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Mycobacteria with a Growth Requirement for Ferric Ammonium Citrate, Identified as *Mycobacterium haemophilum*

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Three previously unidentified strains of pathogenic mycobacteria which had been isolated on media containing ferric ammonium citrate were found to be identical with the new species, *Mycobacterium haemophilum.*

Sompolinsky et al. (7) proposed the name *Mycobacterium haemophilum* for a hitherto unrecognized mycobacterium isolated from subcutaneous granulomata in a 53-year-old Israeli woman receiving immunosuppressive treatment for Hodgkin's disease. The strain was unusual in its growth requirement for either hemin or hemoglobin. So far as is known, there have been no other recorded cases of infection with *M. haemophilum.*

Mezo et al. (3) presented the case histories of five patients from Sydney, Australia, with skin lesions caused by fastidious mycobacteria. Each patient was undergoing immunosuppressive therapy; four had received renal transplants, and the other had lymphocytic lymphoma. Although the cases had been recorded over a period of 9 years, the clinical and microbiological features of the lesions suggested that the same etiological agent was responsible in each case. The causative organism was isolated from the lesions of three patients, but only after the fortuitous discovery that it required a growth supplement of ferric ammonium citrate (FAC).

An apparently similar mycobacterium had been isolated at another Sydney Hospital (9), also from skin lesions of a renal transplant patient. Because of the possibility that the isolates were identical with, or related to, *M. haemophilum,* we conducted comparative studies with a subculture of that organism.

Only three of the four isolates from Australian patients were available for further study, these being strains RM and OBJ from cases 4 and 5 as described by Mezo et al. (3) and strain POW, the isolate reported by Walder et al. (9). All strains had been maintained on Lowenstein-Jensen medium containing 15 mg of FAC per ml (LJ + FAC). A lyophilized culture of *M. haemophilum* was provided by David Sompolinsky. The reconstituted material gave good growth on sheep blood agar in about 2 weeks.

Barely turbid suspensions of the four strains were subcultured onto the following media at 36, 32, and 26°C: (i) LJ; (ii) LJ + 2 mg of sodium pyruvate per ml; (iii) LJ + FAC; (iv) LJ + 60 μM hemin; (v) LJ + 2 μg of mycobactin per ml; (vi) Middlebrook 7H9; (vii) Middlebrook 7H9 + 15 mg of FAC per ml; (viii) Middlebrook 7H9 + 60 μM hemin; (ix) Middlebrook 7H10; (x) Middlebrook 7H10 + 15 mg of FAC per ml; (xi) Middlebrook 7H10 + 60 μM hemin; (xii) chocolate agar, i.e., Oxoid GC agar base plus Oxoid hemoglobin plus Difco supplement B; (xiii) sheep blood agar, i.e., Difco Columbia agar base plus 4% sheep erythrocytes; (xiv) Difco Columbia agar base plus 15 mg of FAC per ml; (xv) Difco Columbia agar base + 60 μM hemin. Similar results were obtained for each of the four strains. Growth appeared earliest (8 to 10 days) and most luxuriantly on LJ + FAC and on sheep blood agar. The strains grew well (in 10 days) in Middlebrook 7H9 containing either hemin or FAC, and although growth was obtained on Middlebrook 7H10 plus hemin and on chocolate agar, colonies were slow to appear. All other media showed little or no growth after 4 weeks of incubation. On those media supporting growth, best results were obtained at 32°C; growth appeared a few days later at 36°C, but was slower at 26°C.

The four strains could not be differentiated in studies of their cellular and colonial morphology. Ziehl-Neelsen smears of growth from LJ + FAC showed deeply stained bacilli of a uniform size, similar to that of *M. tuberculosis,* with some loose cording. Serpentine cords were present in growth from liquid media and were particularly striking in smears of blood agar cultures. Colonies on LJ + FAC were buff colored, showed no pigmentation on exposure to light or on aging, and could be emulsified without difficulty. In these respects they were similar to colonies of smooth varieties of *M. fortuitum.* Colonies on blood agar were colorless, consistently waxlike, and impossible to emulsify.

Each of the four strains gave essentially the same results in a limited panel of standard biochemical tests as listed by Dawson (2). By using growth from LJ + FAC, we found that: (i) the Tween 80 hydrolysis test was negative at 10 days; (ii) the niacin test was negative; (iii) the nitrate reduction test was usually weakly positive, although all strains gave inconsistent results; (iv) the 3-day aryl sulfatase test was negative; (v) the semiquantitative catalase test was negative; (vi) the FAC reduction test was negative; and (vii) the *p*-amino salicylate degradation test was negative. Amidase tests could not be interpreted because the negative control tube, which contained bacteria and buffer but no amide, gave a strong test for ammonia.

Sensitivity tests by the resistance ratio (RR) method were attempted, using LJ + FAC as base medium. However, interpretation was difficult because the anti-mycobacterial effect of some of the compounds was markedly reduced in the presence of 15 mg of FAC per ml. For example, the minimal inhibitory concentration (MIC) of ethambutol for a sensitive strain of *M. tuberculosis* was increased 16-fold. It was subsequently determined that the minimum concentration of FAC necessary for good growth of the test strains was 5 mg/ml, and when sensitivity tests were repeated using base medium containing FAC at this concentration, MICs were increased no more than twofold. Values obtained, along with the RR values derived by comparison with MICs for *M. tuberculosis* under the same conditions, are shown in Table 1. All strains were "sensitive" to rifampin, but "resistant" or "probably resistant" to other compounds. (Sensitivity is indicated by RR values of less than 4.)

