Comparison of 15 Laboratory and Patient-Derived Strains of *Mycobacterium avium* for Ability to Infect and Multiply in Cultured Human Macrophages

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Mycobacterium avium is a cause of nontuberculous chronic granulomatous infections which is attracting increased attention as a frequent opportunistic pathogen in acquired immunodeficiency syndrome. Some important aspects of its human pathogenicity were investigated by using cultured human macrophages infected with it. The uptake and replication of various strains of *M. avium* in the macrophages could be measured by CFU counts of the bacteria in samples of lysed, sonicated macrophages. Microscopic counts of acid-fast bacilli were not useful because the bacteria multiplying in the macrophages were usually not acid fast. Electron microscopy showed the intracellular bacilli to multiply by transverse fission, to be surrounded in individual vacuoles by a broad electronlucent zone, and to have thinner cell walls than extracellularly grown *M. avium*. Fifteen strains, including examples of serovars 1, 2, 4, 8, and 9, were studied for uptake and rate of replication in cultured macrophages from three normal subjects. The strains were isolates from patients with nontuberculous granulomatous infection, acquired immunodeficiency syndrome, or unrelated problems, or they were laboratory reference cultures. There were no differences among them in phagocytosis, but there were differences in intracellular replication. Laboratory strains tended to be avirulent, that is, they did not replicate in the macrophages. Patient isolates usually were virulent and could be compared for virulence by intracellular replication rates. Virulence correlated with flat, transparent bacterial colony morphology on nutrient agar but not with serovar or kind of patient from whom the bacteria were isolated. However, among strains of transparent colony morphology there were wide differences in virulence. Avirulent bacilli generally produced domed, opalescent colonies on nutrient agar. Avirulent bacilli predominated in populations of *M. avium* conditioned to growth in bacteriologic culture medium. Bacilli of virulent colony morphology predominated in populations passaged through cultured macrophages. The model described here presents a new approach to the investigation of the pathogenicity of *M. avium* for human subjects and may be more patient relevant than animal models.

Mycobacterium avium is similar to the closely related bacterium *Mycobacterium intracellulare* and, probably, to *Mycobacterium lepraemurium* and *Mycobacterium paratuberculosis* (20, 41, 42). These bacteria are opportunistic pathogens for people (16, 19, 26, 33, 48) and frequently cause disseminated, fatal infections in acquired immunodeficiency syndrome (AIDS) (21; R. C. Good, M. A. Yakrus, J. O. Kilburn, V. A. Silcox, and W. D. Jones, Proc. Int. Conf. AIDS, Atlanta, Ga., p. 49, 1985; T. P. Mess, W. K. Hadley, and C. B. Wofsy, Proc. Int. Conf. AIDS, Atlanta, Ga., p. 47, 1985). That may be due to profound defects in T4 lymphocytes and associated antimicrobial immunity in AIDS patients (21, 27) because immunity to *M. avium* probably is T cell dependent (37). The bacteria infect the phagocytic monocyte system of the body (30), and then immunity is supposed to suppress the bacteria by proper activation of the phagocytes by T-cell-produced lymphokines (7, 8, 52). An in vitro model of similar cellular immunity for *Mycobacterium tuberculosis* has been developed for use with both mouse (52, 53) and human (9, 52) cells. We now describe the adaptation of the human cell model to experimentation with *M. avium* and factors relating to its human pathogenicity.

MATERIALS AND METHODS

Bacteria. The various *M. avium* strains and their sources and serovars are listed in Table 1. The strains used were from the Serotyping Culture Collection of the National Jewish Center for Immunology and Respiratory Disease and the Trudeau Mycobacterial Culture Collection and also included various cultures submitted for serotyping which were recent isolates from patients. Strain Flat-1 was derived from TMC 724 and consisted of single-culture progeny of a flat transparent colony picked from a plate of Middlebrook 7H10 agar (7H10; Difco Laboratories, Detroit, Mich.). It was chosen for its ability in preliminary experiments to replicate in cultured human macrophages and for its transparent colonial morphology, which was supposed to indicate virulence (34, 43, 50).

