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Treatment of *Mycobacterium haemophilum* Infection in a Murine Model with Clarithromycin, Rifabutin, and Ciprofloxacin

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An animal model of disseminated *Mycobacterium haemophilum* infection was utilized to compare treatment with azithromycin, ciprofloxacin, rifabutin, and the combination of clarithromycin with rifabutin. Following subcutaneous challenge with *M. haemophilum*, local and disseminated infection occurred only in immunosuppressed mice. For disseminated infection, ciprofloxacin was relatively ineffective therapy. Clarithromycin and rifabutin alone significantly reduced the tissue burden in the spleen after 4 weeks of therapy. Combination therapy with rifabutin and clarithromycin was superior to 4 weeks of treatment with the individual agents. When immunosuppressed mice were treated for 20 weeks with the combination of rifabutin and clarithromycin, the tissue burden remained reduced in the spleen at 1 month following the completion of therapy. Combined rifabutin and clarithromycin provide effective treatment for *M. haemophilum* in this model.

Mycobacterium haemophilum has been recently recognized as a pathogen in immunocompromised patients (7, 17, 18). The typical clinical presentation is the appearance of painful cutaneous nodules, subcutaneous abscesses, or plaques on the extremities (5, 7, 13). Disseminated infections include bacteremia, upper respiratory complaints, and infection of the bones, joints, lymph nodes, and lungs (7, 18). Patients do not respond to therapy with standard antituberculous drugs and may experience chronic pain and disability. No standard approach to treatment has been defined, and clinical correlation between the in vitro and in vivo activity of the drugs is unknown. Susceptibility testing of this organism is not presently standardized, as growth requirements differ greatly from those of other mycobacteria (6, 8, 19). Treatment regimens in immunosuppressed patients have included ciprofloxacin, amikacin, streptomycin, rifampin, ethionamide, cycloserine, azithromycin, clarithromycin, imipenem, ethambutol, erythromycin, doxycycline, minocycline, clofazamine, dicloxacillin, isoniazid, and pyrazinamide as monotherapy or in combination (2, 7, 16) and excision (14). At present, ciprofloxacin and rifampin appear efficacious and azithromycin or clarithromycin appears promising (7). Recurrence of disease after treatment has occurred.

In this study, we established an animal model of infection with *M. haemophilum* and compared the in vivo effectiveness of rifabutin, clarithromycin, ciprofloxacin, and the combination of rifabutin plus clarithromycin in the treatment of infection by this organism.

MATERIALS AND METHODS

Mice. Three strains of mice were compared: outbred immunocompetent ICR mice, inbred immunocompetent BALB/c *nu*/+ mice which were slightly more susceptible to *M. haemophilum*, and inbred immunodeficient BALB/c *nu*/*nu* mice. ICR outbred Harlan Sprague-Dawley male mice (Indianapolis, Ind.), euthymic BALB/c *nu*/+ mice, and BALB/c *nu*/*nu* mice (veterinary medical unit breeding colony of the Audie Murphy Veterans Administration Hospital) were housed five per cage with free access to water and food.

Microorganisms. Four clinical isolates of *M. haemophilum*, previously described by Dever et al. (7), were obtained from the clinical microbiology laboratory of J. H. Jorgensen, University of Texas Health Science Center at San Antonio. Isolates were grown on chocolate agar plates (BBL, Baltimore Biologics, Cockeysville, Md.) and incubated at 30°C in 5% CO_2 for 10 days. Colonies were scraped from the agar surface, suspended in 0.9% NaCl, and sonicated until a uniform suspension was obtained. CFU counts were determined by plating 0.1 ml of 10-fold serial dilutions in saline onto chocolate agar plates in duplicate and incubating the plates at 30°C in a CO_2 incubator for 1 month or until growth was observed.

MICs. The MICs were determined by a broth microdilution technique with 7H9 broth containing 50 μg of hemin per ml. The inoculum was 5×10^5 CFU/ml. The tubes were incubated at 30°C in ambient air for 3 to 5 days.

