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Use of Normal C57BL/6 Mice with Established *Mycobacterium avium* Infections as an Alternative Model for Evaluation of Antibiotic Activity

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Several murine models have been used to evaluate the activities of antimicrobial agents against *Mycobacterium avium* infection. The main model used is the beige mouse model, but beige mice are expensive and not easily available. Thus, we developed a model of infection in wild C57BL/6 mice. The drugs that exhibited some activity in a previous model of early infection were evaluated in a new model of established infection. Sparfloxacin (50 mg/kg of body weight), ethambutol (50 mg/kg), minocycline (25 mg/kg), and the inhibitor of the cortisol receptors RU-40555 (100 mg/kg) were compared with clarithromycin (50 mg/kg). Treatments were started 5 weeks after the inoculation and were continued for 21 days. Sparfloxacin and RU-40555, which exhibited a moderate activity in the model of early infection, were not effective in this model of established infection. Clarithromycin and combinations with clarithromycin kept their activities against *M. avium* infection, both in the spleen and in lungs. The present model of established infection of normal C57BL/6 mice is more relevant than the model of early infection for a stringent evaluation of drugs.

Disseminated *Mycobacterium avium* complex (MAC) infection is one of the most common bacterial infections in patients with AIDS (7). The experimental evaluation of the activities of antimicrobial agents against MAC is done in cellular models using macrophages or in animal models. The main animal model used is the beige mouse model (1, 5). This model uses mice that are deficient in natural killer cells, providing a kind of immunodeficiency different from that of AIDS in humans. Beige mice, which often develop spontaneous tumors, are expensive and not easily available. Alternative models of mice with cellular immunodeficiency, such as TxCD4$^-$ mice, Thxb mice, or nude mice, have been suggested (3, 4). TxCD4$^-$ mice are C57BL/6 mice thymectomized at 4 weeks of age and treated intravenously with anti-CD4 antibodies. Thxb mice are adult thymectomized BALB/c mice that are subjected to lethal whole-body gamma irradiation and are reconstituted with syngeneic bone marrow cells. Nude mice require a sterile environment. These models, difficult to realize, are not easily used for routine investigations. We have shown that the CFU counts of MAC in organs of untreated wild C57BL/6 mice progressively rose after an initial 4-week plateau (10). The bacterial load reaches concentrations higher than 10^8 CFU/g of spleen and higher than 10^7 CFU/g of lung after 6 months of infection, without the spontaneous clearance of bacteria observed with Swiss Webster mice (4–6). The model of early infection of normal C57BL/6 mice may be used for a rapid screening of drugs (8, 9, 10). In patients with AIDS, MAC infection has a subacute or a chronic clinical course. Thus, a model of established infection of C57BL/6 mice should be more relevant than the model of early infection for mimicking human infection and should represent a more stringent model for the evaluation of antimicrobial agents.

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Clarithromycin is now considered the reference antibiotic for treatment of MAC infection. The aim of the present study was to evaluate the model of established infection of C57BL/6 mice, in which antimicrobial treatments are started 5 weeks after the challenge with MAC. Several antibiotics (sparfloxacin, minocycline, and ethambutol) and an immunomodulator (RU-40555), already studied in the model of early infection of C57BL/6 mice (8, 9, 10), were studied in this new model, and their activities were compared to that of clarithromycin.

MATERIALS AND METHODS

Bacteria. The MO-1 strain of MAC, used in our previous studies (2, 8–12), was isolated from a patient with AIDS and used after a single subculture on mycobacterial Middlebrook 7H11 agar (Difco Laboratories, Detroit, Mich.) supplemented with Middlebrook OADC enrichment (Difco). One flat transparent colony was picked and cultivated at 37°C in Middlebrook 7H9 broth (Difco) supplemented with ADC enrichment (Difco) in Falcon tissue culture flasks (Becton Dickinson, Oxnard, Calif.). After 21 days of culturing, the bacterial suspension was adjusted to a density of 1 mg/ml with a turbidimeter (Institut Pasteur, Paris, France), and aliquots of the bacterial suspension were frozen at −80°C.

Model of established infection. Seven-week-old female normal C57BL/6 mice from Iffa Credo were used for established infection studies. Mice were challenged with 1.5×10^7 viable bacteria injected intravenously. The spontaneous evolution of infection in organs is presented in Fig. 1. All mice stay alive, even after a 6-month course of infection.