Suspensions of all strains were prepared as follows (10). Each was inoculated into 11 ml of Middlebrook 7H9 broth (7H9; Difco) in a Falcon 3013 (Becton Dickinson Labware, Oxnard, Calif.) disposable tissue culture flask. The flasks were incubated horizontally at 37°C with daily agitation until the bacterial cell density reached between 100 and 200 Klett colorimeter units. The resulting suspensions were frozen in 0.3-ml one-use aliquots at -90°C. For use, an aliquot was thawed in warm water and diluted with RPMI 1640 tissue

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TABLE 1. Strains and sources of *M. avium* used

Designation	Serovar	Source
11907	1	Serotyping Culture Collection ^a
1-51	1	Nonmycobacterial disease
2-82	1	Nontuberculous granulomatous disease
1-81	1	AIDS opportunistic infection
TMC 714	2	Trudeau Mycobacterial Culture Collection
TMC 724	2	Trudeau Mycobacterial Culture Collection
Flat-1	2	Transparent clone of TMC 724
7497	4	AIDS opportunistic infection
13528-1079	4	Serotyping Culture Collection
T-138	8	Nontuberculous granulomatous disease
SJB 2	8	Serotyping Culture Collection
6450	9	Serotyping Culture Collection
11-13	9	Nontuberculous granulomatous disease
12-9	9	AIDS opportunistic infection
11-23	9	Nonmycobacterial disease

^a National Jewish Center for Immunology and Respiratory Disease.

culture medium (GIBCO Laboratories, Grand Island, N.Y.) to a concentration of 10^8 bacilli per ml by Petroff-Hausser microscopic counting. This suspension then was ultrasonicated as described previously (10, 11), but only for 5 s. Dispersion of the bacteria by this sonication into single-cell suspensions was confirmed by Petroff-Hausser counting chamber observations and also by culture counts of CFU on 7H10 medium. This sonication was determined to be less than one-fourth the amount which would begin to kill any of the bacteria. The sonicated suspension, further diluted 1:20 in RPMI 1640 culture medium supplemented with 5% unheated autologous human serum, was used to infect the cultured macrophages. Bacillary concentrations in these infection suspensions were measured by CFU counting of serial 10-fold dilutions of samples on 7H10 plates and usually were between 2×10^7 and 5×10^7 /ml.

Human subjects. Macrophages from three healthy young purified-protein-derivative-negative males were used (informed consent was obtained).

Macrophage isolation and culture. Macrophage isolation and culture were done as previously described (9, 10, 11, 16). Briefly, 50 ml of peripheral blood was drawn; a 40-ml portion was heparinized and centrifuged on Ficoll-Hypaque to obtain purified monocytic cells; the remaining 10 ml was clotted. The monocytic cells were plated at 10^7 /ml in 0.05-ml droplets, 3 droplets per 35-mm bacteriologic-grade plastic petri dish (Falcon 1008). The dishes were incubated for 30 min at 37°C in 7.5% carbon dioxide-air (all incubations were done in this temperature and atmosphere). Nonadherent cells were washed off and discarded, and the adherent cells were incubated for 7 days in 1.5-ml volumes of RPMI 1640 medium supplemented with 50 U of penicillin G per ml–20 mM L-glutamine–1% unheated autologous serum obtained from the sample of clotted blood.

The resulting macrophages then were infected with *M. avium*. This was done by replacing the original culture medium with 1.5 ml of the infecting suspension (see above), in which the culture medium was supplemented with 5% instead of 1% unheated autologous serum to promote phagocytosis. The plates were incubated for 30 min, and the infecting suspension was drawn off and discarded. The macrophages were washed twice with culture medium, and then incubation was resumed and continued for an additional 7 days in the same kind of medium as that used originally.

Enough culture plates were set up to allow taking three plates for each sampling. In initial experiments, two plates

were fixed and stained for acid-fast bacillus (AFB) counts, and one was used for counting CFU. Later, when AFB counts were found to be unreliable, this ratio was reversed. Samples were taken for AFB and CFU counting at 0 h (immediately after infection) and at 4 and 7 days after infection (11). For the acid-fast staining, sample cultures of infected macrophages were rinsed with warm physiologic saline, fixed with warm glutaraldehyde (9), and then rinsed with distilled water and stained by the Kinyoun method as previously described (47).

AFB counts. The bacilli were counted microscopically, and the numbers of bacilli per macrophage and percentages of macrophages infected were calculated as described earlier (9, 10, 11, 13). A total of 600 consecutive macrophages were counted, 200 from each of three spots of macrophages on each petri dish, and the values reported therefore are means for 1,200 counted macrophages per experimental condition. The data are reported as means $\pm$ the standard error of the mean (SEM).

