Therapeutic Efficacy of Liposomal Clofazimine against *Mycobacterium avium* Complex in Mice Depends on Size of Initial Inoculum and Duration of Infection

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The therapeutic efficacy of liposomal clofazimine (L-CLF) against *Mycobacterium avium* complex (MAC) was evaluated in the acute and chronic infection models of the beige mouse (C57BL/6J *bg^j bg^j*). The maximum tolerated dose of L-CLF was inversely proportional to the infection level. L-CLF showed higher antibacterial activity than free clofazimine. Treatment with 25 mg of L-CLF per kg of body weight (intravenously) was started at days 1, 8, 15, and 22 postinfection and was studied at three levels of MAC infection (10^4 , 10^5 , and 10^6 bacilli/mouse). L-CLF treatment caused a significant ($P < 0.05$ to 0.001) reduction in the numbers of viable bacteria in lung, liver, and spleen at all infection levels, irrespective of time of treatment. However, the best results were obtained when an already established infection was treated (day 22). The organ-related differences in response to the treatment were also affected by the level of infection. A marked reduction in the numbers of CFU was observed in the lungs of mice with lower infection levels, whereas liver and spleen were treated more efficiently at higher infection levels. These studies might help in evaluations of host responses to therapy.

Disseminated *Mycobacterium avium* complex (MAC) infection is the most common complication of advanced human immunodeficiency virus disease (22, 28, 31, 38). About 50% of patients with human immunodeficiency virus infection are estimated to become infected with MAC (16, 37). Chemotherapy remains the only ray of hope for terminally ill patients (8). Management of antimicrobial chemotherapy for *M. avium* infection is difficult and is still the subject of clinical trials. Most of these trials use multiple-drug combinations without promising results (14). The search for new drugs and treatment modalities is therefore essential.

Clofazimine is a substituted iminophenazine originally developed for the treatment of tuberculosis (7) and later approved for the treatment of leprosy (6). It is also an accepted component of many multidrug combinations prescribed for the treatment of disseminated MAC infection in AIDS patients (1). Despite being an active component of multidrug therapies for MAC infection, the efficacy of clofazimine against MAC infection is not well documented. In vitro, this drug has been shown to inhibit the growth of *M. avium* and *Mycobacterium intracellulare* at concentrations of 0.1 to 1.0 $\mu\text{g/ml}$ (1, 18). Because of its good in vitro activity against MAC and acceptable in vivo tolerance, clofazimine could become one of the prospective drugs used in chemotherapy for MAC.

Liposome encapsulation is an effective tool for delivering drugs to the cells of the reticuloendothelial system (2, 3). Liposomes are thus of particular interest as vectors for agents intended to target MAC, which, as an intracellular pathogen, invades, resides, and multiplies in the resident macrophages of various tissues. Liposome encapsulation has been shown to improve the therapeutic efficacies of many antimicrobial agents against a number of facultative intracellular pathogens

(12, 15, 34), including MAC (15, 23, 35). Since clofazimine has already been shown to maintain its in vitro (30) as well as in vivo (29) antibacterial activities against MAC after liposome encapsulation, we planned this study to further investigate how other factors like the extent of infection as well as the time of treatment would affect the therapeutic potential of liposomal clofazimine (L-CLF) in the widely accepted beige mouse (C57BL/6J *bg^j bg^j*) model of disseminated MAC infection (20).

In most of the earlier studies in which the acute infection model was used to screen various drugs, emphasis was always given to the chronic disease model that simulates the human process more closely (20). With this point of view, we planned an extensive therapeutic study involving acute as well as chronic disease models corresponding to low-, moderate-, or high-grade infections treated with L-CLF on identical schedules. The results presented here might help us understand many difficult aspects of the complex host-pathogen relationship in patients with MAC infections.

MATERIALS AND METHODS

Animals. Female C57 BL/6J *bg^j bg^j* mice (age, 4 to 6 weeks; weight, 14 to 16 g) were obtained from Jackson Laboratories (Bar Harbor, Maine). The animals received water and food ad libitum. They were used according to a protocol approved by Institutional Animal Care and Use Committee. Beige mice have been widely accepted as a model for studying MAC infection (20, 29).