Infection. In the pilot study to determine the mouse model, ICR, BALB/c *nu*/+, or BALB/c *nu*/*nu* mice were challenged with the four isolates of *M. haemophilum*. Five mice per isolate were infected at four sites on the back with 0.05 ml of 6.7×10^6 to 6×10^7 CFU of *M. haemophilum* per ml, both undiluted and in twofold dilutions (1:2, 1:4, and 1:8) of the inoculum. The mice were observed for lesions and sacrificed at 8 weeks. A sample of spleen and one of skin from the injection site were cultured and sent for histologic examination. In a subsequent experiment, the mice were challenged subcutaneously with an undiluted inoculum (1.0×10^6 CFU of isolate 2 per ml) into the right shoulder. CFU per gram of spleen and cultures of the skin were obtained at 2, 4, 6, and 8 weeks. Additionally at 8 weeks, heart blood was obtained from anesthetized mice and inoculated into isolator culture tubes (Wampole Laboratories, Cranbury, N.J.) and plated onto chocolate agar plates. Lung tissue was also excised at 8 weeks, and CFU per gram of tissue was determined. Skin lesions were photographed, and tissue from the injection site, spleen, kidneys, and lungs was sent for histologic examination.

Intravenous challenge. ICR outbred mice and immune-deficient BALB/c *nu*/*nu* mice were challenged with a 0.1-ml suspension of 10^6 CFU of *M. haemophilum* (isolate 2) per ml intravenously via the lateral tail vein. One hour after challenge, some of the mice were sacrificed, the spleens were weighed, and CFU per gram tissue was determined for assessment of tissue burden.

Antimicrobial agents. Clarithromycin (Abbott Laboratories, North Chicago, Ill.) was dissolved in methanol, diluted in phosphate-buffered saline at pH 7.4, and refrigerated for not more than 2 weeks. Rifabutin (Adria Laboratories, Columbus, Ohio) was prepared daily by dissolving in methanol and then diluting in sterile water. Ciprofloxacin for intravenous infusion was obtained from Miles Laboratories, West Haven, Conn.

Treatment regimens. Two weeks after a subcutaneous challenge with a ciprofloxacin-susceptible isolate of *M. haemophilum* (isolate 2), BALB/c *nu*/*nu* mice (five mice per group) were randomized and treated for 4 weeks with either clarithromycin (200 mg/kg of body weight per day), rifabutin (40 mg/kg/day), or the combination of clarithromycin plus rifabutin administered per os via gavage or ciprofloxacin (100 mg/kg/day) given intraperitoneally in a volume of 0.2 ml per dose. At 2, 4, and 8 weeks, 48 h after therapy, groups of mice were anesthetized with methoxyflurane (Metofane; Pitman-Moore, Washington Crossing, N.J.) and

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TABLE 1. MICs (micrograms per milliliter) of antimicrobial agents for three isolates of *M. haemophilum*

Antimicrobial agent	MIC for isolate:		
	1	2	3
Amikacin	32	32	>32
Amoxicillin	16	8	
Azithromycin	>8	8	>8
Ciprofloxacin	8	2	>8
Clarithromycin	1	0.5	2
Doxycycline	8	>16	
Imipenem	2	8	>16
Rifampin	1	0.25	0.5
Temofloxacin	8	4	
TMP-SMZ[a]	<0.25	0.25	>8

[a] TMP-SMZ, trimethoprim-sulfamethoxazole.

sacrificed. Heart blood was obtained and placed into pediatric 1.5-ml isolator tubes (Wampole) for quantitative blood culture. The spleens were excised, weighed, and sampled for histologic examination. The spleens were reweighed and homogenized in 2 ml of sterile normal saline. The homogenates were diluted by serial 10-fold dilutions in saline. Each dilution (0.1 ml) and the undiluted homogenate were plated on chocolate agar plates in duplicate and incubated in 5% CO_2 at 30°C for 4 to 6 weeks. CFU counts were determined, and the CFU per gram of tissue was calculated. The minimum count was 18 CFU/g of tissue. The lesions were photographed and observed daily for regression with therapy.