CFU counts. CFU counts were done as described previously (11, 13), except that sodium dodecyl sulfate macrophage lysates, after having been neutralized with bovine serum albumin, were ultrasonicated for 5 s instead of the 10 s used for tubercle bacilli. This sonication efficiently distributed the bacilli and was twofold less than the minimum needed to begin to kill any of these bacilli. Briefly, the CFU procedure was as follows.

Supernatant culture medium from a macrophage culture plate to be sampled was withdrawn and stored aseptically at -90°C . The culture plate also was frozen. When an appropriate number of these samples and plates had accumulated, they were all processed for CFU counts. The medium supernatants were adjusted to 2-ml volumes with 7H9 medium. The plates were treated for 10 min with 1.5-ml volumes of sodium dodecyl sulfate, and the resulting macrophage lysates were neutralized with 0.5 ml of 20% bovine serum albumin in distilled water. The 2-ml volumes of supernatant or neutralized cell lysate were sonicated and then diluted serially in 10-fold steps in 7H9 medium. Five 20- μl volumes of each dilution were plated as droplets on half the area of a petri dish (diameter, 9 cm) of 7H10. These plates were incubated at 37°C for 2 weeks, and then the bacterial colonies developing where the droplets had been deposited were counted as CFU (11, 31). The data are reported as CFU per milliliter of lysate or supernatant ($\pm$ SEM), which represented the number of bacteria associated with 10^5 macrophages (15).

Initial experiments with serovars 2, 4, and 8 included separate CFU counts of macrophages and their culture supernatants to measure the proportions of non-macrophage-associated bacterial replication. In later experiments, macrophage lysates and their culture medium supernatants were combined to obtain total counts of CFU for each macrophage culture.

The data were plotted semilogarithmically (log mean CFU per milliliter versus time, in days, after infection of the macrophages) to demonstrate exponential bacillary replication and facilitate calculation of bacterial dividing times. These were determined by calculating the average slope of bacillary replication (means of height/base ratios at 4 and 7 days) and then reading the intersections of the averaged slope with horizontal intercepts for a doubling of the bacillary population on the ordinate of the data plotted against time in hours on the abscissa.

Growth of *M. avium* in tissue culture medium. The ability of RPMI 1640 culture medium alone or conditioned by macro-

FIG. 1. Percent of subject 1 human macrophages (Mφ) infected and mean AFB per macrophage at 0, 4, and 7 days after infection of the macrophages with *M. avium* serovar 2 strains TMC 724 and TMC 714 and a flat, transparent clonal isolate from TMC 724 (Flat-1), as determined by AFB counts. Each datum point is the mean of counts for 600 macrophages constituted of 100 from each of six separate monolayer cultures. Maximum SEMs (not shown) for percentages of macrophages infected were 0.10, 0.10, and 0.11 of the indicated values at 0, 4, and 7 days and 0.12, 0.10, and 0.19 at 0, 4, and 7 days for AFB per macrophage.

phages or other factors to support the replication of *M. avium* was tested as follows.

The medium to be tested was filtered through a membrane (pore size, 0.22 μ m; Millex-GV, SLGV025LS; Millipore Corp., Bedford, Mass.). Then, one 0.2-ml sample was placed in the first of four wells of a 96-well sterile culture tray with round-bottomed wells (Corning 25850; Corning Glass Works, Corning, N.Y.), and 0.1-ml samples were placed in each of the next three wells. Sample 1 was inoculated with 5 μ l of strain Flat-1 bacilli from a freshly thawed one-use frozen aliquot, diluted to a concentration equivalent to 20 Klett colorimeter units, and ultrasonicated for 5 s, to make a final concentration in each inoculated sample of approximately 2×10^6 CFU/ml by actual 7H10 plate count. Well 2 in the series received 0.1 ml of the bacillus-inoculated fluid from well 1, well 3 received 0.1 ml from well 2, and so on, so that the last well received 1:8 as much 7H9 (and bacilli) as well 1. This was done because 7H9 culture medium alone would encourage bacillary growth in the RPMI 1640 medium to which it was added. The plate of 96 wells always included a 7H9-medium-positive control similarly inoculated with bacilli. Zero-hour CFU concentrations were determined by freezing a 20- μ l sample from the just-inoculated 7H9 well in a 2-ml volume of 7H9 medium at -90°C . This sample was thawed and plated 7 days later, when incubation of the test and control samples was completed.

