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# Efficacies of cyclodextrin-complexed and liposome-encapsulated clarithromycin against *Mycobacterium avium* complex infection in human macrophages

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**Abstract**

Cyclodextrins and liposomes have been used in recent years as drug delivery vehicles, improving the bioavailability and therapeutic efficacy of many poorly water-soluble drugs. In this study, we used two approaches to enhance the availability of the poorly water-soluble antibiotic, clarithromycin, by inclusion complex formation and by liposome-encapsulation. We examined the efficacies of these formulations against *Mycobacterium avium* complex (MAC) in human peripheral blood monocyte-derived macrophages. The water solubility of clarithromycin was enhanced by about 700-fold by complexation with cyclodextrin. The use of a rapid radiometric (BACTEC) method for the detection of MAC growth and susceptibility showed identical MICs against MAC for both the free and complexed drug. The anti-MAC efficacy of the cyclodextrin complex of clarithromycin in macrophages was slightly lower than the free drug, probably due to the high stability of the inclusion complex. At higher drug concentrations, Liposome-encapsulated clarithromycin was slightly more effective against intracellular MAC growth than the free drug.


*Keywords:* Clarithromycin-cyclodextrin; Liposomes; Efficacy; Mycobacterium; Macrophages; Solubility; HPLC

## 1. Introduction

*Mycobacterium avium* complex (MAC) can cause pulmonary disease, subacute lymphadenitis and disseminated disease (Inderlied et al., 1993). In AIDS patients with disseminated infection, the mononuclear phagocyte system is the predominant site of infection, but other organ systems such as the skin, bone and joints, eyes, thyroid, adrenals, testis and the central nervous system (Eng et al., 1989; Horsburgh, 1991). Bacteremia occurs in most of these patients, the organism being pre-

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dominantly in circulating monocytes. Monocytes and fixed tissue macrophages are replete with MAC in AIDS patients, indication of the immune deficiency in these individuals (Spencer and Jackson, 1989; Horsburgh, 1991, 1999; Chin et al., 1994; Bartley et al., 1999).

A wide variety of antimicrobial agents, alone or in combination have been used in the treatment of MAC infections, among which clarithromycin has proved to be an active agent both in vitro and in vivo (Rastogi and Labrousse, 1991; Mor and Heifets, 1993; Dautzenberg, 1994; Alvarez-Elcoro and Enzler, 1999; Hewitt et al., 1999). Clarithromycin is a semi-synthetic 14-member macrolide exhibiting a broad in vitro antibacterial spectrum. Structurally, it differs from erythromycin only in the substitution of an O-methyl group for the OH group at position six of the lactone (Piscitelli et al., 1992). It is practically insoluble in water and its poor solubility is pH dependent (Salem, 1996). Although clarithromycin accumulates in macrophages its MIC against MAC is higher in macrophages than in broth (Mor et al., 1994). Thus, it is likely that the antibiotic accumulates in lysosomes, but has limited access to the phagosomes containing MAC.

Several approaches have been adopted in order to overcome solubility and bioavailability limitations of hydrophobic drugs and to guarantee drug effectiveness and safety. One of these is by enhancing the solubility, and, hence, the bioavailability, via complexing hydrophobic drugs with soluble cyclodextrins. The resulting complexes generally show favorable changes of the characteristics of the guest molecule, such as increased solubility, enhanced stability, reduced side effects, and moreover, a general improvement in the bioavailability (Duchene, 1987; Loftsson and Brewster, 1996; Rajewski and Stella, 1996).

Another method to overcome the above mentioned limitation is by drug association to an effective transportation system 'vehicle' that could minimize drug toxicity, increase drug bioavailability and specificity of action. Liposomes, in this context, are by far the most studied and suitable vehicles to carry hydrophobic drugs in their lipid bilayer. On the other hand, due to the ability of MAC to evade macrophage antibacterial mechanisms, being shielded by the host cell from both immune surveillance and extracellular antibiotics (Oh et al., 1995), an effective therapy could utilize the potential ability of liposomes to target antibacterial agents to macrophages (Düzgünes et al., 1991), because they are avidly taken up by phagocytic cells of the reticuloendothelial system and can release their contents intracellularly (Majumdar et al., 1992; Popescu et al., 1987).

We have shown that certain liposome-encapsulated drugs have better efficacy than the free drugs in vitro or in animal models of MAC infection (Kesavalu et al., 1990; Ashtekar et al., 1991; Gangadharam et al., 1991; Düzgünes et al., 1991, 1996; Majumdar et al., 1992; Gangadharam et al., 1995). We have also demonstrated the enhancement in the solubility of hydrophobic drugs via cyclodextrin complexing procedures (Steffan et al., 2002). In this study, we examined and compared these two approaches to enhance clarithromycin availability using MAC-infected macrophages derived from human peripheral blood monocytes. Part of this work was presented earlier in preliminary form Salem and Düzgünes (1999).