Antibiotics. Clarithromycin (Abbott France, Rungis, France), sparfloxacin (Rhône DPC Europe, Antony, France), and minocycline and ethambutol (Lederle Laboratories, Oullins, France) were provided by the manufacturers. Clarithromycin was diluted in sterile water; sparfloxacin was dissolved in 0.1 N NaOH and diluted in phosphate-buffered saline. Minocycline and ethambutol were diluted in sterile water.

Immunomodulator. RU-40555 is an inhibitor of the cortisol receptors that was previously tested in the early-infection model (Roussel-UCLAF, Romainville, France). RU-40555 was provided by the manufacturer and suspended in carboxymethyl cellulose.

Antibiotic susceptibility testing. The MICs of clarithromycin, sparfloxacin, and ethambutol were determined by the agar macrodilution method. Serial twofold dilutions of each antimicrobial agent were incorporated into 7H11 agar medium plated in quadrant petri dishes. The inoculum was made from a 7-day-old culture in Dubos-Tween medium and was adjusted to 1 mg (wet weight)/ml and diluted to 10^{-3} and 10^{-5}. From each dilution, 0.05 ml was plated on one quadrant. Every

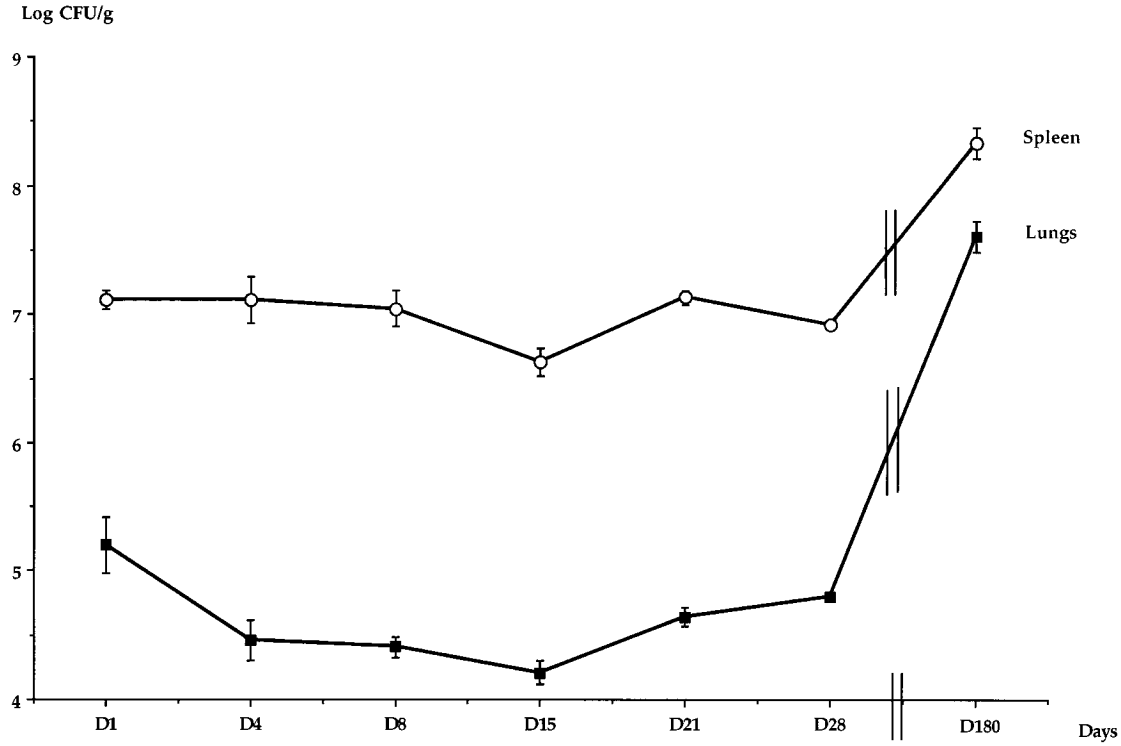

FIG. 1. Time course of MAC CFU (spontaneous evolution during 6 months) in the spleens and lungs of untreated C57BL/6 mice inoculated at day 0 with 1.5×10^7 CFU by intravenous injection. The data are means ± standard errors of the means (bars).

assay was duplicated. Plates were incubated at 37°C, and colonies were counted after 14 days of culturing. The lowest concentration of the drug that inhibited more than 99% of the bacterial population was considered to be the MIC. Since the pH of the medium modifies the MICs of macrolides, the MIC of clarithromycin was determined in Mueller-Hinton agar medium (pH 7.4) supplemented with Middlebrook OADC enrichment (10, 13).

