Mycobacterial Agglutination-Inhibition Test

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The mycobacterial agglutination-inhibition test (MAIT) is described as a modification of the mycobacterial agglutination of Schaefer (MATS). It is an organized assemblage of absorption and agglutination tests which is especially useful in the identification of Mycobacterium avium isolates normally excluded from serological identification because of unstable cell suspension characteristics. The identity of 100 unstable M. avium and 3 unstable Mycobacterium intracellulare isolates was determined by the MAIT method and by animal pathogenicity tests. The comparability of the MAIT and MATS methods was demonstrated by testing 10 stable M. avium and 10 stable M. intracellulare isolates by both methods and obtaining the same serological identification for each culture. The reproducibility of the MAIT was confirmed when the same result was obtained in three consecutive tests on each of the 123 cultures used in this evaluation.

Mycobacterium avium and Runyon group mycobacteria are routinely identified at this laboratory by Schaefer’s (6) method of agglutination of mycobacteria with type-specific antisera. An important limitation of the test is the requirement for a stable (dispersed) suspension of the isolate being examined. M. avium and Mycobacterium intracellulare (3) isolates generally provide stable cell suspensions but some do not. Yoder (9) reported several M. avium and M. intracellulare isolates, of bovine origin, which gave unstable cell suspensions. Routine serological identification was precluded and the more costly animal pathogenicity test was used for identification.

Absorption tests were used by Schaefer (7) in 1967 to confirm the antigenic identity of M. avium and mycobacteria of Runyon groups I, II, III, and IV. Nearly complete absorption of the type-specific agglutinins by isolates of the homologous serotype was reported. The direct agglutination test was recommended for the identification of most isolates but absorption was necessary in certain instances.

The mycobacterial agglutination test described by Schaefer (MATS) (6) is routinely used in this laboratory (8) to identify isolates of M. avium and M. intracellulare which give stable cell suspensions. Isolates which give unstable cell suspensions are identified (or separated) by a new test described here as the mycobacterial agglutination-inhibition test (MAIT).

MATERIALS AND METHODS

Cultures. One hundred twenty-three mycobacterial test cultures were used: 110 M. avium and 13 M. intracellulare. All but two were isolated at this laboratory from tissues of infected animals. M. avium strain D4 was isolated from a fowl and supplied to this laboratory by the Ministry of Agriculture and Fisheries, Weybridge, England. M. avium strain St. Elizabeth was isolated from a chicken at the St. Elizabeth Hospital Farm in Washington, D.C. Both of these stock cultures are used in the production of tuberculins.

Cell Suspensions of test cultures. Each of the test cultures was transferred from the primary colony to Dubos broth supplemented with Tween 80 and albumin (Bacto) and incubated for 14 days at 37 C. The fluid culture was transferred in 1.0-ml samples to each of 10 plates containing Middlebrook 7H10 agar enriched with 4.1 g of sodium pyruvate per liter of medium. The plates were incubated at 37 C for 14 days. The growth was harvested by adding 2.0 ml of phenolized phosphate-buffered saline (PPBS: Na2HPO4, 0.14%; NaCl, 0.8%; phenol, 0.5%) to each plate and removing the confluent growth with a rubber policeman. The cell-buffer mixture was removed with a sterile Pasteur pipette and suspended in PPBS to an optical density (OD) of 0.4 (at 525 nm) on a Bausch & Lomb model 340 spectrophotometer. The mixture, contained in a flask, was rotated occasionally during a 5-day period in which all bacterial cells were killed by the phenol. A sufficient volume of killed cells in PPBS were transferred to a 50-ml conical centrifuge tube to provide approximately 1.0 cm3 of packed cells following centrifugation at 680 RCF (relative centrifugal force) for 20 min. The supernatant fluid was discarded and the packed
cells were retained in the centrifuge tube where they were subsequently used to absorb antisera.

**Reference antigen.** *M. avium* serotype 1 reference antigen (Av1Ag) was prepared from *M. avium* cultures 33965 and 35360. Phenol-killed cells were suspended in PPBS to an OD of 0.4 (at 525 nm). *M. avium* serotype 2 reference antigen (Av2Ag) was prepared by the same methods, except *M. avium* cultures 34809 and 35378 were used. The exact methods used in preparing these antigens are published elsewhere (6, 8). Reference culture 35360 was isolated at this laboratory from a tuberculous chicken, and the other three were isolated at this laboratory from cattle. The cultures used for reference antigens were selected because they adequately represented the cultures isolated here, they were stable in suspension, and their serological activity was discrete, specific, and reproducible.