The 96-well tray was then incubated at 37°C in 7.5% carbon dioxide for 7 days, with gross reading of bacillary growth by pellet size made at 4 and 7 days. At 7 days, 20- μ l samples were taken, after thorough mixing, from the undiluted sample wells. The wells with serially diluted inocula of *M. avium* did not have to be used, for there was no evidence by this dilution testing that the very small amount of 7H9

medium in the bacillary inoculum influenced the results. The 20- μ l sample was mixed with 2 ml of 7H9 medium, ultrasonicated for 5 s, diluted in four succeeding 10-fold steps, and then plated from the various dilutions produced in quintuplet in droplets (20- μ l volume) on 7H10 plates for CFU counts (11).

The results are reported as CFU per milliliter of the sample being tested for mycobacterial growth, $\pm$ SEM.

Specimen preparation and electron microscopy. Infected macrophage cultures were processed in situ in the culture dishes by using the fixation procedures of either Silva and Macedo (45) or Hirsch and Fedorko (22). The methods yielded similar images. Typically, the macrophage culture was first fixed for 1.5 h at room temperature in a mixture of 4% formaldehyde (prepared paraformaldehyde) and 1.25% glutaraldehyde in 0.5 M cacodylate buffer (pH 7.3). CaCl_2 (10 mM) was added to all solutions, except when otherwise noted. The fixation was followed by rinsing with 0.5 M cacodylate buffer, and the cells were postfixed in cold 1% OsO_4 buffered at pH 7.3 for 30 min, rinsed, and treated with 0.5% uranyl acetate in 0.1 M calcium-free acetate buffer (pH 6.3) for 45 min, followed by two rinsings with calcium-free 0.1 M acetate buffer. Specimens, still in situ, were dehydrated in graded concentrations of ethanol and embedded in Epon. After polymerization of the resin, areas of the dish with specimens were cut out and sectioned, and the sections were mounted on 1-mm aperture grids coated with a Formvar support film. Specimens were observed with and without lead staining.

To compare microbial anatomy, *M. avium* replicating both extracellularly and in 7H9 were observed. A 1-ml sample of the broth was pelleted in an Eppendorf high-speed centrifuge in a polyethylene tube, and the pellet was processed in the

FIG. 2. Mean log CFU counts for lysed subject 1 macrophages (MΦ) (cells; A) and macrophage culture supernatant medium (supernate; B) at 0, 4, and 7 days for the same experiment as that shown in Fig. 1. Each datum point is the mean of five values. Maximum SEMs (not shown) for cells were 0.18, 0.14, and 0.10 of the values for 0, 4, and 7 days, respectively, and for the supernatant they were 0.19, 0.27, and 0.11 for 0, 4, and 7 days. Panel A also shows CFU counts in petri dishes of culture medium inoculated with *M. avium* Flat-1 but lacking macrophages. The 4-day value for these counts was lost by contamination. D, mean dividing time.

FIG. 3. Growth of *M. avium* in tissue culture medium (RPMI 1640 and serum) compared with unsupplemented culture medium (alone), the supplemented culture medium conditioned (cond.) by macrophages (MΦ), macrophages infected with *M. avium*, or an equivalent number of *M. avium* without macrophages, and bacteriologic culture medium (7H9). Growth of the bacilli in the various media was measured as described in the text by plating appropriate dilutions that had been incubated for 7 days in the various media being compared for log CFU counting in comparison with zero-time counts (suspension kept frozen while other samples were being incubated) in bacteriologic culture medium. Small bars at tips of large bars are SEMs. D, where shown, is estimated bacterial dividing time.

FIG. 4. Photomicrographs of Ziehl-Neelsen-stained cultures of human macrophages at 7 days after infection with an average of 1 to 2 bacilli per macrophage using a suspension of bacilli made of cloned transparent colony morphology *M. avium* 7497, serovar 4. Even macrophages accumulating very heavy loads of bacilli (e.g., >60 per cell, panel A) remained healthy. The cell-associated coccobacilli were very small and scattered and stained blue (non-acid fast). The load of bacilli was considerably higher in panel A macrophages than normal (panel B), owing to mycobacterial-growth-enhancing factor (Crowle et al., Proc. Int. Conf. AIDS, Atlanta, Ga., p. 32, 1985).

tube. The method of preparation was the same as that used for the macrophage cultures described above. Epon and Spurr resins used for embedding produced essentially the same results. After polymerization of the resin, the centrifuge tube was split open and the exposed pellet was sectioned.

RESULTS

Uptake and growth of *M. avium* in human macrophages.