Administration of drugs. Mice were divided into seven groups. The clarithromycin group received clarithromycin, 50 mg/kg once daily (o.d.) in 0.1 ml given subcutaneously (s.c.). The sparfloxacin group received sparfloxacin, 50 mg/kg of body weight o.d. in 0.2 ml given s.c. The other treatments were RU-40555, 100 mg/kg o.d. in 0.4 ml given intraperitoneally, ethambutol, 50 mg/kg o.d. in 0.1 ml given s.c., and minocycline, 25 mg/kg o.d. in 0.1 ml given s.c. The following groups received the same regimen of each drug as did the single-drug groups: clarithromycin plus minocycline, clarithromycin plus RU-40555, clarithromycin plus RU-40555 plus minocycline, and sparfloxacin plus RU-40555 plus ethambutol. Control groups received saline. Treatments were started 5 weeks after the inoculation and were continued for 21 days.

Quantitation of mycobacteria in the spleen and lung. Treated and control mice were sacrificed at days 7, 14, and 21 of treatment, 24 h after the last injection of the drug(s). Control mice were also sacrificed the first day of treatment. At least four animals were sacrificed at each time point. The spleen and the right lung of each mouse were removed aseptically, weighted, and homogenized in sterile water with a glass homogenizer. Serial 10-fold dilutions were plated onto 7H11 agar (Difco) supplemented with OADC enrichment. After 14 days at 37°C, colonies were counted and the number of CFU per gram of tissue was calculated.

Statistical analysis. Means ± standard errors of the means were determined. Comparisons between groups were made by Student's *t* test.

RESULTS

In vitro susceptibility to antimicrobial agents. The MICs for MAC strain MO-1 were 0.5 μg of sparfloxacin per ml, 8 μg of ethambutol per ml, 64 μg of minocycline per ml, and 2 μg of clarithromycin per ml (at pH 7.4).

Effect of treatment on infection in mice. CFU counts in the spleen are shown in Fig. 2. Compared with saline, clarithromycin alone and the combinations of clarithromycin plus RU-40555, clarithromycin plus minocycline, and clarithromycin plus minocycline plus RU-40555 decreased the CFU in the spleen ($P < 0.02$). Sparfloxacin alone or its combination with ethambutol plus RU-40555 did not decrease the CFU in the spleen.

Compared with sparfloxacin, clarithromycin ($P < 0.001$) and the combinations of clarithromycin plus RU-40555, clarithromycin plus minocycline, and clarithromycin plus minocycline plus RU-40555 decreased the CFU counts in the spleen ($P < 0.02$). Clarithromycin was more effective than the combination of sparfloxacin plus ethambutol plus RU-40555 in decreasing the CFU counts in the spleen ($P < 0.05$). Clarithromycin and the combinations of clarithromycin plus minocycline and clarithromycin plus minocycline plus RU-40555 were more effective than the combination of clarithromycin plus RU-40555 ($P < 0.02$).

CFU counts in the lung are shown in Fig. 3. Compared with saline, clarithromycin ($P < 0.05$) and the combinations of clarithromycin plus RU 40555 ($P < 0.05$), sparfloxacin plus ethambutol plus RU-40555 ($P < 0.001$), clarithromycin plus minocycline ($P < 0.001$), and clarithromycin plus minocycline plus RU-40555 ($P < 0.001$) decreased the CFU counts in the lungs.

Among the treated mice, the combinations of clarithromycin plus minocycline and clarithromycin plus minocycline plus RU-40555 were more effective than sparfloxacin alone ($P < 0.02$).