## 2. Materials and methods

### 2.1. Reagents

Clarithromycin was supplied by Abbott Laboratories (Abbott Park, IL). βCD-cyclodextrin was purchased from Cyclodextrin Technologies Development Inc. (Gainesville, FL). Egg yolk phosphatidylcholine (PC), and phosphatidylglycerol (PG) derived from PC by transphosphatidylation were from Avanti Polar Lipids (Alabaster, AL), and cholesterol was from Calbiochem (La Jolla, CA). Bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), dimethylsulfoxide (DMSO), ammonium sulfate and HEPES were obtained from Sigma (St Louis, MO). Other chemicals were supplied by Merck. Solutions were prepared in distilled water further purified in a Barnstead Nanopure filtration apparatus.

### 2.2. Clarithromycin and clarithromycin-βCD complex preparation for solubility study and MIC determination

As a starting point for this study, the solubility as a function of pH and salt concentration of clarithromycin was determined. The water solubility profile of clarithromycin was determined in the pH range between 2.4 and 7.4 in water and in 0.05 and 0.1 M of phosphate buffers. Clarithromycin was added in excess to saturate each solution; tubes were then shaken for 24 h at 25 °C. To avoid changes in concentration due to evaporation, solution tubes were sealed with Teflon-lined screw-caps and wrapped with Parafilm. At the end of the vortexing period, the samples were centrifuged at 10.000 rpm for 5 min and the supernatants were clarified through 0.45 μm pore-diameter membranes. The clarithromycin concentrations were determined by HPLC as described below. All assays were conducted in triplicate.

The inclusion complex (clarithromycin-βCD) was formed in an equimolar concentration of clarithromycin and β-cyclodextrin (13.0 mM) by dissolving the corresponding concentration of clarithromycin in chloroform in round-bottom tubes. The organic solvent was eliminated under a steam of nitrogen, forming a uniform film. The clarithromycin layer was then dried under low pressure at 37 °C for 24 h. Stock solutions of βCD were prepared by accurately dissolving cyclodextrin in water or in water containing phosphate buffers at different pH, ranging from 2.4 to 7.4. These solutions were then added to the clarithromycin layer and vortexed vigorously for 48 h at room temperature. The solution was filtered through 0.22 μm pore-diameter membranes and the filtrate was lyophilized. The lyophilized materials were reconstituted to final dilution as demanded by the experiment. βCD-clarithromycin complex solubility was also submitted to HPLC study under the same conditions.

Since the aqueous solubility limit of clarithromycin is very low (Piscitelli et al., 1992), for MIC determinations the drug was initially dissolved in DMSO (Mor et al., 1994) and then diluted in distilled water to at least 40 times the required final concentration (as BACTEC vials contain 4 ml of medium).

The accurate determination of clarithromycin concentrations for the solubility study, the determination of complexation rates, or MIC assessment, was realized in triplicate using a Rabbit-HP (Rainin, Emeryville, CA) liquid chromatograph and a prepacked (30 cm × 3.9 mm I.D.) $C_{18}$ Mirosorb column (Rainin Instruments, Woburn, MA). The column's back pressures ranged between 1800 and 2000 p.s.i. A pre-column was also used. Sample analysis was performed at room temperature using a mobile phase that consisted of 65% methanol and 35% (v/v) 0.05 M monobasic sodium phosphate buffer. The pH was adjusted to 4.0 using orthophosphoric acid. Flow rates of 1.0 ml/min achieved good resolution. Monitoring and concentrations determination was realized using an UV detector fixed at 210 nm. Method linearity was established for the range of concentrations 0.10–20 μg/ml with a regression factor of 0.9998. The limit of quantitation was identifiable and reproducible at 0.10 μg/ml with a precision of 7.34%. The between days precision at low concentration (0.2 μg/ml) was found to be 6.18%, at medium concentration (8 μg/ml) was 5.45% and at high concentration (18 μg/ml) was 5.71%. The method was proved to be sensitive and specific.

### 2.3. Preparation of liposomes

Clarithromycin encapsulation in liposomes was achieved by either incorporating the drug into (i) the lipid solution before formation of a thin dried film, by (ii) including the drug in the hydration solution, or by (iii) utilizing both methods at the same time (Table 1). In the first experiments where clarithromycin was incorporated in the membrane phase of the liposomes, and due to the low aqueous solubility limit of clarithromycin, the drug was initially dissolved in $CHCl_3$ at three different ratios together with the phospholipids and cholesterol: Chloroform solutions of PG, PC and cholesterol at a molar ratio of 1:9:5 with 10, 20 or 30% of clarithromycin, were prepared in glass tubes, dried to a thin film on a Büchi rotary evaporator, and then placed in a vacuum oven at room temperature to remove any residual $CHCl_3$.