**Reference antisera.** *M. avium* strains 33965 and 35360 were used for the production of *M. avium* serotype 1 reference antisera (Av1As). *M. avium* strains 34809 and 35378 were used for the production of *M. avium* serotype 2 reference antisera (Av2As). The reference antisera were produced in adult New Zealand White rabbits. Each rabbit received a series of eight 1.5-ml intravenous injections of antigen during a 4-week period. The antigen was a 0.4 OD (at 525 nm) suspension of the appropriate mycobacterial cells in PPBS. The rabbits were exsanguinated 6 days after the last injection. The undiluted antisera were preserved by adding sufficient 10.0% 8-hydroxyquinoline sulfate to produce a 1:10,000 final dilution of the preservative and then stored at −20 C. The titers of the antisera were determined on serial twofold dilutions using conventional MATS procedures. The titer was designated as the highest twofold dilution which caused complete agglutination (at 18 hr) of a cell suspension of the same strain. The titer of each reference antisem was 1:160 when tested with the homologous reference antigen. Specificity was determined by reacting each antisem (using the MATS) with standardized cell suspensions of four heterologous *M. avium* strains (including serotype 3), 34 *M. intracellulare* strains, and 6 Runyon group II strains. Minimal cross agglutination occurred. All reference antisera were made specific, without loss of titer, by absorbing with packed cells of the cross-reacting serotypes.

Antisera used in the MAIT were thawed and adjusted to the 1:160 working dilution. Detailed instructions regarding titration and absorption are available in other publications (6, 8).

**Sero logical tests.** The MATS was performed according to procedures published by Schaefer (6) and by this laboratory (8). The MAIT was performed as illustrated in Fig. 1 and described here. The absorption of Av1As and Av2As was accomplished by adding 1.5 ml of a 1:160 dilution of each to approximately 1 ml of packed cells of the test culture retained in the 50-ml centrifuge tube. The absorption of specific agglutinins was enhanced by maintaining the temperature of the cells and serum mixture at 4 C for 18 hr. Following absorption the mixture was centrifuged at 680 RCF for 20 min. The absorbed serum was retained, and the cells were discarded.

Five-tenths milliliter of the absorbed antisera mixture was transferred to each of two test tubes (11 by 100 mm) labeled C and D. Five-tenths milliliter of Av1Ag was added to tube C, and 0.5 ml of Av2Ag was added to tube D. The contents of each tube were mixed by gently rotating and incubated at 37 C. Each suspension was observed for agglutination at 3 hr and again at 18 hr of incubation.

Results were recorded as follows: N, no agglutinated cells, suspending fluid opaque; 1, very small clumps of agglutinated cells throughout opaque suspending fluid; 2, small clumps of agglutinated cells throughout slightly opaque suspending fluid; 3, most cells agglutinated and sedimented, suspending fluid nearly transparent; 4, all cells agglutinated and sedimented, suspending fluid transparent.

Cell suspensions (antisera) of two *M. avium* strains were used to represent a single serotype. Equivalent agglutination of both cell suspensions was required for an acceptable test. The *M. avium* serotype 1 cultures 33965 and 35360 gave consistently similar agglutination as did *M. avium* serotype 2 cultures 34809 and 35378. This procedure is routinely used in this laboratory (8), although it is not indicated in Fig. 1.

Controls were provided by four test tubes (11 by 100 mm) designated as tubes A, B, E, F in Fig. 1. Control tubes for A, B, E, and F contained, respectively, 0.5 ml of unabsorbed Av1As and 0.5 ml of Av1Ag, 0.5 ml of unabsorbed Av1As and 0.5 ml of Av2Ag, 0.5 ml of unabsorbed Av2As and 0.5 ml of Av1Ag, 0.5 ml of unabsorbed Av2As and 0.5 ml of Av2Ag. Incubation, observation, and recording were conducted as described above for the MAIT method.

The determinations made by the MATS and the controls for the MAIT were performed in duplicate. The homologous reagents in test tubes A and F and the heterologous reagents in test tubes B and E were tested once each day as a control for tests completed during that period. The MAIT was performed in triplicate on all 123 test cultures.