Additionally, five animals per group were treated for 20 weeks with the combination of clarithromycin and rifabutin. CFU per gram of spleen was compared with those for untreated mice at 4, 12, 20, and 24 weeks (4 weeks posttreatment).

RESULTS

MIC. The MICs of antimicrobial agents for three isolates of *M. haemophilum* are shown in Table 1. Isolate 2 appeared to be the most susceptible to ciprofloxacin, clarithromycin, and rifampin and was therefore used in the treatment studies.

Intravenous challenge. Both ICR and BALB/c *nu*/*nu* mice were sacrificed 1 h after intravenous challenge with 10^6 CFU of *M. haemophilum* isolate 2 per ml. The average CFU per gram of spleen was 5.6×10^5 CFU/ml in BALB/c *nu*/*nu* mice.

Infection. Eight weeks after subcutaneous challenge, all four isolates of *M. haemophilum* were recovered from both the spleen and the skin of BALB/c *nu*/*nu* mice but not from the ICR or BALB/c *nu*/+ immunocompetent mice. Acid-fast bacilli were observed in poorly formed granulomas from both the skin and the spleens of mice infected with each of the four isolates.

Table 2 shows the recovery of *M. haemophilum* from skin, spleens, blood, and lung at 8 weeks following subcutaneous injection into the right shoulder with isolate 2. Skin lesions were observed in the BALB/c *nu*/*nu* mice, but no lesions were observed in the ICR mice. *M. haemophilum* was recovered from the spleens of the four BALB/c *nu*/*nu* and *nu*/+ mice but from only one spleen of immunocompetent ICR mice. Lungs were also excised at 8 weeks, and no growth was observed in the ICR mice; two of five BALB/c *nu*/*nu* mice had 5.8×10^2 and 3.5×10^4 CFU/g of lung. None of the heart blood from the ICR mice which was plated directly on the chocolate agar grew *M. haemophilum*; all of the BALB/c *nu*/*nu* mice grew 1 to 200 CFU/ml.

TABLE 2. Recovery of *M. haemophilum* from skin, spleen, blood, and lung 8 weeks after subcutaneous infection

Tissue	No. of animals with positive culture (n = 5) for mouse type		
	nu/*nu*	*nu*/+	ICR
Blood	5	0	0
Spleen	4	4	1
Lung	2	0	0
Skin	4	0	1

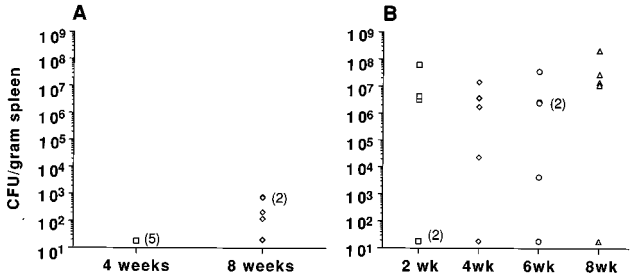

FIG. 1. (A) CFU per gram of spleen after subcutaneous infection with *M. haemophilum* at 4 and 8 weeks in BALB/c *nu*/+ mice. (B) CFU per gram of spleen after subcutaneous infection with *M. haemophilum* at 2, 4, 6, and 8 weeks in athymic BALB/c *nu*/*nu* mice. Numbers in parentheses are the numbers of animals per group that were tested.

The results of the determinations of CFU per gram of spleen are shown in Fig. 1. BALB/c *nu*/+ mice (five per group) were also challenged subcutaneously with the same inoculum. No growth was observed from the spleens at 4 weeks; however, at 8 weeks the CFU per gram of spleen ranged from 2.2×10^2 to 1.4×10^4 CFU/g of tissue (Fig. 1A). CFU/gram of tissue was greater than 10^6 CFU/ml for the BALB/c *nu*/*nu* mice (Fig. 1B).