Table 1
Encapsulation of clarithromycin by lipid film hydration

| Composition (PG:PC:CH) | Drug added (% phospholipid) | Drug in liposomes (μg/μmol phospholipid) | Drug in solution (μg/μmol phospholipid) |
|---|---|---|---|
| 1:9:5 | 10 | 4.99 | 0 |
| 1.9:5 | 20 | 5.33 | 1.49 |
| 1:9:5 | 30 | 17.74 | 6.72 |
| 1:1:1 | 30 | 143.72 | – |
| 2:0:1 | 30 | 185.22 | – |

The dried films were hydrated with 1–2 ml of 0.05 M citrate buffer at pH 5.16 by vortexing under an argon atmosphere. Unincorporated clarithromycin was removed by centrifugation of the multilamellar liposomes at 3000 rpm for 10 min in an Eppendorf centrifuge equilibrated at 4 °C, and resuspension in ice-cold citrate buffer (three times). Finally, the liposomes were resuspended in HEPES buffered saline (HBS, 140 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.4 and 303 mOsm).

In the second series of experiments, clarithromycin corresponding to the 10, 20 or 30% mole ratios was incorporated into the hydration solution, rather than the liposome membrane. These liposomes were submitted to five freeze-thaw cycles at −80 and 37 °C, respectively. In the third series of experiments, a formulation was elaborated based on 30% clarithromycin incorporated in the lipid film and in the hydration solution at the same time. Unencapsulted drug was removed in both cases by centrifugation as described above. In all these experiments, the amount of encapsulated clarithromycin was quantitated by HPLC after dissolving an aliquot of the liposomes in an excess of methanol and normalized to the lipid concentration determined by phosphate assay (Bartlett, 1959). Control liposomes were prepared similarly, but without the clarithromycin.

### 2.4. Microorganisms

The *M. avium–M. intracellulare* complex strain MAC 11 obtained from Dr K. Hadley and Dr D. Yajko (San Francisco General Hospital), was stored in 20% (v/v) glycerol in Middlebrook 7H9 medium in polypropylene vials at −70 °C until use. MAC was thawed and cultured in 7H9 broth and after 7 days of incubation, the bacterial suspension was subcultured 24 h before the infection of the macrophage monolayer. The bacterial suspension was adjusted to $10^7$ per ml by using a McFarland standard.

### 2.5. MIC determination by BACTEC

Growth of MAC was monitored radiometrically by means of a BACTEC 460-TB instrument (Becton Dickinson). Growth was measured as a function of the release of $^{14}C$-labeled $CO_2$ resulting from the metabolism of $^{14}C$-labeled palmitate in Middlebrook 7H12 broth (Siddiqi et al., 1981). Growth was then expressed as a numerical value referred to as the 'growth index' (GI) that ranges from 1 to 900 (Siddiqi et al., 1981). The data are presented in each case as the mean of three determinations.

After 7 days of incubation of MAC in 7H9 broth, the bacterial suspension was adjusted by using a McFarland standard. From this, 10-fold dilutions were made in BACTEC medium from $10^{-1}$ to $10^{-5}$. Clarithromycin βCD-complex and drug containing vials (1.0, 2.0 and 4.0 μg/ml), and drug-free control vials were injected with 200 μl of the suspension from the $10^{-3}$ dilution. A 1/100 control vial was inoculated with 200 μl of the $10^{-5}$ dilution. Since free clarithromycin is initially dissolved in DMSO, the possible inhibitory effects of this compound on MAC growth was investigated. Samples of 20% DMSO were injected directly into the BACTEC vials. The vials were read at the same time every day until the 1/100 control showed a $GI > 30$, with an increase in $GI > 10$ for 3 consecutive days. All measurements were carried out in triplicate.

### 2.6. Infection and treatment of human monocyte-derived macrophages

Human peripheral blood was obtained from a healthy donor and peripheral blood mononuclear cells were isolated by Histopaque-1077 (Sigma) density gradient centrifugation. The cells were then cultured at a density of $2 \times 10^6$ per well in 48-well tissue culture plates (Falcon) at 37 °C and 5% $CO_2$ in a cell culture incubator. The medium and nonadherent cells were aspirated after 24 h, and the adherent monocyte monolayer was washed twice with 1 ml of DME-HG. The cell layer was incubated in 1 ml of cell differentiation medium (DME-HG-L-glutamine + 20% fetal bovine serum + 10% human serum) with a change to a maintenance medium (DME-HG-L-glutamine + 20% fetal bovine serum) on day 3. The Trypan Blue exclusion technique was used to determine the viability of the cells after washing. Adherent cells developed morphologic characteristics of macrophages on day 4.