DISCUSSION

In vivo assessment of potential chemotherapeutic drugs against MAC remains difficult, and to establish the complete chemotherapeutic efficacy of any compound, the animal model must closely resemble human infection. Gangadharam et al. have shown, at 4 weeks after challenge, that the lungs of un-

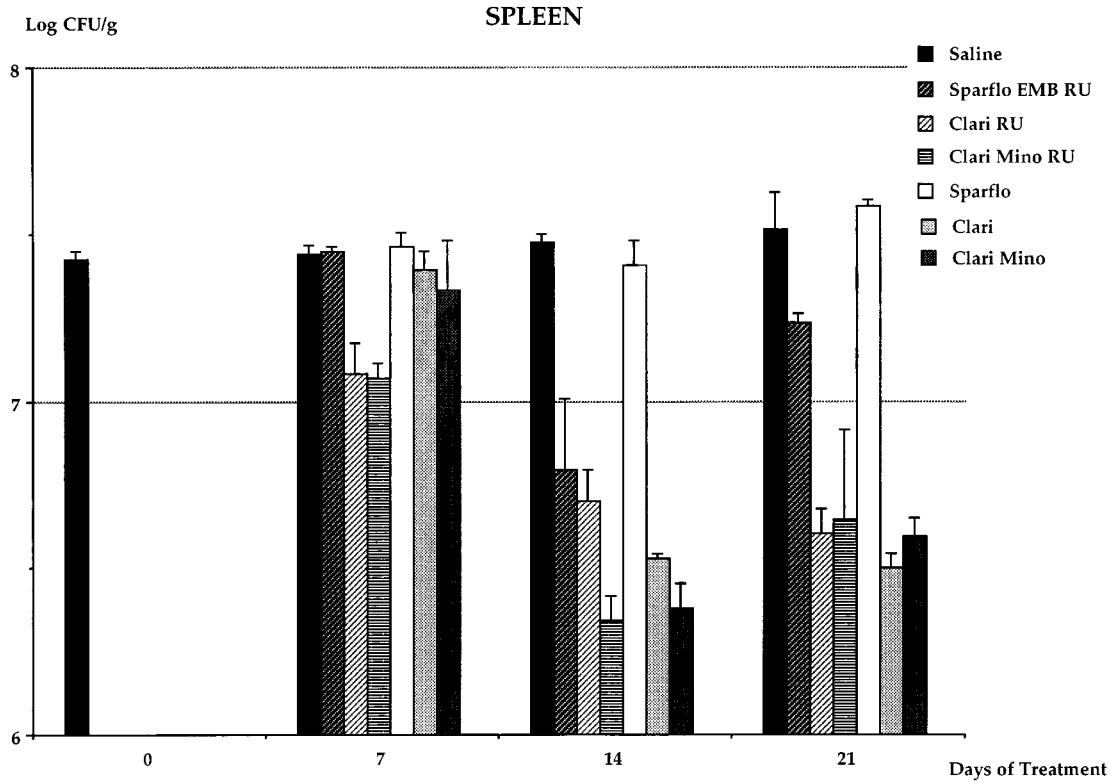

FIG. 2. Time course of MAC CFU in the spleens of C57BL/6 mice treated with sparfloxacin (Sparflo; 50 mg/kg/day s.c.), ethambutol (EMB; 50 mg/kg/day s.c.), RU-40555 (RU; 100 mg/kg/day intraperitoneally), clarithromycin (Clari; 50 mg/kg/day s.c.), and minocycline (Mino; 25 mg/kg/day s.c.). These drugs were used alone or in combination. Control mice were treated with saline. The data are means ± standard errors of the means (bars).