Treatment. Treatments for 4 weeks with ciprofloxacin, rifabutin, clarithromycin, and the combination of clarithromycin and rifabutin were compared (Fig. 2). At 2 weeks (Fig. 2A), growth appeared in the clarithromycin- and ciprofloxacin-treated mice but not in the rifabutin- or rifabutin-plus-clarithromycin-treated mice. At 4 weeks (Fig. 2B), growth was observed in only the ciprofloxacin-treated and control mice. One month posttreatment, growth of *M. haemophilum* was observed in the rifabutin-, clarithromycin-, and ciprofloxacin-treated mice but not in the combined rifabutin-and-clarithromycin-treated mice (Fig. 2C). When animals were treated with combination therapy of clarithromycin and rifabutin for 20 weeks with evaluation at 4, 12, 20, and 24 weeks, *M. haemophilum* was not recovered from any of the treated mice. Lesions (Fig. 3) disappeared after 5 to 7 days in treated groups except those treated with ciprofloxacin. All untreated, control mice died before completion of the treatment period; the majority were dead before 12 weeks. Consequently, there were no evaluable controls at 20 or 24 weeks. Five treated mice were available for evaluation at both 20 and 24 weeks; no *M. haemophilum* isolates were recovered from blood, skin, or spleens and no acid-fast bacilli were observed in any tissue.

Pathology. The results of the histologic findings for the skin and spleens of the untreated and treated mice are shown in Table 3.

DISCUSSION

M. haemophilum, a newly recognized pathogen first described in 1978 (15), has been reported with increasing frequency in immunosuppressed patients, especially those with AIDS (3, 4, 7, 9–11, 16–18). Severely immunocompromised patients may present with skin lesions and severe multisystem infection including arthritis, osteomyelitis (20), and pneumonitis (18). The organism fails to grow on routine mycobacterial media and requires hemin-supplemented media (chocolate agar), ferric ammonium citrate, or 7H11 agar with an X-factor

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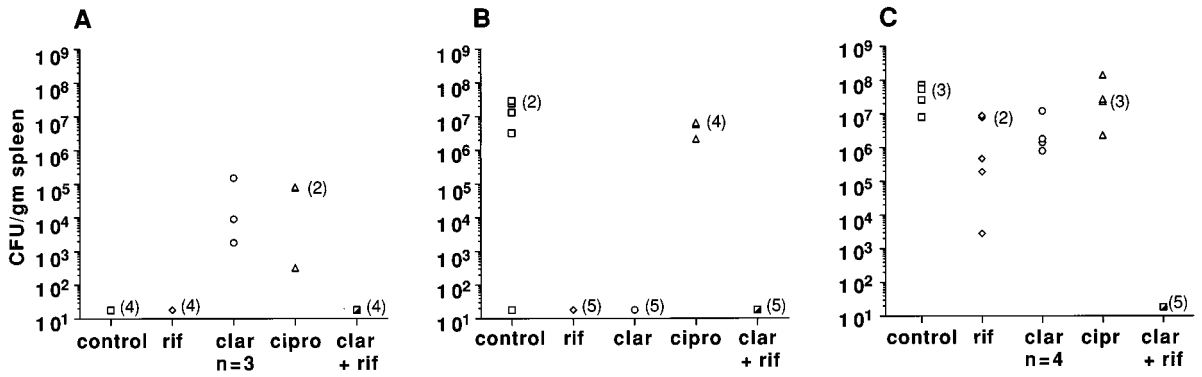

FIG. 2. Comparison of CFU per gram of spleen in athymic mice treated with rifabutin (rif), clarithromycin (clar), ciprofloxacin (cipro), and the combination of rifabutin and clarithromycin (clar + rif) after 2 weeks ($n = 4$) (A), 4 weeks ($n = 4$) (B), and 8 weeks (4 weeks posttherapy) ($n = 5$) (C).

strip for growth with an optimal incubation temperature of 32°C (6, 12).

Effective treatment strategies are poorly defined because of the small number of reported cases and the absence of comparative treatment evaluations. The relationship between in vitro drug susceptibilities and clinical response is not known. Susceptibility data are not standardized although the majority of isolates appear susceptible to rifampin and resistant to isoniazid, ethambutol, and pyrazinamide. Reports indicate that small numbers of isolates are susceptible to rifabutin and ciprofloxacin (2, 3), which is in agreement with our results. Combination therapy has been used to treat *M. haemophilum* infections, but recurrence of disease has occurred. Our animal data show that long-term combined therapy was superior to monotherapy for continued inhibition of the organism.