Drug, lipids, and reagents. Clofazimine was kindly provided by S. Franzblau (Hansen Disease Center, Baton Rouge, La.) and by CIBA-GEIGY, Basel, Switzerland. Soft-gel capsules of clofazimine (Lamprene; Geigy Pharmaceuticals) were purchased from The University of Texas M. D. Anderson Cancer center pharmacy. L- α -Dimyristoylphosphatidyl choline (DMPC) and L- α -dimyristoylphosphatidyl glycerol (DMPG) were obtained from Avanti-Polar Lipids Inc., Alabaster, Ala. All other reagents and chemicals were of analytical grade.

MAC isolate. *M. avium*-*M. intracellulare* complex strain 101 (serovar 1), an isolate obtained from a patient at Children's Hospital, Los Angeles, was maintained on slants of Lowenstein-Jensen medium (Remel, Lenexa, Kans.). Culture suspensions were prepared by growing an initial inoculum on Middlebrook 7H11 agar (Difco Laboratories, Detroit, Mich.) supplemented with oleate, albumin, and dextrose-catalase enrichment (Remel) and glycerol (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The transparent colonies from these plates were subcultured in Middlebrook 7H9 broth (Difco Laboratories) supplemented with oleate, albumin, and dextrose-catalase enrichment and glycerol and were

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allowed to grow at 37°C on a rotary shaker for 3 days. The culture suspensions were shaken and sonicated for 90 s with a bath-type sonicator (Laboratory Supplies Co., Inc., Hicksville, N.Y.) and were adjusted to the desired concentrations with a Klett-Summerson colorimeter (Klett Manufacturing, Brooklyn, N.Y.) or a Spectronic 20 spectrophotometer (Milton Roy, Rochester, N.Y.). The size of the inoculum was determined by titration, plating and counting of the numbers of CFU on 7H11 agar plates after 2 weeks of incubation at 37°C.

Preparation of clofazimine formulations. (i) Free clofazimine. Free clofazimine was prepared by dissolving 10 mg of clofazimine in 1 ml of acidified dimethyl sulfoxide as described earlier (29). Just before the injections, the stock solution of drug in dimethyl sulfoxide was diluted with sterile water to achieve the desired concentration.

(ii) Oral clofazimine. For oral administration, soft-gel capsules of clofazimine were used. One 50-mg capsule was dissolved in 48 ml of mineral oil and 2 ml of water, resulting in a drug concentration of 1 mg/ml of the oil and water emulsion. An aliquot of 0.1 ml of the final solution (≈ 5 to 6 mg/kg of body weight) was fed to each mouse every day with an appropriate gavage needle. This corresponds to a daily dose of approximately 300 mg for humans.

(iii) L-CLF. Multilamellar liposomes containing clofazimine were prepared by dissolving DMPC-DMPG (7:3) and clofazimine (drug:lipid ratio, 1:15) in 80% tertiary butanol (Fisher Scientific). The drug-lipid solution was then sonicated, frozen with a dry ice-acetone mixture, and lyophilized for 2 days by using a freeze-dryer (Labconco Co., Kansas City, Mo.). The preliposomal powder was stored at -20°C until use. Empty liposomes were prepared in a similar way by excluding the drug from the lipid mixture. The liposomes were prepared by reconstituting the lyophilized powder in the required volume of sterile saline. Encapsulation efficiency was more than 95%, as determined spectrophotometrically at 284 nm.

Infection of animals. The animals were infected by injecting the required number of viable bacteria via the tail vein. Four different concentrations of MAC (approximately 5×10^7 , 5×10^6 [high-grade infection], 5×10^5 [medium-grade infection], and 5×10^4 [low-grade infection] viable CFU per mouse) were injected to groups of animals. All MAC suspensions were obtained by serially diluting the highest suspension containing 5×10^8 CFU/ml.

Determination of MTD of L-CLF in infected mice. A minimum of 40 mice were infected with each dose of MAC. On day 15 postinfection, the mice in each group were further distributed into groups of five mice each and were given L-CLF at doses ranging from 5 to 50 mg/kg of body weight via the tail vein injection. The mice were observed for mortality or any visual impairment. The maximum tolerated dose (MTD) of L-CLF is the dose which could be safely injected into animals without causing any mortality. The drug concentrations equal to and lower than the MTD were used further to study the dose-response of treatment.

