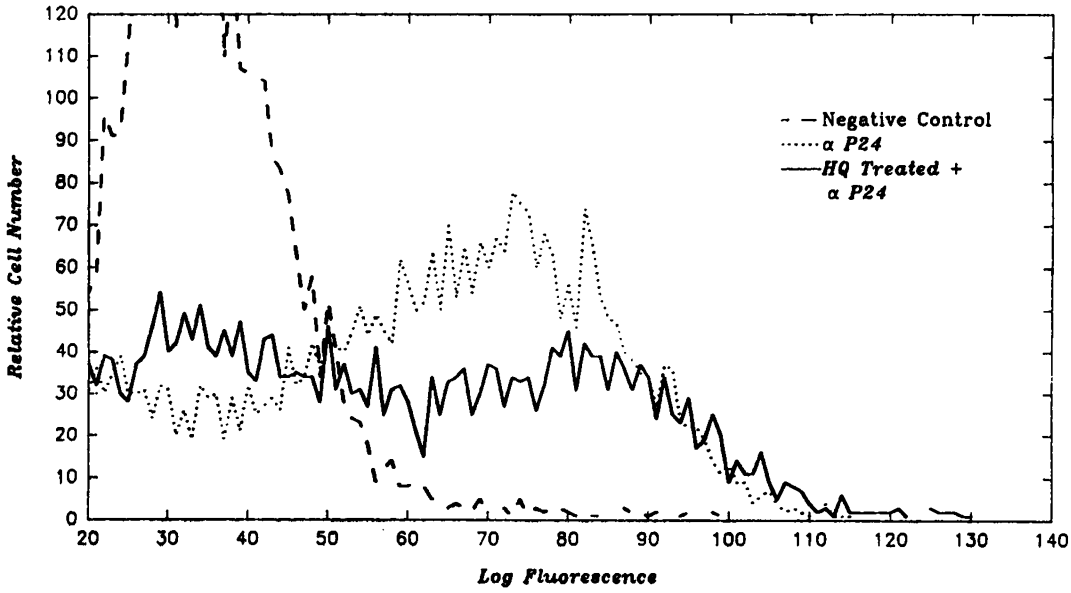

FIG. 4. p24 antigen can be detected intracytoplasmically in HCQ-treated and untreated cells. In this cytofluorographic analysis (gating on live cells) using an anti–p24 monoclonal antibody, comparable peak channel shifts corresponding to the presence of HIV-1–specific protein were demonstrated in both treated and untreated cells.

HIV-1 derived from HCQ-treated cells with that from untreated cells, the efficiency of infection was reduced by 77% and 80% in CEM and in U-937, respectively, as determined by p24 antigen assay (Table 1).

DISCUSSION

This report documents that pretreatment of primary T cells and monocytes and T cell and monocytic cell lines with HCQ inhibits HIV-1 replication as determined by RT activity (Figs. 1, 2 and 3). HCQ has no anti-RT activity itself and, at concentrations that inhibit HIV-1 replication, is not toxic to either T cell or monocytic cell lines. The definitive mechanism of action of inhibition of HIV-1 replication is uncertain but lysosomotropic agents such as HCQ have multiple cellular effects which could account for their anti–HIV-1 properties.[5] HCQ inhibits viral fusion with cell membranes, inhibits DNA and RNA synthesis (10^{-1} M) and endosomal acidification (10^{-3} M),[5,16] blocks receptor-mediated endocytosis, and prevents entry of several viruses including influenza and adenovirus into cells.[5] Despite these numerous properties, the ability of HCQ to block HIV-1 replication may not be explained by any of them. The inhibition of DNA/RNA synthesis by HCQ occurs at concentrations not achieved in T cells (although attainable in monocytes).[20] Despite this, comparable anti–HIV-1 effects were observed (Figs. 1, 2, and 3) in primary T cells and monocytes, and T cell and monocytic cell lines. In addition, it has been shown that HIV-1 entry into cells proceeds by a pH-independent pathway[21] so that inhibition of endosomal acidification and endocytosis may not explain HCQ's effect. Furthermore, HCQ did not prevent HIV-1 entry into the CEM cells inasmuch as we were able to demonstrate the presence of intracytoplasmic p24 antigen (Fig. 4) in the treated cells.