MAC was cultured in Middlebrook 7H9 broth. After 7 days of incubation, the bacterial suspension was subcultured 24 h before the infection of the macrophages monolayer, and the bacterial suspension was adjusted to $10^7$/ml by using a McFarland standard. Macrophages were inoculated with the MAC suspension at a ratio of 5:1. The cells were incubated for 24 h at 37 °C in 5% $CO_2$ and moist air. After 24 h, the monolayer was washed three times with Hank's Balanced Salt Solution (HPBS) to remove the extracellular MAC. After the first day of infection, the number and viability of the cells were ascertained by Naphtol Blue Black staining of nuclei and by Trypan blue exclusion, respectively.

The effects of free and clarithromycin-containing complex were compared at three different concentrations clinically achievable in the serum of treated patients. Generally, peak plasma concentrations of clarithromycin in HIV-infected adults receiving 0.5 or 1 g doses of the drug orally every 12 h range from 2 to 4 or 5 to 10 μg/ml, respectively (Abbott Laboratories, 1996). Thus, infected macrophages were treated with 2.0, 4.0 and 8.0 μg/ml of either free clarithromycin, clarithromycin-βCD complex or clarithromycin-loaded liposomes for 24 h. The medium was then removed, and the cells were washed three times with HBS and incubated for 7 days in DME-HG-L-glu + 20% FBS.

About 7 days after the treatment, the GI of MAC was determined after lysis of the macrophages with 0.25% SDS, which was subsequently neutralized with 10% BSA. The lysate was diluted serially to $10^3$ and aliquots of the diluted lysates (200 μl) were injected into the BACTEC vials. The BACTEC vials were incubated at 37 °C and assayed every 24 h. In all the macrophage experiments, triplicate wells of macrophages were utilized for each condition. The GI of MAC obtained from each of the wells was determined in duplicate.

## 3. Results

### 3.1. Solubility behavior

Fig. 1 illustrates the relationship between pH and clarithromycin solubility at 25 °C. A significant increase in solubility was attained with pH adjustment. The solubility was significantly increased when the medium pH was decreased (9.22 mg/ml at pH 2.4). Increasing the phosphate buffer concentration increased the solubility, especially at low pH (12.1 mg/ml in 0.1 M phosphate buffer at pH 2.4).

The solubility profile of clarithromycin in βCD inclusion complex is also shown in Fig. 1. Clarithromycin solubility in βCD at pH 7.4 increased by approximately 700-fold compared with its solubility in water, while at pH 5.4 this increase was minimal. At pH 2.4 the solubility of clarithromycin in the βCD complex reached 3.7 mg/ml, which means an approximately 60% decrease of its water solubility at this pH. Conversely, an increase in the salt concentration produced a significant decrease of drug solubility throughout the pH range studied (data not shown). Solubility profiles of clarithromycin in both cases are consistent with expectations based on the mechanism of cyclodextrin inclusion formation which predicts higher complexation rates, thus, solubility, for highly hydrophobic drugs. The equilibrium during com-

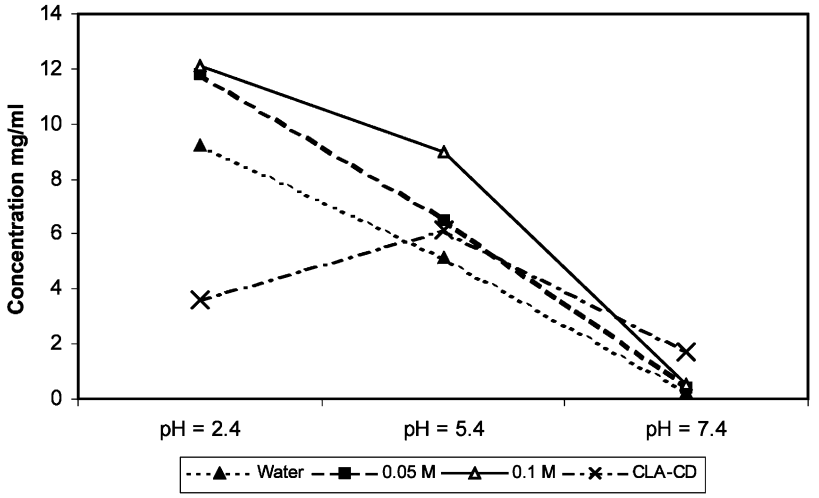


Fig. 1. Solubility of clarithromycin as a function of pH, phosphate buffer concentration and β-cyclodextrin at 25 °C.

plex formation at low pH is shifted toward the free form of the drug due to its higher solubility at this pH. Although clarithromycin solubility is greater at lower pH in comparison with the highest solubility obtained with the βCD complex at pH 7.4, the solubility obtained at a neutral pH represents better physicochemical properties and stability for the molecule, and, accordingly, better therapeutic utility.