Dose-response of L-CLF in mice infected with various number of bacteria. Groups of 40 mice each were infected with each dose of MAC mentioned above. Therapy was initiated 15 days postinfection, and five mice each were treated with free clofazimine (5 mg/kg of body weight) or L-CLF (5, 10, 15, 25, or 50 mg/kg of body weight). In all, a total of six doses (twice weekly) were administered. Control animals were injected with empty liposomes containing the lipid content equivalent to the 25-mg dose of L-CLF. The positive controls in this study received daily oral treatment for the entire period with soft-gel capsules of clofazimine at a dosage of 100 μg per mouse per day, corresponding to the 300-mg dose used for patients. The dose of L-CLF showing a significant reduction in the number of infecting bacilli in all groups was then used to study the response of treatment in early and delayed treatment schedules.

Effect of time of treatment on therapeutic response of clofazimine. Clofazimine at a dose of 25 mg/kg of body weight was selected from the dose-response study to study the effect of time of treatment on the outcome of the infection. All the animals were infected on day 0 and were further divided into four groups. One group each was treated on day 1, 8, 15, or 22 postinfection with 25 mg of L-CLF per kg or empty liposomes as vehicle controls, and treatment was continued twice a week (total of six doses). The animals in each group were sacrificed 1 day after the last treatment. The numbers of CFU from tissues of L-CLF-treated animals were compared with those from the tissues of their respective controls injected with empty liposomes.

Assessment of infection. One day after the last treatment, five animals from the control group (receiving empty liposomes) as well as those treated with liposomal drug were sacrificed and the vital organs such as lungs, liver, and spleen were removed aseptically. Part of each tissue was fixed in 10% formal saline for histopathological observations with hematoxylin-eosin and acid-fast staining. The remaining tissues were weighed and homogenized in a known volume of phosphate-buffered saline with an electric homogenizer (Omni International Inc., Gainesville, Va.). The numbers of CFU were determined after appropriate dilutions were plated onto 7H11 agar medium. The plates were incubated at 37°C, and the numbers of CFU were counted after 2 weeks of incubation. The results were expressed as the numbers of CFU per gram of tissue.

Measurement of clofazimine levels in tissues. The animals sacrificed in the experiment with various doses of drug were used to quantitate drug levels. These mice were treated at day 15 postinfection with six doses of clofazimine as explained before and were sacrificed 1 day after administration of the last treatment dose. The tissues were processed as described above. A measured

TABLE 1. Effect of the infecting dose of MAC on the MTD of L-CLF^a

Infecting dose of MAC/mouse	MTD of L-CLF (mg/kg)
None	100
5×10^4	50 ^b
5×10^5	50 ^b
5×10^6	25 ^b
5×10^7	15 ^b

^a MTD is the dose of L-CLF which could be injected safely to animals without causing any mortality. Forty mice were infected with each dose of MAC. At day 15 postinfection, the animals in each group were further divided into groups of five mice each, treated with L-CLF (at doses of 5, 10, 15, 25, or 50 mg/kg of body weight), and observed for mortality.

^b Mice injected with a single dose of L-CLF higher than the indicated dose died within 24 h.

volume from each tissue homogenate was used to extract drug from tissues and to quantitate the drug levels in tissues from various treatment groups. Each tissue homogenate was extracted twice with methanol. The supernatants from the two extractions were pooled and dried under vacuum in a Speedvac (RC 10.10 concentrator system). The pellet containing drug was reconstituted in 500 μl of methanol, and a 25- μl aliquot was analyzed by high-performance liquid chromatography (HPLC). The HPLC system consisted of a Waters solvent delivery system (model 510), a Waters autoinjector (model 712 WISP), a variable-wavelength UV detector (model 481), a data module (model 745 B), and a Waters $\mu\text{Bondapak C}_{18}$ column (3.9 by 300 mm). The mobile phase consisted of methanol-0.0425 M phosphoric acid (70:30; vol/vol) pumped at a flow rate of 1 ml/min as described earlier (29, 33), with slight modifications. The column effluent was monitored for the absorbance at 284 nm, and the drug concentrations were calculated by using appropriate standard curves prepared for each tissue. The drug levels were expressed as micrograms per gram of tissue.

Statistical analysis. The data were expressed as means $\pm$ standard deviations for three to five animals. Values were compared by using Student's *t* test. The Bonferroni correction was performed to keep the global *P* value at 0.05; all values lower than 0.05 were considered significant.