The strains were also studied by sero-agglutination. Although growth from Middlebrook 7H10 plus hemin was rough, the supernatants of suspensions prepared from cultures on LJ + FAC and allowed to sediment for several hours provided material suitable for agglutination tests. Procedures used were the same as those for the *M. avium-M. intracellulare-M. scrofulaceum* complex (4, 5). None of the four strains was agglutinated by appropriately diluted antisera specific for *M. szulgai*, *M. malmoense*, *M. simiae* serovars 1 and 2, and the 30 recognized serovars of the *M. avium-M. intracellulare-M. scrofulaceum* complex. Hyperimmune antisera to *M. haemophilum* (titer = 2,560) and to strain RM (titer = 320) were prepared in rabbits, and both antisera agglutinated suspensions of *M. haemophilum*, RM, OBJ, and POW, to titer. Furthermore, absorption of low dilutions of anti-*M. haemophilum* and anti-RM (6) with the three heterologous strains resulted in the removal of all homologous agglutinins. On the other hand, the antisera failed to agglutinate bacterial suspensions of representatives of *M. szulgai*, *M. malmoense*, *M. asiaticum*, *M. simiae*, and the *M. avium-M. intracellulare-M. scrofulaceum* complex.

From the results of our investigations, we conclude that each of the strains RM, OBJ, and POW belongs to the species *M. haemophilum.*

Our study has shown that FAC is a suitable supplement in egg-based media for culture of *M. haemophilum.* Sompolinsky et al. (7) found that ferric chloride did not promote growth in Middlebrook 7H9, but did not test FAC. In conflict with the findings of Sompolinsky et al., we were unable to demonstrate enhanced growth on hemin-containing LJ, even though Middlebrook 7H10 (and 7H9) plus hemin supported growth. Also, the failure of FAC to cause growth on 7H10 in our study came as a surprise, since we found that the strains grew well in Middlebrook 7H9 in the presence of FAC. The requirement for iron-containing complexes raises the possibility that *M. haemophilum* might also grow on media containing mycobactin, particularly since *M. paratuberculosis*, which is mycobactin-dependent, grows on LJ + FAC on subculture. Furthermore, Walder et al. (9) found that strain POW grew on media supplemented with mycobactin. Our studies indicate that mycobactin does not promote growth of any of the four strains on LJ. The mycobactin used in our tests was prepared by standard methods (1) from a

TABLE 1. *MICs and resistance ratios determined in sensitivity tests using LJ + FAC*[a]

Antimycobacterial compound	*M. haemophilum*		RM		OBJ		POW		*M. tuberculosis* (wild strain)
	MIC	RR	MIC	RR	MIC	RR	MIC	RR	MIC
Streptomycin	>80	>8	>80	>8	>80	>8	>80	>8	10
p-Amino salicylic acid	0.25	4	>1	>16	0.5	8	>1	>16	0.06
iso-Nicotinic acid hydrazide	>1	>8	>1	>8	>1	>8	>1	>8	0.12
Ethambutol	>8	>4	>8	>4	>8	>4	>8	>4	2
Rifampin	20	0.5	80	2	80	2	40	1	40

[a] MICs represent micrograms of compound per milliliter in LJ + FAC. RR values represent (MIC for test strain)/(MIC for wild strain of *M. tuberculosis*); values <4 usually indicate sensitivity of the test strain to that compound.

soil strain of *M. phlei*; the source of the material used by Walder et al. is not known.

In sensitivity tests on Middlebrook 7H10 plus hemin, Sompolinsky et al. (7) found *M. haemophilum* to be sensitive to *p*-amino salicylic acid, whereas our results indicate probable resistance to that compound. Rifampin seems effective in vitro, although its clinical value remains to be proven.

Sompolinsky et al. (8) investigated the serological properties of *M. haemophilum* by direct agglutination as well as by reaction with antibody-coated cells of *Staphylococcus aureus*. They found low degrees of cross-reactivity with some *M. avium-M. intracellulare-M. scrofulaceum* serovars, but absorption studies demonstrated the uniqueness of the surface antigens of *M. haemophilum*. The present study supports those observations and suggests that there might be only one serovar of *M. haemophilum*.

Increased awareness of the pathogenic potential of many species of mycobacteria has been the result of improved procedures for their primary isolation. So that *M. haemophilum* will be provided for, tuberculosis laboratories should include slopes of blood agar or LJ + FAC in the set of media for cultural examinations of skin lesions. In addition to *M. haemophilum*, *M. chelonei* and *M. fortuitum* will be isolated from skin lesions by general bacteriology laboratories if the incubation time for blood agar cultures is extended. Experiences with *M. haemophilum* give further support to the contention that a mycobacterial etiology must be considered in unexplained infective lesions.

M. haemophilum appears to be a potential pathogen of low virulence whose natural habitat is the environment. Although the only cases of infection recorded in Australia have occurred in a defined locality, i.e., Sydney, temporal features do not suggest communicability. Since each of the four renal transplant patients first developed lesions 5 to 6 months after surgery (3), an incubation period of 2 to 4 months seems most likely.

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