### 3.2. Formulation of clarithromycin liposomes

Several methods were employed to encapsulate clarithromycin in liposomes: (i) The drug was incorporated in the lipid mixture used to form liposomes; (ii) the drug was dissolved in the aqueous solution used to hydrate the dried film; (iii) the drug was included both in the lipid film and in the aqueous medium. Hydration of drug-containing thin lipid films (composed of PG:PC:cholesterol at a molar ratio of 1:9:5) yielded encapsulation efficiencies between 3.6 and 6.7%. When the drug was incorporated into the hydration solution the efficiency was even lower, between 0 and 2%. A slightly higher percentage (7.2%) was obtained when 30% of drug was included in both the lipid film and an equivalent amount the hydration solution. In contrast, when 30% drug was incorporated in a lipid film composed of PG:PC:cholesterol at a ratio of 5:5:5, the encapsulation efficiency increased up to 47.9%. Furthermore, when the composition was changed to 10:0:5 (PG:PC:CH), the efficiency increased up to 61.7%.

### 3.3. MIC determination

The MICs for both clarithromycin and the clarithromycin-βCD complex were 1.0 μg/ml (Fig. 2), indicating that the cyclodextrin structure has had no negative effects on the activity of clarithromycin. The βCD proved not to interfere with microbial growth. Since DMSO was used to solubilize the free drug, its possible inhibiting effects on MAC growth was also investigated. Nevertheless, no negative effects were found at the highest concentration normally used to dissolve the free clarithromycin (Fig. 2).

The MICs found for clarithromycin and for its complex were within the range of clarithromycin MIC observed previously, although these vary depending on the susceptibility testing method employed, the composition and pH of the media, and use of nutritional supplements (Barradell et al., 1993). Values representing susceptibility of *M. avium* complex isolates to clarithromycin are between 0.1 and 4 μg/ml (Fernandes et al., 1989; Naik and Ruck, 1989; Perronne et al., 1990;

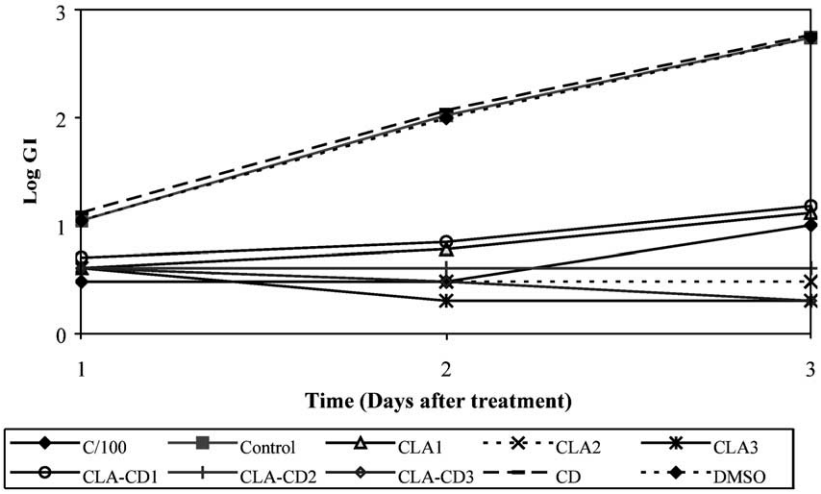


Fig. 2. Representative radiometric data showing the effects of free or β-cyclodextrin-complexed clarithromycin on the growth of *M. avium–intracellulare* complex. These data were used to establish the MICs for free clarithromycin (CLA, 1, 2, and 3, corresponding to 1, 2 and 4 μg/ml of drug, respectively), or the clarithromycin-β-cyclodextrin complex (CLA-CD 1, 2, and 3, corresponding to 1, 2, and 4 μg/ml of drug, respectively). Growth is represented as log GI values obtained with the BACTEC instrument for 3 days following the exposure to drug, as described in Section 2.

Heifets et al., 1993; Mor et al., 1994; Furney et al., 1995). Once MICs were established, higher concentrations of either the free drug, the complex, or liposomes containing clarithromycin were tested in the MAC-infected macrophage model.