RESULTS

MTD of L-CLF for infected mice. During our dose-response study with L-CLF, we observed that the MTD of the drug varied among the animals infected with different doses of MAC. Careful analysis of the data showed an inverse correlation between the extent of infection and the clofazimine dose tolerated (Table 1). Animals infected with the maximum number of MAC bacilli tolerated the least amount of drug. A single injection of L-CLF at a concentration of 25 mg/kg of body weight to animals infected with highest (5×10^7) number of bacilli resulted in 100% mortality within 24 h. Animals infected with 5×10^6 viable bacteria responded similarly to the dose of 50 mg/kg of body weight. Animals infected with low (5×10^4) and medium (5×10^5) doses, however, tolerated up to 50 mg/kg of body weight. The MTD of L-CLF for the uninfected control animals was 100 mg/kg.

Dose-response study of L-CLF treatment on MAC infection in beige mice. In the dose-response study, responses to various doses of L-CLF treatment were studied against four different levels of infection. Figure 1a to d indicates the effect of treatment on the multiplication of MAC in beige mice infected with various doses of the bacteria. It was noteworthy that the responses of different organs to treatment varied with the dose of initial infection. At the low infection dose (5×10^4), for example, a statistically significant ($P \leq 0.05$) difference in the numbers of CFU for control versus treated animals could be seen in lungs and livers with 5 mg of free as well as liposomal drug per kg; much higher doses, however, were required to obtain a similar reduction in spleens. In contrast, at a high infection dose (5×10^6), a higher drug concentration was needed for lungs, compared to that needed for livers and

FIG. 1. Effect of treatment with various concentrations of L-CLF on the multiplication of MAC in lung (■), liver (▨), and spleen (□) of animals infected with 5×10^4 CFU/mouse (a), 5×10^5 CFU/mouse (b), 5×10^6 CFU/mouse (c), and 5×10^7 CFU/mouse (d). Treatment was started on day 15 postinfection, and a total of six doses of the indicated drug concentration was administered intravenously to each animal. *, $P < 0.05$ compared with controls receiving empty liposomes. †, $P < 0.05$ compared with animals treated with 5 mg of L-CLF per kg.

spleens, before a significant reduction in the number of MAC CFU was seen. At the highest infection level, i.e., 5×10^7 bacteria per mouse, the lungs seemed almost resistant to therapy (Fig. 1d). At that infection level, 15 mg/kg was the highest dose that could be injected without causing significant mortality (Table 1). These results demonstrate the differential responses of various organs to therapy with L-CLF.

Although the therapeutic efficacy of free clofazimine as well as L-CLF did not appear to be significantly different at the 5-mg/kg dose, L-CLF definitely showed an advantage over the free drug because higher doses of L-CLF could be injected, resulting in better therapeutic efficacy. Compared with the orally administered drug, treatment with even the lowest dose of L-CLF (5 mg/kg) resulted in a significant reduction in the numbers of CFU; higher doses achieved a greater response (Fig. 1c).

Clofazimine levels in tissues. Intravenously injected free clofazimine or L-CLF was distributed to all major organs studied. Increasing levels of the drug were found with increasing doses of clofazimine in the three organs studied (Table 2). The maximum amount of drug could be detected in the lungs on a per-gram basis, while the liver showed the maximum amount of drug per tissue.

The level of infection did not affect the drug distribution in the lungs and liver. In the spleen, however, the trend suggested that the drug levels increased with increasing levels of infection. When the distribution of free versus liposomal drug at the 5-mg/kg concentration was compared, significantly higher levels of the drug were detected in the lungs of the animals injected with free clofazimine rather than L-CLF; the livers and spleens, however, did not show a similar trend. Higher levels of drug in all the tissues could be achieved in mice injected with 10 and 25 mg of L-CLF per kg. Significant levels of drug were also detected in the tissues of mice treated daily with oral doses of clofazimine, with the lungs showing the minimum concentration of the drug.

Effect of initial inoculum and duration of infection on treatment with L-CLF. Our observations that the infection level affected the response to treatment and that various organs responded differently to the treatment prompted us to examine the effect of treatment in various organs in relation to the initial infection and the time of treatment onset. Because of the differences in drug tolerance as well as organ-related differences in response to various doses of treatment, we selected the 25-mg/kg dose, which resulted in a significant reduction in the level of infection in all organs.