Few animal studies have been reported. Sompolinsky et al. (15) reported that a heavy inoculum of organisms injected intravenously, intramuscularly, and subcutaneously into mice and guinea pigs did not cause obvious pathological changes and that most of the animals survived a 3-month observation period with some mice dying within 2 to 4 weeks. However, large numbers of mycobacteria were found in smears of the liver, kidneys, and spleen. Intramuscular injection of 10^6 to 10^7 cells of *M. haemophilum* into the thighs of frogs was without effect when the animals were kept at room temperature; however, when animals were kept at 30°C, the animals died within 8 to 20 days and clumps of bacteria were found in smears of the liver and kidneys. Abbott and Smith (1) reported distinctive skin lesions containing acid-fast organisms on the ears of 12 of 30 prednisolone-treated outbred mice at 2 months following a challenge with 2.4×10^8 CFU of *M. haemophilum* per ml. No lesions were present on the animals not immunosuppressed with prednisolone.

We have established infection of *M. haemophilum* in a genetically immunosuppressed mouse model. Infection was not established in the immunocompetent outbred ICR mice. Infection was not observed until 8 weeks after inoculation in immunocompetent inbred BALB/c *nu*/+ mice, and there were significantly fewer CFU per gram of spleen compared with the BALB/c *nu*/*nu* mice, for which disseminated infection occurred in 2 weeks. This suggests that *M. haemophilum* requires an immunosuppressed state for infection, a finding consistent with that usually observed for humans. Clarithromycin and rifabutin alone (but not ciprofloxacin) were effective in suppressing growth for 4 weeks but failed to suppress the growth of organisms when treatment was discontinued. In contrast, the combination of rifabutin and clarithromycin was effective in sustaining the suppressed state. Combination therapy for 20

FIG. 3. Nodular lesions following subcutaneous injection of *M. haemophilum* into the shoulder of an athymic mouse.

TABLE 3. Histopathology of skin and spleen after treatment with ciprofloxacin, rifabutin, clarithromycin, and the combination of rifabutin and clarithromycin

Treatment	Tissue	Macrophages with AFB[a]	Infiltrates with epitheloid macrophages
Control	Skin	Many	Focal areas
	Spleen	Moderate	Focal areas
Ciprofloxacin	Skin	Rare	Rare
	Spleen	Moderate	Rare
Rifabutin	Skin	None	None
	Spleen	Rare	Rare
Clarithromycin	Skin	None	Rare
	Spleen	Rare	Rare
Clarithromycin + rifampin	Skin	None	None
	Spleen	None	None

[a] AFB, acid-fast bacilli.

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weeks followed by no treatment for 4 weeks confirmed the suppression of infection. This suggests that combination therapy for an extended period of time may be necessary for long-term suppression of this organism in the immunosuppressed patient. Our model offers some indication of appropriate therapy for *M. haemophilum* infection. Further studies with other combination antimycobacterial agents and treatment in patients are needed.