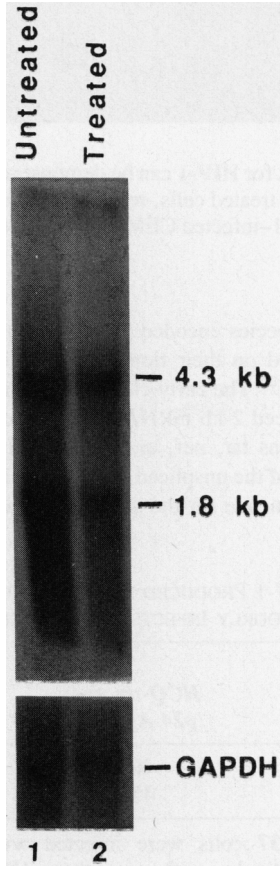

FIG. 5. HIV-1_{III-B}–infected CEM cells, treated and untreated with HCQ. Comparable amounts of HIV-1 mRNA are detected in both HCQ treated and untreated cells. Lanes 1 and 2 (HCQ untreated and -treated HIV-1–infected CEM cells) demonstrate the 4.3- and 1.8-kb bands corresponding to HIV-1 mRNA.

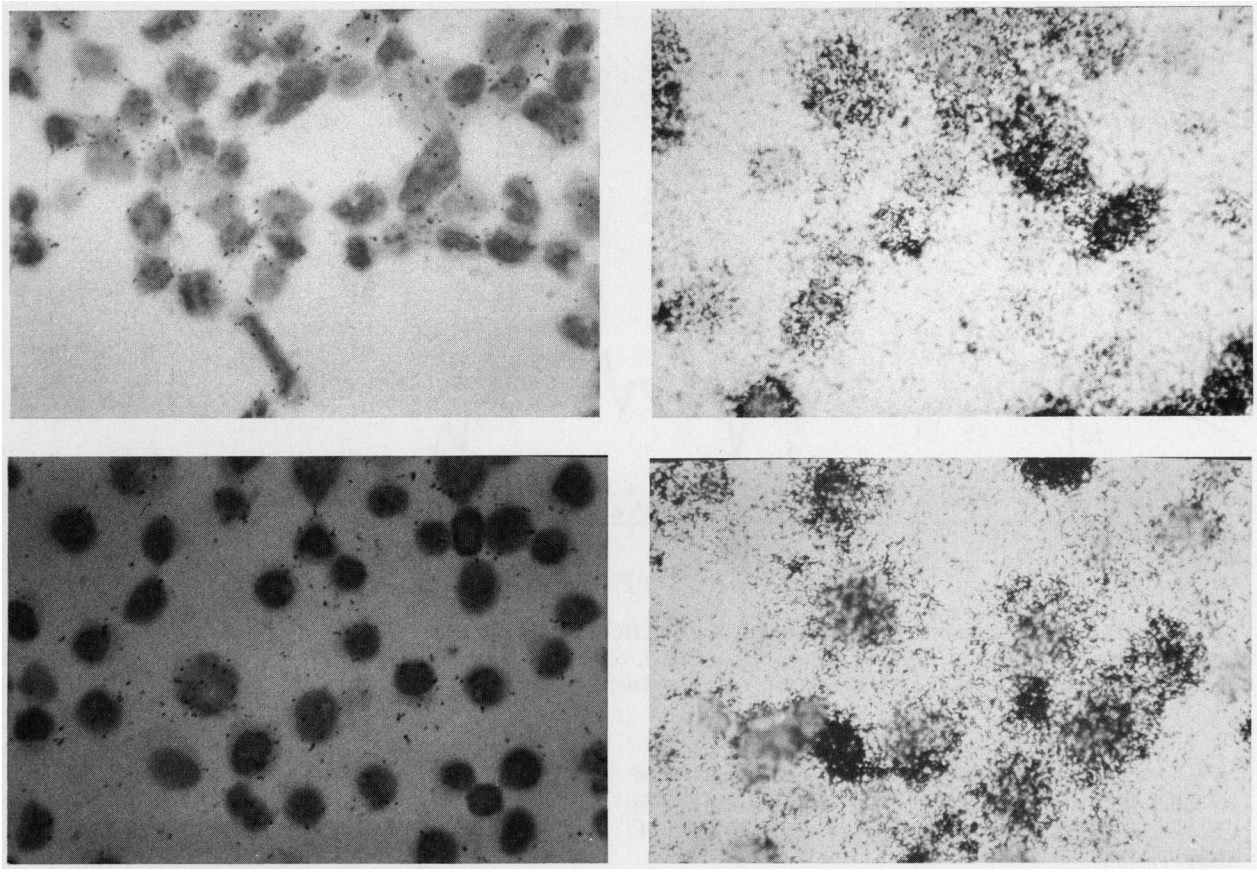

FIG. 6. mRNA for HIV-1 can be demonstrated in HCQ-treated and untreated cells by in situ hybridization. (A and C) Controls in the untreated and treated cells, respectively, using the sense probe; (B and D) comparable levels of mRNA in both HCQ-untreated and treated HIV-1–infected CEM cells using the anti-sense RNA probe. ×25.