TABLE 2. Levels of clofazimine in organs of mice infected with various loads of MAC^a

Formulation and dose (mg/kg)	Clofazimine concn (μg/g of tissue) ^b								
	Lung			Liver			Spleen		
	Low	Med	High	Low	Med	High	Low	Med	High
Free clofazimine 5	6.8 ^c	17.4 ± 2.0	1.3 ± 0.9	UD ^d	0.3 ± 0.1	0.5 ± 0.3	UD	UD	2.3 ^c
L-CLF									
5	UD	0.53 ^c	0.43 ^c	0.25 ^c	0.2 ± 0.02	1.49 ^c	0.6 ^c	UD	UD
10	5.26 ± 1.9	2.46 ± 3.6	6.9 ± 1.7	0.6 ± 0.4	1.3 ± 1.2	5.9 ± 1.9	UD	10.9 ^e	6.1 ^c
25	27.8 ± 6.4	30.9 ± 5.8	42.4 ^c	2.9 ± 1.1	38.1 ± 11.1	9.5 ± 4.7	UD	26.9 ± 4.3	134.5 ^e
50	47.3 ± 23.5	82.9 ± 19.4	TD ^f	45.7 ± 2.0	79.2 ± 15.5	TD ^f	30.4 ± 5.9	117.0 ± 15.6	TD ^f
Oral clofazimine, 5 ^g	ND ^h	ND	2.3 ± 1.01	ND	ND	9.8 ± 2.1	ND	ND	4.5 ± 1.02

^a The animals treated at 15 days postinfection were sacrificed 1 day after administration of the last dose, and tissue were collected, processed, and analyzed as mentioned in the text.

^b Low, Med, and High, 10⁴, 10⁵, and 10⁶ CFU of MAC injected per mouse, respectively.

^c Drug was detected in one of three animals analyzed.

^d UD, undetectable.

^e Average of two values.

^f TD, toxic dose for this group.

^g Given daily by gavage.

^h ND, not done.

Effect of time lapse between infection and onset of treatment. The animals infected at various levels (low, medium, and high) and treated with six doses of 25 mg of L-CLF per kg responded to the treatment quite well (Fig. 2 to 4). Irrespective of the infection level, a significant ($P \leq 0.05$ to 0.001) reduction in the numbers of viable bacteria was seen in all tissues. A consistent observation at all infection levels was that the reduction in the numbers of CFU was higher when the treatment was started at a later time point after infection compared with that when the treatment was started early (Table 3). Treatment at day 22 postinfection showed higher log differences in control versus treated animals compared with the differences in those treated on days 1, 8, and 15 postinfection. These differences were statistically significant ($P \leq 0.05$; the Bonferroni correction was applied).

Responses of different Organs to therapy. Significant organ-related differences in response to treatment were observed; these changes were also affected by the level of infection. A detailed study of the effects on organs yielded the following observations.

(i) Lungs. The results obtained for the lungs (Fig. 2) were of particular interest because most of the earlier studies with liposome-encapsulated antibiotics had not been able to demonstrate significant reductions in the numbers of infecting bacilli. Also in our study, the L-CLF treatment schedule failed to significantly reduce the number of MAC organisms in the lungs of mice infected with the highest MAC dose (Fig. 1d). However, at low and medium infection doses of MAC, the drug response was much better (log differences, 2.43 and 2.33, respectively) compared to that at the high infection level (log difference, 1.37) when the treatment was started at day 22 postinfection.

(ii) Liver. For mice infected with MAC at 10⁴, 10⁵, and 10⁶ CFU/mouse and treated, L-CLF started showing its effect from day 1 postinfection (log difference, ≥ 0.4), but the best effect was seen when the treatment was started at day 22 postinfection. The log differences in the numbers of CFU observed in tissues from animals infected with low, medium, or high doses were 1.1, 1.8, and 2.49, respectively. Thus, the efficacy of L-CLF in liver was highest when the animals were infected with

the maximum number of bacilli and treatment was started at day 22 postinfection (Fig. 3).

(iii) Spleen. Considering a log difference of 0.5 to be biologically significant, reductions in the numbers of CFU in the spleens was significant, irrespective of the amount of inoculum or the time of treatment (Table 3; Fig. 4). However, there was a slight trend which indicates that the treatment was more effective (in terms of a reduction in the number of infecting bacilli) when it was started at a later time point, i.e., at day 22 postinfection.