The uptake of *M. avium* and its growth in human macrophages were initially studied with strains TMC 714, TMC 724, and Flat-1. The results from a typical experiment, with macrophages from subject 1, are shown in Fig. 1 for AFB counts and in Fig. 2 for CFU counts. By both types of count, phagocytosis of the three varieties of *M. avium* was good and similar. However, the AFB counts (Fig. 1A) showed no changes in percentage of macrophages infected, and there was no change in mean numbers of bacilli per macrophage (Fig. 1B) during the next 7 days of culture.

CFU counts for the same experiment are shown in Fig. 2. The data in Fig. 2A demonstrate substantial bacillary replication associated with the macrophages. The data in Fig. 2B show increases of *M. avium* also in the extracellular culture fluid. This effect was examined by testing the ability of *M. avium* to grow in the tissue culture medium alone (Fig. 3). The bacilli were unable to multiply in tissue culture medium in the absence of macrophages (Fig. 2A; 14) but could

multiply some in such medium conditioned by human macrophages (A. J. Crowle, M. H. May, and G. S. Douvas, Proc. Int. Conf. AIDS, Atlanta, Ga., p. 32, 1985). The observed extracellular accumulation of *M. avium* therefore was macrophage dependent.

Seven other experiments with macrophages from subjects 2 and 3 produced AFB and CFU results essentially the same as those shown in Fig. 1 and 2. Since the macrophage-dependent growth of *M. avium* tended to be exponential, it was possible and convenient to compare *M. avium* strains for rates of growth (virulence) in human macrophages by mean dividing time. For instance, the mean dividing time for strain Flat-1 (Fig. 2A) was 40 h, as compared with 20 h in 7H9 bacteriologic culture medium (Fig. 3).

Morphology of *M. avium*-human macrophage association. Two typical optical microscope views of *M. avium*-human macrophage association in 7-day cultures (advanced infection) are shown in Fig. 4. The acid-fast coccobacilli as grown for infection in 7H9 medium were taken up well. Once in the macrophages, they rapidly lost acid-fastness. Intracellularly, they replicated as dispersed, very small, pleomorphic coccobacilli that usually appeared to be blue, i.e., non-acid fast. Only in cultures with very great numbers of bacilli did some of the bacteria retain a faint red indicating weak acid fastness. All but the most heavily infected macrophages appeared to be healthy (Fig. 4), and this was confirmed by electron microscopy (see below).

FIG. 5. Transmission electron micrographs of intramacrophage *M. avium* bacilli (b in panel A; bar = 1 μ m), extracellularly growing bacilli (panel B; bar = 0.1 μ m), intracellular bacillary division (panel C, arrow; bar = 1 μ m), and well-developed mesosomal apparatus (m) of an intracellular bacillus (panel D; bar = 0.1 μ m) suggesting impending division. Primitive budding or branching could be found among in vitro-grown forms (B) but not among bacilli replicating in macrophages. Cell wall complexes were thicker for extracellular bacilli (3 nm) than for intracellular bacilli (<1.5 nm). The bacilli shown here are serovar 4, strain 7497. In panel A, n indicates the nucleus.

Some electron microscopic images of the *M. avium*-infected human macrophage relationship are shown in Fig. 5. These show the intracellular bacilli to be predominantly short rods. They tended to be separate from each other within individual vacuoles and surrounded by a broad electronlucent zone (1, 17, 35, 38). This ultramicroscopic visualization of the distribution of most of the bacilli as individuals accounts for their dispersed distribution seen with the light microscope and confirmed a similar observation by Wheeler and Hanks for *M. paratuberculosis* (49). A remarkable thinness of the cell wall complex of these intramacrophage bacilli was seen as compared with extracellularly grown *M. avium*. Intracellularly, the bacteria never exhibited any tendency toward branching; however,

rudimentary tendencies toward branching sometimes could be seen among *M. avium* grown in nutrient broth (Fig. 5B). They appeared to replicate within the macrophages by normal transverse fission (Fig. 5C). Sometimes they produced rounded bodies which Kölbel (25) believes may be related to spores.

Replication of different serovars of *M. avium* in human macrophages. Two sets of data on the replication of different *M. avium* serovars in human macrophages are shown. One set is shown in Fig. 6, and the other is shown in Tables 2 to 4.

Three separate experiments produced very similar results. The data graphed in Fig. 6 are from one experiment of the three which compared laboratory strains with AIDS-patient-

derived strains of the two serovars most often isolated from human infections, 4 and 8 (32; Good et al., Proc. Int. Conf. AIDS, Atlanta, Ga., p. 46, 1985). These data are presented to further illustrate the growth characteristics of *M. avium* in human macrophages and to show how the macrophages can distinguish among strains of the bacilli for virulence. They demonstrate that the two laboratory strains were avirulent, Flat-1 was modestly virulent, and the strains isolated from patients with AIDS were more virulent.