### 3.4. Intracellular activity

We compared the effects of free clarithromycin and the clarithromycin-βCD complex, at a concentration range clinically achievable in the serum of treated patients, on the survival and replication of MAC within human macrophages. The results indicated that at the end of the third day of incubation period following the treatment, MAC growth decreased with the increase in clarithromycin and complex concentrations, in comparison with the untreated controls (Fig. 3). Both treatments were effective in slowing the intracellular multiplication of MAC as compared with controls without antimicrobial agents. In our model, the free and βCD-complexed clarithromycin, at an extracellular concentration four times the MIC, were able to exhibit inhibitory activity against intracellular MAC. The anti-MAC effect was also observed at drug lower concentration. Although there were slight differences in the GI readings between the clarithromycin-βCD complex and the free drug throughout the concentration range studied (Fig. 3), these differences were not statistically significant. Treatment with β-cyclodextrin alone did not affect MAC growth compared with untreated controls in the range of concentrations present in the clarithromycin complex. Similarly, free DMSO, at concentrations corresponding to those present in cultures treated with free clarithromycin did not inhibit MAC growth in macrophages.

The effects of different concentrations of clarithromycin-loaded liposomes on intracellular MAC growth were examined (Fig. 4). These concentration of clarithromycin were in the range achievable in the serum of treated patients. The liposome composition selected for these studies, PG:PC:cholesterol (1:9:5) was similar to that employed in our previous in vivo studies with other antibiotics (Düzgünes et al., 1991; Gangadharam et al., 1991, 1995). As shown in Fig. 4, free and liposome-encapsulated clarithromycin have similar effects on the growth of intracellular MAC at the lower concentrations. Liposome-encapsulated clarithromycin at 8 μg/ml reduced the GI by

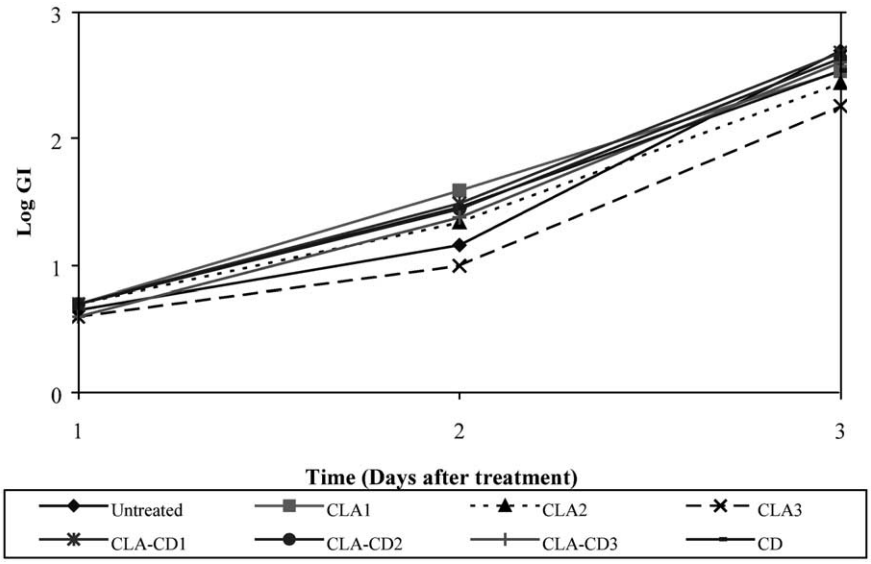


Fig. 3. Treatment of MAC-infected macrophages with free or clarithromycin-β-cyclodextrin complex. Following infection, the cells were treated for 24 h with free (CLA, 1, 2, and 3, corresponding to 2, 4 and 8 μg/ml of drug, respectively) clarithromycin-β-cyclodextrin complex (CLA-CD 1, 2, and 3, corresponding to 2, 4 and 8 μg/ml of drug, respectively). The macrophages were lysed on day 7 and the GI was determined by BACTEC, as described in Section 2.

0.3 log orders of magnitude compared with the GI obtained with the free drug (Fig. 5). Buffer-loaded (empty) liposomes had no effect on the intracellular growth of MAC or macrophage viability.