Histopathology. The extent of infection, reflected by the number of bacteria seen in the histological sections, was reduced as the drug concentration increased. These results

TABLE 3. Log differences in numbers of CFU of MAC recovered from various organs of control versus treated mice^a

Infection dose	Day of treatment	Log CFU differences		
		Lung	Liver	Spleen
5 × 10 ⁴ (low)	1	1.07 ^b	0.41 ^b	0.70
	8	1.02 ^b	0.46 ^{b,c}	0.37 ^c
	15	1.39 ^b	-0.27 ^c	0.62 ^c
	22	2.43	1.00	1.00 ^c
5 × 10 ⁵ (medium)	1	1.75	0.80 ^b	0.99 ^b
	8	1.35	0.31 ^{b,c}	0.81 ^{b,c}
	15	0.36 ^b	0.73 ^{b,c}	0.88 ^{b,c}
	22	2.33	1.80	1.72
5 × 10 ⁶ (high)	1	0.87	0.41 ^b	0.70 ^b
	8	1.64	1.24 ^b	1.51 ^b
	15	1.20	1.81 ^b	2.45
	22	1.37	2.49	1.82

^a The treatment was started on days 1, 8, 15, and 22, and the animals were sacrificed 1 day after administration of the last dose. Log differences are between treated mice versus respective control mice (receiving empty liposomes).

^b Significant change compared to the numbers of CFU on day 22 in respective groups ($P < 0.05$).

^c Statistically lower compared to the log differences for high infection ($P < 0.05$; the Bonferroni correction was applied).

FIG. 2. Effect of treatment with L-CLF on the multiplication of MAC in the lungs of control (■) and treated (▨) animals infected with low (a), medium (b), or high (c) doses of MAC. Treatment was started on the indicated days, and each animal received a total of six doses of L-CLF at a dose of 25 mg/kg of body weight. *, $P < 0.05$ compared with controls receiving empty liposomes.

therefore supported the bacteriological observations, including the higher efficacy of L-CLF than that of the free clofazimine or oral treatment regimens. None of the tissue sections showed the presence of clofazimine crystals.

DISCUSSION

Although clofazimine is an active component of many multidrug therapies for MAC, its efficacy in treating MAC infection in patients with AIDS is not well documented. Oral administration of this drug to patients has led to many side effects such as gastrointestinal intolerance, severe and life-threatening abdominal pain, and organ damage caused by clofazimine crystal deposition (17). Some of these adverse effects can be overcome by using a nontoxic, injectable formulation of the drug. Intravenous administration is a potential route for treatment of an intracellular pathogen like MAC, which causes disseminated infection. The drug's lipophilic nature, however, restricts

its intravenous administration. In this study, the MTD of free clofazimine was 5 mg/kg of body weight, which was not enough to cause a significant reduction in the number of viable bacteria in all organs studied (Fig. 1a to d). Moreover, even this concentration of free drug cannot be administered to patients, for obvious reasons, such as the presence of organic solvents and crystallization in the presence of the aqueous phase. Therefore, it was imperative to look for alternate modes of delivery to increase the availability of clofazimine as well as make it acceptable for use in patients. Encapsulation of clofazimine in multilamellar vesicles composed of DMPC-DMPG (7:3) allowed for the intravenous administration of clofazimine, thereby reducing the number of doses and enhancing therapeutic efficacy, supporting our earlier observations (29). Liposomes composed of the lipids used in this study have been proven to be safe for use in humans (26, 27).

Our data indicate that L-CLF is more effective than free clofazimine injected intravenously or administered orally in

FIG. 3. Effect of treatment with L-CLF on multiplication of MAC in the livers of control (■) and treated (▨) animals infected with low (a), medium (b), or high (c) doses of MAC. Treatment was started on the indicated days, and each animal received a total of six doses of L-CLF at a dose of 25 mg/kg of body weight. *, $P < 0.05$ compared with controls receiving empty liposomes.