These strains of *M. avium*, as well as the ones whose behavior is described in Tables 2 to 4, were also tested for rates of growth in 7H9 medium. The results generally were that the rates of growth in macrophages as compared with 7H9 were inversely related, with the strains growing fastest in the macrophages generally growing slowest in the broth (data not shown).

Replication of other *M. avium* serovars in human macrophages. Macrophages from two normal subjects were used in two experiments per donor to test a variety of additional strains of *M. avium* for correlations between virulence and serovar or source. The results of these experiments are best summarized for comparison by mean dividing time (D), as previously mentioned. In each experiment, an infection with strain Flat-1 was included for comparison and interpretation.

The results are shown in Table 2. Virulence of the bacteria was exhibited in a range of complete killing by the macrophages (avirulence) to a D of 18.5 h. Strain Flat-1 exhibited moderate virulence, with a D of 51.8 h. The most virulent was strain 1-51, with a D of 18.5 h. The least virulent strains were 11907, 6450, 1-81, and 12-9. Strains 11907 and 6450 were laboratory strains, whereas strains 1-81 and 12-9 were isolates from patients with AIDS.

The bacteria were then ranked (1+ to 4+) according to virulence. A 4+ indicated the most virulent, with Ds of 15 to 29 h; 3+ was for Ds of 30 to 45 h; 2+ was for Ds of 45 to 60 h; 1+ was for Ds of 61 to 75 h; +/- was for Ds of 76 to 136 h; and - was for bacteria killed by macrophages. In addition, colonies were counted and transparent and opaque morphologies were compared and expressed as ratios. These data are assembled in Tables 3 and 4 and indicate the following. Virulent bacilli had colonial morphologies which were flat and transparent. Avirulent bacilli had colonial morphologies which were domed and white. There was little relationship between virulence and pathologic association with AIDS, although virulent strains tended to have been isolated from patients. However, the most virulent strain, 1-51, was isolated from a patient in whom it was judged to have been clinically unimportant.

Two additional important observations can be made on the basis of these data. One is that there was a correlation between lesser virulence and increasing variation in the mean dividing times, which would indicate that the establishment of infection of low-virulence strains is very tenuous and pivots greatly on responses of the infected macrophages at the time of their infection (see, for instance, responses to strain 6450, Table 2).

The other observation is that bacteria of the flat transparent colony morphology survive and multiply in the cultured macrophages, whereas bacteria of the round white colony morphology die (cf. strains 2-82 and 11907 in Tables 3 and 4). However, the data in Table 4 also show that flat transparent colonies are not equally virulent (cf. strains 2-82 and 11-13 in Table 3) and that macrophages from one donor may eliminate the domed white colony morphology faster than macrophages from another (cf. subject 1 and 2 macrophages infected with strain 2-82; Table 4).

FIG. 6. Growth in human macrophages of five different strains of *M. avium* (Table 1). The values shown are from combination of both macrophages and their supernatants. Maximum SEMs were 0.22, 0.29, and 0.17 of the values shown at 0, 4, and 7 days. ····, T-138 (mean dividing time [see the text], 21 h); ·····, 7497 (mean dividing time, 33 h); —, Flat-1 (mean dividing time, 63 h); — — — (upper), SJB 2 (mean dividing time, 7 days); — — — (lower), 1079 (mean dividing time, >7 days).

DISCUSSION

The in vitro model of human macrophages previously used to study tuberculosis can be readily adapted for the study of *M. avium* infections.

With the model, we found some notable differences between *M. avium* and *M. tuberculosis* infection of cultured human macrophages. The most striking difference was an apparent loss of acid fastness by intramacrophage *M. avium*. As a consequence, while the replication of *M. tuberculosis* in human macrophages can be monitored reasonably well by both AFB and CFU counts (11, 12), for *M. avium* only the CFU counts were useful (cf. Fig. 1 and 2). Macrophage-associated coccobacilli were present and in advanced infections were numerous (e.g., 40 to 60 per macrophage; Fig. 4) but only stained blue by the Kinyoun variation of Ziehl-Neelsen technique we used (11, 47). This technique has regularly stained extracellular *M. avium* and both extra- and intracellular *M. tuberculosis* red and unequivocally acid fast.