The mRNA species encoded by HIV-1 can be divided into two classes based on their time of expression in the HIV-1 replication cycle.[22] The early class of viral mRNA consists of the multiply spliced 2-kb mRNA species that encode the viral regulatory proteins tat, nef, and rev. The late class of viral mRNA consists of the unspliced 9.2-kb and singly spliced 4-kb transcripts that encode for the viral structural proteins. Fig. 5 demonstrates the presence of only the 4- and 2-kb species but not the 9.2-kb species. In a previous report, a similar pattern of HIV-1 mRNA expression has been reported in the chronically HIV-1–infected ACH T cell line in which the 9.2-kb species was present only after PMA and PHA stimulation.[19] In Fig. 6 comparable numbers of cells expressing HIV-1 mRNA are observed in both the treated and untreated cells. The lack of an effect of HCQ on steady-state HIV-1 mRNA levels suggests that the drug inhibits posttranscriptional production of functional virions since HIV-1 (Table 1) derived from HCQ-treated cells loses its ability to infect the CEM T cell line. These findings are consistent with the previous study of Tsai et al.,[23] who reported that chloroquine, which is structurally related to HCQ,[24] inhibits posttranslational glycosylation of gp120 in the CEM T cell line, reducing the number of viruses produced by the infected cells.

TABLE 1. HIV-1 PRODUCED FROM HCQ-TREATED CELLS POORLY INFECTS OTHER CELLS

Source of cells infected with HIV	*HCQ-treated p24 Ag (pg)*	*Untreated p24 Ag (pg)*
CEM	460	2457
U-937	450	2245

CEM or U-937 cells were infected with HIV-1_{III-B} or HIV_{BR-1}, respectively, with or without HCQ pretreatment. Cell-free supernatants from CEM infected with HIV-1_{III-B} or U-937 infected with HIV-1_{BR-1} were harvested on day 7 and used to infect CEM or U-937 cells, respectively, as described in Materials and Methods. p24 antigen production was measured on day 7 after infection.

Weak bases like HCQ are known to affect acid vesicles leading to dysfunction of enzymes necessary for protein synthesis.[25–30] Our data are consistent with these observations and suggest that HCQ inhibits a viral or cellular enzyme that is essential for the production of an infectious virion. This novel site of action is distinct from 3′-azido-3′-deoxythymidine[31] (which inhibits viral nucleic acid synthesis) or soluble CD-4 (which blocks viral entry into the cell),[32] making this drug potentially useful in combination therapy. Additionally, the

inhibition of HIV-1 replication does not appear to be cell lineage restricted. HIV-1 infection of primary monocytes and monocytic cell lines appears to be inhibited equally when compared to primary T cells and T cell lines. Studies are underway to determine if HIV-1 infection can be inhibited in other cell types (e.g., astrocytes and glial cells) known to be susceptible to infection.

The most important findings in this study are that (1) HCQ has the ability to inhibit in vitro replication of various strains of HIV-1 including two laboratory strains and five "wild" strains (the laboratory strains include HIV-1_{III-B}, primarily a T cell tropic virus, HIV-1_{BR-1}, primarily a monocytotropic virus, and several patient isolates that have varying abilities to infect T cells and monocytes) in T cell and monocytic cell lines; and (2) the HIV-1 produced from HCQ pretreated cells poorly infects other cells. A similar antiretroviral effect has been previously demonstrated with chloroquine in another retroviral system, avian reticuloendotheliosis virus,[23] suggesting that this antiretroviral effect is not specific for HIV-1. Studies are presently underway to compare the specificity and inhibitory potency of HCQ for HIV-1 vs HIV-2. The cellular concentration of HCQ in the monocyte is 100-fold higher than in T cells.[20] This difference in intracellular HCQ concentration may be reflected in the ED_{50} The ED_{50} for the CEM cells (10 μM) is 10-fold higher than the ED_{50} (1 μM) for the U-937 cells. Although an anti-HIV-1 effect was demonstrated in both T cell and monocytic cell lines, different mechanisms of action (inhibition of DNA or RNA synthesis vs. inhibition of HIV-1 peptide synthesis) may be responsible. These potential novel mechanisms of action, distinct from other anti–HIV-1 agents, may make HCQ a potentially useful drug either as a sole agent or in combination with other antiretrovirals.

ACKNOWLEDGMENTS

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