FIG. 4. Effect of treatment with L-CLF on the multiplication of MAC in the spleens of control (■) and treated (▨) animals infected with low (a), medium (b), or high (c) doses of MAC. Treatment was started on the indicated days, and each animal received a total of six doses of L-CLF at a dose of 25 mg/kg of body weight. *, $P < 0.05$ compared with controls receiving empty liposomes.

beige mice for the treatment of MAC infection. The therapeutic efficacy of L-CLF was statistically significant ($P \leq 0.05$ to 0.001) in all the organs at all infection levels, irrespective of the time of treatment. The best efficacy was observed, however, at the delayed onset of treatment, when the infection was already established. This enhanced antibacterial activity of clofazimine on day 22 can be attributed to the synergistic effect of the drug and certain cytokines released by activated T lymphocytes as a result of the acquired specific immunity. It is well established that the specific acquired immunity takes at least 3 weeks to develop (9). It is therefore possible that the cytokines released by activated lymphocytes or macrophages may be acting synergistically with clofazimine to produce an enhanced therapeutic effect. Moreover, the combination of antimycobacterial drugs and cytokines has been reported to be effective against MAC infection (4, 9, 10, 32). How the cytokines may potentiate the antibacterial activity of clofazimine is a subject for future studies. Preliminary studies conducted in our laboratory

showed that preactivation of macrophages with gamma interferon leads to the enhanced efficacy of L-CLF (21). Furthermore tumor necrosis factor alpha has been reported to be able to potentiate the pro-oxidative effects of clofazimine (24) which might be involved in the antimycobacterial activity of macrophages. The organ-related differences that we observed in response to the treatment (Fig. 1a to d and 2 to 4) were also affected by the level of infection. In the lungs, for example, at lower and medium infection levels (10^4 and 10^5 CFU per mouse, respectively) the reduction in the numbers of CFU was significantly more (log differences, 2.43 and 2.33, respectively) compared to that in the mice infected with the high dose (log difference, 1.37). In another study (29), the lungs failed to respond to the L-CLF therapy when the animals were infected with higher numbers of bacteria. On the other hand, the liver showed a greater reduction in the numbers of CFU with the high level of infection.

The drug levels attained in tissues (Table 2) suggested that increased levels of clofazimine can be obtained in tissues by injecting higher doses of L-CLF. Although the level of infection did not affect drug levels in all organs, we observed a significant reduction in the numbers of CFU with increasing doses of L-CLF at all infection levels. The fact that the drug levels did not change significantly with an increasing level of infection might help to explain the peculiar situation seen in the lung, where a higher level of infection was difficult to treat. The drug concentration obtained in the lung was enough to kill only a small number of bacteria, and as the number of infecting bacteria increased, the drug was not able to reduce the bacterial load significantly. The spleen, in contrast, showed higher concentrations of drug with higher infection levels; hence, the reduction in the numbers of CFU was observed even at the highest infection level (Fig. 1d). These organ-related differences therefore seem to result from either the relative distribution of bacteria versus drug, the localization of bacteria and the drug in various organs at various times, or both of these. The indirect effect of various cytokines under the influence of the dose of the inoculum as well as organ type might also contribute to these differences (5).

Most of the earlier studies with liposomal aminoglycosides (11, 13, 15, 19, 36) or other drugs like capreomycin (25) at an infection level of approximately 10^7 bacteria per mouse could not demonstrate a significant reduction in the numbers of bacteria in the lungs, which is similar to our observations in animals infected with a similar dose. Since the lower infection levels have never been used before with a late onset of treatment after infection is established, it is difficult for us to compare our observations with published data.

Keeping in mind the differential organ responses to treatment, we emphasize the importance of examining all major organs involved in MAC infection, especially the lung, liver, and spleen, which are expected to be more affected by liposomal drugs, in evaluations of the efficacies of such drugs. It may at times also be important to evaluate drug efficacy in a clinically more relevant situation, one in which treatment is started when the infection has been established, as was done in this study during the late onset of treatment. Although this might take longer time for the evaluation process, it may change the outcome of therapy to a significant extent, making it more relevant to the clinical situation.

Our study showed that L-CLF is highly effective against MAC infection, as was evident from the significant reduction in the numbers of bacterial CFU in beige mice. The effect of L-CLF is more pronounced when it is given at a later stage of infection, a situation similar to the clinical experience with AIDS patients. The enhanced therapeutic effect may be caused

by the combined effect of clofazimine and certain cytokines produced by activated T lymphocytes (21). In view of the reduced numbers of T-helper cells in AIDS patients, the combination of L-CLF with cytokines might be a plausible prospective regimen for treating disseminated MAC infection in AIDS patients.

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