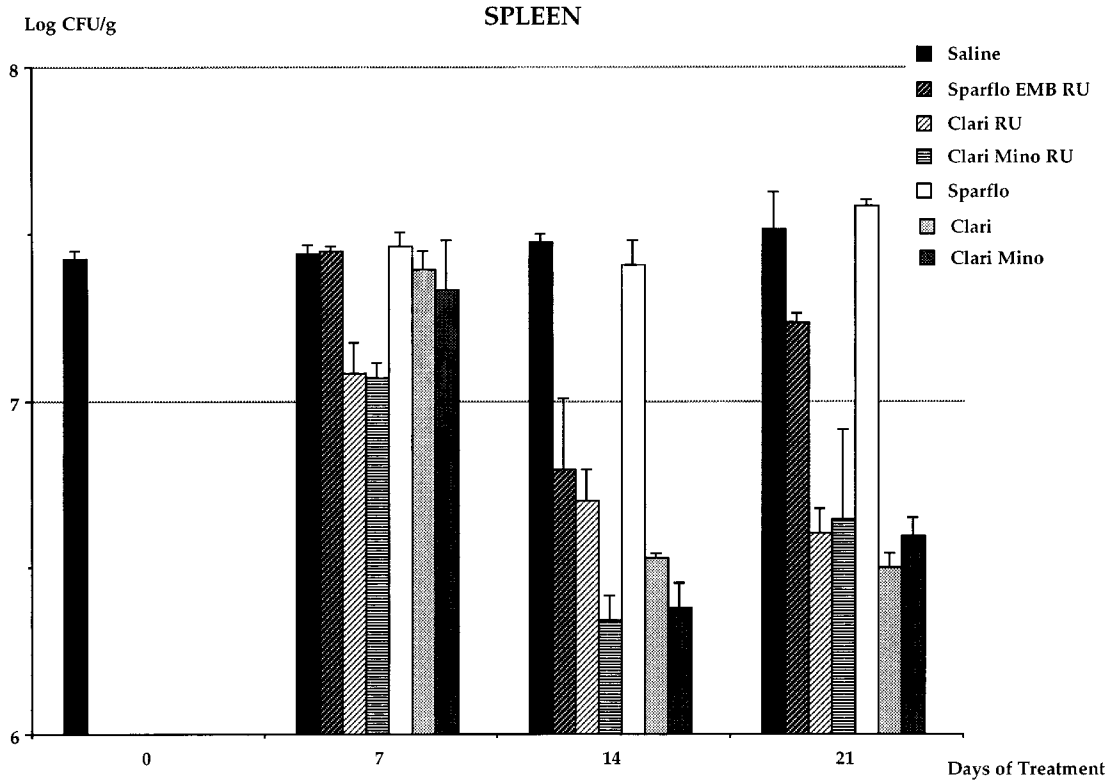

FIG. 3. Time course of MAC CFU in the lungs of C57BL/6 mice treated with sparfloxacin (Sparflo; 50 mg/kg/day s.c.), ethambutol (EMB; 50 mg/kg/day s.c.), RU 40555 (RU; 100 mg/kg/day intraperitoneally), clarithromycin (Clari; 50 mg/kg/day s.c.), and minocycline (Mino; 25 mg/kg/day s.c.). These drugs were used alone or in combination. Control mice were treated with saline. The data are means ± standard errors of the means (bars).

treated infected mice exhibited histiocytic proliferation in the alveoli and alveolar septa, involving approximately 50% of the parenchymal area (6). Thus, the activities of antibiotics in these conditions might be different from those in an early infection. A chronic established infection, closer to the human infection, is more difficult to eradicate than an early acute infection.

We evaluated in this model of established infection of C57BL/6 mice activities of drugs that exhibited some activity in the model of early infection.

The new fluoroquinolone sparfloxacin, which exhibited a moderate activity against MAC infection in the model of early infection of C57BL/6 mice, was not effective in the present model of established infection. Furthermore, the combination of sparfloxacin plus ethambutol plus RU-40555, which exhibited a good bactericidal activity in the model of early infection, was not effective in the spleen in the model of established infection.

RU-40555, an immunomodulator able to enhance the inflammatory reaction, provided discordant results in the model of early infection: when started after the bacterial challenge, RU-40555 treatment could slightly enhance the activity of antibiotics, but this benefit was not present when the treatment was started before challenge (8, 10). In the model of established infection, RU-40555 did not exhibit any activity.

It is important that clarithromycin kept its activity against MAC infection, either in the spleen or in lungs, in this model of established infection. The combination of clarithromycin and minocycline was also effective in this model, with the combination being slightly more effective than clarithromycin alone in the lungs. However, this difference did not reach statistical significance.

Our study confirms that normal C57BL/6 mice may be used for the evaluation of antimicrobial agents. We have previously shown that the model of early infection, which is simple and rapid, may be useful for an initial screening of drugs (8–10). The present model of established infection is more relevant than the model of early infection for a stringent evaluation of drugs. For the future development of the model, several strains with different susceptibility patterns should be used. The discriminant power of this model is confirmed by the good activity of the reference compound, clarithromycin, against established experimental infection.

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