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REFERENCES

1. **Abbott, M. F., and D. D. Smith.** 1980. The pathogenic effects of *Mycobacterium haemophilum* in immunosuppressed albino mice. J. Med. Microbiol. **13:**535–540.
2. **Bernard, E. M., F. F. Edwards, T. E. Kiehn, S. T. Brown, and D. Armstrong.** 1992. Activity of clarithromycin and other agents against isolates of *Mycobacterium haemophilum*, abstr. 1332, p. 333. *In* Program and Abstracts of the 32nd Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
3. **Centers for Disease Control.** 1991. *Mycobacterium haemophilum* infections—New York City metropolitan area, 1990–1991. Morbid. Mortal. Weekly Rep. **40:**636–637, 643.
4. **Centers for Disease Control.** 1992. *Mycobacterium haemophilum* infections—New York City metropolitan area, 1990–1991. JAMA **67:**215–216.
5. **Davis, B. R., J. Brumbach, W. J. Sanders, and E. Wolinsky.** 1982. Skin lesions caused by *Mycobacterium haemophilum*. Ann. Intern. Med. **97:**723–724.
6. **Dawson, D. T., and F. Jennis.** 1980. Mycobacteria with a growth requirement for ferric ammonium citrate, identified as *Mycobacterium haemophilum*. J. Clin. Microbiol. **11:**190–192.
7. **Dever, L. L., J. W. Martin, B. Seaworth, and J. H. Jorgensen.** 1992. Varied presentations and responses to treatment of infections caused by *Mycobacterium haemophilum* in patients with AIDS. Clin. Infect. Dis. **14:**1195–1200.
8. **Hawkins, J. E., R. J. Wallace, Jr., and B. A. Brown.** 1991. Antibacterial susceptibility tests: mycobacteria, p. 1138–1152. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
9. **Kiehn, T. E., M. White, K. J. Pursell, N. Boone, M. Tsivitis, A. E. Brown, B. Polsky, and D. Armstrong.** 1993. A cluster of four cases of *Mycobacterium haemophilum* infection. Eur. J. Clin. Microbiol. Infect. Dis. **12:**114–117.
10. **Kristjansson, M., V. M. Bieluch, and P. D. Byeff.** 1991. *Mycobacterium haemophilum* infection in immunocompromised patients: case report and review of the literature. Rev. Infect. Dis. **13:**906–910.
11. **Males, B. M., T. E. West, and W. R. Bartholomew.** 1987. *Mycobacterium haemophilum* infection in a patient with acquired immune deficiency syndrome. J. Clin. Microbiol. **25:**186–190.
12. **McBride, J. A., M. E. Mc Bride, and J. E. Worf.** 1992. Evaluation of commercial blood-containing media for cultivation of *Mycobacterium haemophilum*. Am. J. Clin. Pathol. **98:**282–286.
13. **McBride, M. E., A. H. Rudolph, J. A. Tschen, P. Cernoch, J. Davis, B. A. Brown, and R. J. Wallace.** 1991. Diagnostic and therapeutic considerations for cutaneous *Mycobacterium haemophilum* infections. Arch. Dermatol. **127:**276–277.
14. **McGovern, J., B. C. Bix, and G. Webster.** 1994. *Mycobacterium haemophilum* skin disease successfully treated with excision. J. Am. Acad. Dermatol. **30:**269–270.
15. **Sompolinsky, D., A. Lagziel, D. Naveh, and T. Yanhilevitz.** 1978. *Mycobacterium haemophilum* sp. nov., a new pathogen of humans. Int. J. Syst. Bacteriol. **28:**67–75.
16. **Soubani, A. O., M. Al-Marri, and S. Forlenza.** 1994. Successful treatment of disseminated *Mycobacterium haemophilum* infection in a patient with AIDS. Clin. Infect. Dis. **18:**475.
17. **Straus, W., S. Ostroff, V. Silcox, J. Kilburn, T. Kiehn, D. Armstrong, E. Sordillo, V. LaBombardi, and R. Good.** *Mycobacterium haemophilum*: an emerging pathogen in the immunocompromised, abstr. 668, p. 222. *In* Program and Abstracts of the 32nd Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
18. **Straus, W. L., S. M. Ostroff, D. B. Jernigan, T. E. Kiehn, E. M. Sordillo, D. Armstrong, N. Boone, N. Schneider, J. O. Kilburn, V. A. Silcox, V. LaBombardi, and R. C. Good.** 1994. Clinical and epidemiological characteristics of *Mycobacterium haemophilum*, an emerging pathogen in immunocompromised patients. Ann. Intern. Med. **120:**118–125.
19. **Vadney, F., and J. E. Hawkins.** 1985. Evaluation of a simple method for growing *Mycobacterium haemophilum*. J. Clin. Microbiol. **22:**884–885.
20. **Yarrish, R. L., W. Shay, V. LaBombardi, M. Meyerson, D. K. Miller, and D. Larone.** 1992. Osteomyelitis caused by *Mycobacterium haemophilum*: successful therapy in two patients with AIDS. AIDS **6:**557–561.

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