The effect of the various treatments on macrophage viability was investigated (Fig. 6). On day 7 post-infection, macrophage viability was comparable in infected but untreated controls, and MAC-

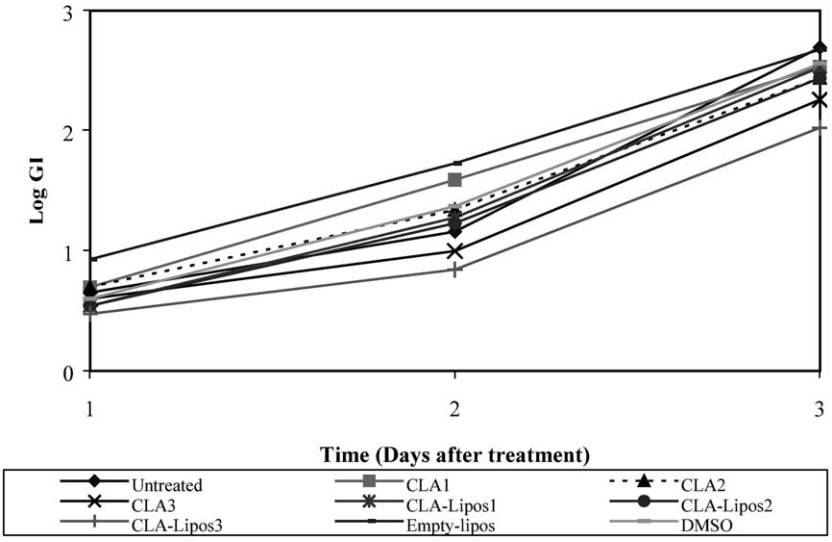


Fig. 4. Effects of free and liposome-encapsulated clarithromycin against MAC growth inside human macrophages. Infected cells were incubated for 24 h with free clarithromycin (CLA 1, 2 and 3, corresponding to 2, 4 and 8 μg/ml of drug, respectively) or liposome-encapsulated clarithromycin (CLA-lipos 1, 2, and 3, corresponding to 2, 4 and 8 μg/ml of drug, respectively). After 7 days, cells were lysed to determine MAC growth by BACTEC.

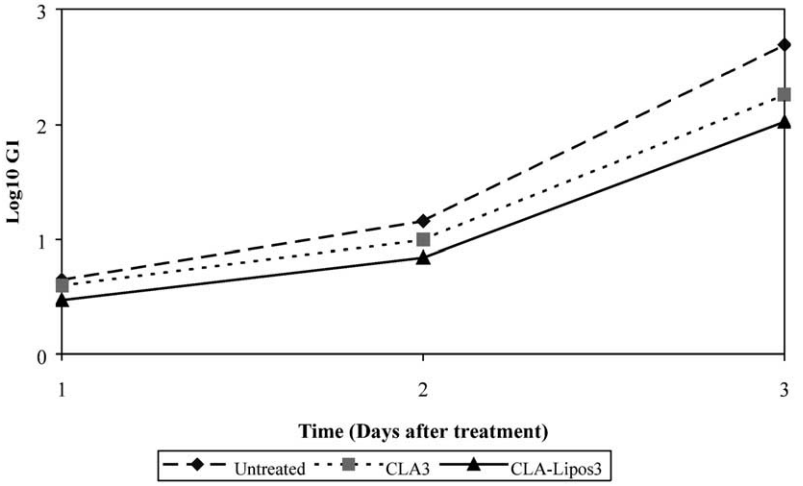


Fig. 5. Comparison of the activity of free and liposome-encapsulated clarithromycin against MAC growth inside human macrophages. Infected cells were incubated for 24 h with 8 μg/ml of free (CLA3) or liposome-encapsulated clarithromycin. Growth is represented as log GI values obtained with the BACTEC instrument for 3 days following the exposure to drug, as described in Section 2.

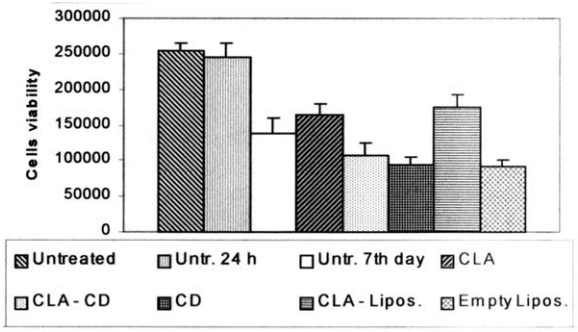


Fig. 6. Relative viability of MAC infected macrophages using the Trypan Blue exclusion technique. Viability was determined by counting viable cells in infected but untreated control wells, as well as in wells containing MAC-infected cells treated with βCD-clarithromycin, free drug or uncomplexed βCD. Counts were performed just before cell lysis for growth determination by BACTEC.

infected cells treated with clarithromycin-βCD, free drug, clarithromycin-loaded liposomes, βCD alone or empty liposomes. Thus, the decrease in the MAC GI observed in macrophages treated with clarithromycin formulations cannot be attributed to the decrease in the number of macrophages in the cultures due to cell death or removal of the cells from the monolayer.