This unexpected finding of non-acid fastness is not unprecedented. Mycobacteria, specifically *M. avium* (30), pass through stages of growth in which they exhibit various degrees of acid fastness (16). This variability may be found

TABLE 2. Dividing times of different strains of *M. avium* in cultures of human macrophages

Expt no.	Subject for macrophages	Mean dividing time (h) ^a of strain								
		Flat-1	2-82	1-51	11-23	11-13	11907	6450	1-81	12-9
14	1	60					78	43	64	38
18	1	(223)					C	C	C	291
19	1	38	13	11	19	17				
21	1	38	23	18	31	37				
15	2	53					C	S	C	78
16	2	78					S	136	C	S
17	2	44	21	27	48	36				
20	2	(52)	22	(18)	22	45				
Mean ± SEM		51.8 ± 6.3	19.8 ± 2.3	18.5 ± 2.9	30.3 ± 6.5	33.8 ± 5.9	NA	NA	NA	>136 ± 79

^a See the text. Figures in parentheses are estimates (incomplete data). C, Bactericidal action of macrophages; S, bacteriostatic action of macrophages; NA, not applicable (bactericidal effect in one or more experiments).

more frequently in infected cells than in bacteriologic culture (4, 20, 39, 46). Often, the non-acid fastness of *M. avium* may have gone unmentioned because of preoccupation with acid fastness as a diagnostic sign of mycobacterial infection. Non-acid-fast mycobacteria in infected tissues probably are frequently overlooked or dismissed as contaminants (46). In fatal human infections with *M. avium* there can be a scarcity of acid-fast rods in biopsy tissues and fluids which should be rich in them (23, 48). When the bacilli are found in massive numbers in human specimens (e.g., autopsy of AIDS patients infected with *M. avium*; 27, 28, 40), they can also be acid fast. But it is difficult to know where these acid-fast forms actually multiplied, i.e., intra- or extracellularly, and our data suggest that extracellularly, or in the last stages of massive accumulation in infected macrophages, they become acid fast (Fig. 4).

Mycobacterial acid fastness is abrogated by drugs like ethambutol (12) which exclusively interfere with the manufacture of mycolic acid, and its use in cell wall maintenance, and the bacteria then die from cell wall deterioration. Considering that ethambutol enters and functions in macrophages very efficiently (12), our recent finding that extramacrophage (acid-fast) *M. avium* is susceptible to it but intramacrophage (non-acid-fast) *M. avium* is not (L. B. Heifets, M. D. Iseman, A. J. Crowle, and P. J. Lindholm-Levy, Am. Rev. Respir. Dis., in press) provides some independent evidence for a constitutional change in the cell walls of intramacrophage *M. avium* which directly relates to and could account for its appearing to be non-acid fast.

In contrast to these possibilities of non-acid-fast forms in

human specimens and our cultured human macrophages, the bacilli have been described as acid fast in various tissue culture and animal experiments. Cultures of HeLa (human) cells infected with *M. avium* produced groups of branched, mycelioid AFB (5). But the published photomicrographs of these cultures suggest that these groups of bacilli were extracellular. *M. lepraemurium* and *M. intracellulare* are probably the same species as *M. avium* (20). In cultures of mouse peritoneal macrophages these appear generally to be acid fast (6, 18). In the study using *M. lepraemurium* (6), non-acid-fast forms were seen early in the cultures. In the *M. intracellulare* study (18), the tissue culture medium was supplemented with fetal bovine serum, which supports extracellular replication of the bacilli. The AFB seen in the mouse macrophages of this study were in chains that were difficult to disperse. In our experiments, the tissue culture medium alone would not support the replication of *M. avium* (Fig. 2 and 3), and the bacilli tended to be single and easily dispersed. Whether these marked differences are trivially due to differences in culture conditions or more importantly due to differences between mouse and human macrophages—in human macrophages the non-acid-fast forms persist and predominate—remains to be determined. These contrasts suggest that *M. avium* isolated from patients or grown in cultured human macrophages differs substantially from in vitro- or animal model-cultured bacilli. Such contrasts for in vivo and in vitro forms of *M. tuberculosis* are well established (44).