## 4. Discussion

Our results indicate that clarithromycin forms an inclusion complex with cyclodextrin, where the complex was less crystalline, and, therefore, more soluble than the pure drug. An enhancement of clarithromycin water solubility of approximately 700-fold was achieved at pH 7.4. To our knowledge, this is the first time that clarithromycin is reported to form an inclusion complex, with a significant enhancement of its physicochemical properties. Furthermore, the complex matched the pure drug MIC against MAC in vitro, which indicates that the cyclodextrin did not interfere with the mechanism of action of the drug against MAC. The higher MIC of clarithromycin against intracellular MAC, despite accumulation within macrophages, may be partially the result of the lower antimycobacterial activity of clarithromycin at the mildly acidic conditions (pH 5.7–6.3) found within MAC-infected phagosomes (Crowle et al., 1991; Mor et al., 1994; Oh and Straubinger, 1996; Schaible et al., 1998).

Taking into account these finding, the slightly lower activity of βCD-clarithromycin against MAC inside macrophages could be attributed primarily to the mechanism of drug release from

the inclusion complex. The association-dissociation process in the aqueous substrate-cyclodextrin system is a very dynamic one and is a function of drug and cyclodextrin concentrations, binding constant, and dilution (Uekama et al., 1994). The binding equilibrium of drug to cyclodextrin is established usually with half-lives of much less than 1 s. The kinetics of dissociation, through which the drug becomes bioavailable, is much faster than most physiological processes (Cramer et al., 1967; Rohrbach and Wojcik, 1981; Hersey et al., 1986). It is likely that, in this particular case, the availability of the free form of clarithromycin was lower than expected due to the elevated stability of the clarithromycin–cyclodextrin complex.

The observation that the anti-MAC effect of clarithromycin-containing complex was lower than the free antibiotic is in apparent contradiction to the MIC results obtained by directly incubating the complex with MAC in broth. In the latter study, the MICs obtained with both were identical, and equal to 1 μg/ml, probably because clarithromycin was directly available to MAC, and so was equally effective in inhibiting the bacteria at the lower concentration. The ability of macrophages to uptake the drug most likely depends on the mechanism of drug release from the inclusion complex.

On the other hand, in our cell model, results at lower drug concentrations indicate that free and liposome-encapsulated clarithromycin have similar effects on the growth of intracellular MAC. The difference between both treatments was not statistically significant. It seems that macrophage uptake rates of the free and encapsulated drug are similar. Despite clarithromycin's capacity to concentrate into phagocytic cells, the enhancement in efficacy was not as expected, yet results at 8 μg/ml indicate that liposome-encapsulated clarithromycin is slightly more effective against MAC growth than the free drug (by approximately 0.3 log GI units), this small difference observed between the two treatments was statistically significant ($P \leq 0.001$). This observation is partially in agreement with previous results obtained with ciprofloxacin, streptomycin and amikacin, showing that the liposome-encapsulated drugs were more effective than the free drug against MAC inside murine peritoneal or human monocyte-derived macrophages (Kesavalu et al., 1990; Ashtekar et al., 1991; Majumdar et al., 1992). The slightly higher anti-MAC effect of clarithromycin-loaded liposomes compared with that of the free drug (Fig. 5), may be the result of a greater accumulation and availability of the drug in the compartments containing MAC, which is in accordance with the liposomes' ability to deliver encapsulated antibiotics to MAC bacteria inside endosomes or in phagosomes (Düzgünes, 1998). In this case, however, the enhancement in efficacy was not as expected, probably due to the higher efficiency of the uptake of the free drug by the infected macrophages in comparison with that of the encapsulated from. It is possible, therefore, that the advantage conferred by liposome-mediated uptake was obviated. An additional factor that plays a role in the relative activity of the free and encapsulated clarithromycin is the relative inactivity of clarithromycin in the acidic milieu within phagolysosomes (Mor et al., 1994).

## 5. Conclusion

Cyclodextrins and liposomes have been used in recent years as drug delivery systems, improving the bioavailability and therapeutic efficacy of many poorly water-soluble drugs. In this study, we have investigated the means to enhance the availability (thus, efficacy) of clarithromycin against MAC infection using both systems. Although cyclodextrin and liposome-encapsulated clarithromycin showed a modest enhancement in efficacy against MAC in macrophages in culture, additional factors, such as the stability of liposomes during circulation in the bloodstream, their passive targeting to infected macrophages in the liver and spleen, or the solubility properties of the clarithromycin complex may influence the bioavailability of the antibiotic. On the other hand, the appreciable improvement in clarithromycin solubility by complexation with cyclodextrin may have a positive impact on the in vivo bioavailability of clarithromycin.

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