Electron microscopy verified that the bacilli were intracellular and healthy and replicated in our experiments. They

TABLE 3. Characteristics of the *M. avium* strains tested in relation to relative virulence

Virulence	Mean dividing time (h) ^a	SEM	Strain	Serovar	Colony ratio (T:O) ^b	Source
4+	18.5	2.9	1-51	1	100:1	Other disease
4+	19.8	2.3	2-82	1	7:1	Nontuberculous granuloma
4+	20.0	2.4	T-138	8	10:1	AIDS
4+	21.8	5.4	7497	4	10:1	AIDS
3+	30.3	6.5	11-23	9	10:1	Other disease
3+	33.8	5.9	11-13	9	15:1	Nontuberculous granuloma
2+	52.6	4.3	Flat-1	2	All T	T clone
+/-	>54	NA	SJB 2	8	All O	Laboratory strain
+/-	>124	NA	1079	4	All O	Laboratory strain
+/-	>136	NA	12-9	9	1:20	AIDS
+/-	NA	NA	11907	1	1:100	Laboratory strain
+/-	NA	NA	6450	9	All O	Laboratory strain
+/-	NA	NA	1-81	1	All O	AIDS

^a From Table 2, Fig. 6, and related experiments.

^b Of infecting suspension; T, transparent; O, opaque.

TABLE 4. Ratios of transparent-to-opaque colony morphology for *M. avium* 2-82 and 11907 in separate experiments with macrophages from subjects 1 and 2

Strain	Subject	Expt no.	T:O ratio ^a			
			Culture	0 Time	4 Days	7 Days
2-82	1	19	7:1	20:1	All T	All T
	1	21	7:1	20:1	All T	All T
	2	17	7:1	50:1	All T	All T
	2	20	7:1	20:1	100:1	All T
11907	1	14	All O	All O	2:3	1:9
	1	18	All O	All O	1:20	1:10
	2	15	All O	ND	ND	1:100
	2	16	All O	1:20	1:20	1:20

^a T, Smooth transparent morphology on 7H10; O, domed opaque colony morphology on 7H10; ND, not done; Culture, infection suspension; 0 time, 4 days, and 7 days, times relative to time of infection of macrophages (0 time was immediately after infection). The morphologies were of colonies cultured on 7H10 from lysates of the macrophages cultured for CFU counts.

also indicated that the intracellular bacilli had thinner walls than extracellular *M. avium* or intra- or extracellular *M. tuberculosis*. This might account for their non-acid fastness. As has been well documented for *M. lepraemurium*, a close relative of *M. avium* (20), individual intracellular bacilli were surrounded by large electronlucent zones which other workers have interpreted as protective bacillary envelopes of crystalloid mycoside C (1, 2, 15, 16, 24). Our data confirmed a dispersion of the intracellular bacilli in individual vacuoles (1). Both optical and electron microscopy confirmed that *M. avium*, unlike *M. tuberculosis* (8, 9, 14, 42), is not toxic for infected macrophages (1, 41, 42).

Differences among the *M. avium* strains in virulence for human macrophages, when virulence was defined as the ability to survive and multiply in human macrophages (30), were readily discerned. We were able to confirm with direct human relevance evidence from animal data that opaque colony morphology correlates with avirulence and transparent colony morphology correlates with virulence (19, 20, 29, 36, 43, 51). As is best illustrated by the data for strain 2-82 in Table 4, the macrophages tended to eliminate opaque colony forms but could not stop the growth of transparent colony forms.

This species of mycobacteria is divided into serovars 1 to 3 (*M. avium*) as animal pathogens and higher-number serovars (*M. intracellulare*) as human opportunistic pathogens (19, 20, 51). According to our results, however, there was no relationship between serovar and virulence for human macrophages. Consequently, finding serovars 4 and 8 most frequently in AIDS (21; Good et al., Proc. Int. Conf. AIDS, Atlanta, Ga., p. 46, 1985) and non-AIDS mycobacterial granulomatous infections (32) probably relates more to chance of exposure than virulence, as recent statistical evidence further suggests (3).

Sometimes, strain virulence did not correlate with clinical pathogenicity. For example, the most virulent strain, 1-51, was isolated from a patient in whom it was considered clinically unimportant. Furthermore, two strains which our data indicate to be essentially avirulent, 12-9 and 1-81, were isolated as opportunistic *M. avium* infections in patients with AIDS. The avirulence of 12-9 and 1-81 might have been selected for by in vitro cultivation in transit to us for testing (29). Alternative explanations are that patients with AIDS may be so severely compromised immunologically (27) that even avirulent *M. avium* can colonize them and that their

macrophages may be producing mycobacterial-growth-enhancing factors which encourage such colonization (8, 14; Crowle et al., Proc. Int. Conf. AIDS, Atlanta, Ga., p. 32, 1985).

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