**Animal pathogenicity tests.** The pathogenicity of each of the 123 test cultures was determined by inoculating two chickens intraperitoneally with 1.0 ml of a suspension containing 0.1 mg (wt) of bacterial cells per ml of Butterfield buffer (42.5 mg of KH₂PO₄ per liter of distilled water). The chickens were 9-week-old White Leghorn pullets. They were necropsied 8 weeks after inoculation or at the time of death due to mycobacterial infection. The presence or absence of tuberculous lesions was noted and recorded. Cases of minimal pathological changes in the viscera were further evaluated by microscopy examination of smears stained by the Ziehl-Neelsen method.

**RESULTS**

Each of the 100 unstable *M. avium* cultures
was confirmed as *M. avium* by three separate MAIT trials and by animal pathogenicity tests. Seventy-six were serotype 1 and 24 were serotype 2. There were no exceptions to the expected results; viz., agglutination of either the Av1Ag or the Av2Ag, but not both. Lesions were observed in all chickens inoculated with cultures of this group. The lesions were found consistently in the liver and spleen, frequently in the intestines, and occasionally throughout the viscera.

The 10 *M. avium* cultures which gave stable cell suspensions were confirmed as *M. avium* in three separate MAIT trials, 5 were serotype 1, and 5 were serotype 2. Tuberculous lesions were observed in all chickens inoculated with cultures of this group. Lesions were distributed as in the first group.

*M. avium* strains D4 and St. Elizabeth absorbed the specific agglutinins from Av1As in the MAIT, clearly indicating *M. avium* serotype 1. Both strains were pathogenic for chickens, causing extensive lesions in liver and spleen.

The 10 stable and 3 unstable cell suspensions of *M. intracellulare* were all confirmed as *M. intracellulare* in three separate MAIT trials. No lesions were found in any of the chickens inoculated with cultures of this group.

*M. avium* serotype 1 test cultures generally absorbed all of the measurable homologous agglutinins from Av1As. This is indicated in Table 1 by a complete lack of agglutination (100% "N") of the Av1Ag. The *M. avium* serotype 1 test cultures absorbed a minimal amount of the heterologous agglutinins from Av2As. This is indicated by a high proportion
(96.3%) of “4” agglutination of Av2Ag with Av1As-Av2As mixture following absorption with *M. avium* serotype 1 test cultures. The remaining 3.7% of reactions were rated at “2” agglutination.

*M. avium* serotype 2 test cultures were equally effective in absorbing all of the measurable homologous agglutinins from Av2As. This is indicated in Table 1, by a complete lack of agglutination (100% “N”) of the Av2Ag. The *M. avium* serotype 2 test cultures differed from *M. avium* serotype 1 test cultures by absorbing a considerable portion of the measurable heterologous agglutinins. This is indicated by a high proportion of intermediate reactions (17.3% “1”, 58.6% “2” and 24.1% “3” agglutination) when Av1Ag was reacted with Av1As-Av2As mixture following absorption with *M. avium* serotype 2 test cultures.

*M. intracellulare* test cultures apparently absorb none of the heterologous agglutinins from Av1As or Av2As. This is indicated by complete agglutination (“4”) of both Av1Ag and Av2Ag. Complete agglutination occurred in all 13 (100%) trials in this group.

**DISCUSSION**

The MAIT may be described as an organized assemblage of absorption and agglutination tests. The basic principle is one of specific absorption of agglutinins from reference antisera followed by a manifestation of the remaining agglutinins. When the agglutinins of one serotype are absorbed by the test culture from the mixture of reference antisera, they are unavailable for subsequent agglutination of the homologous reference antigen. However, the heterologous reference antigen is readily agglutinated because its specific agglutinins were not absorbed by the test culture. The final phase of the test always utilizes reference antigens and mixed reference antisera, thereby assuring vigorous, discrete, and reproducible reactions.

Typical reactions in the MAIT are shown in Table 2. A requisite for a satisfactory test is complete (“4”) agglutination of reference antigens by their homologous, unabsorbed reference antisera and the complete absence of agglutination (“N”) of reference antigens by their heterologous, unabsorbed reference antisera. It is also essential that the reference sera mixture, previously absorbed with the test culture, cause the agglutination of only one reference antigen (indicating *M. avium* serotype 1 or 2) or both reference antigens (indicating *M. intracellulare*). If neither reference antigen is agglutinated, a mixed *M. avium* serotype would be indicated, and that individual test would be disqualified; none were observed in the tests reported here. A high level of agglutination of the appropriate reference antigen is desirable but not essential.

The MAIT is not applicable for determining the serotype of *M. intracellulare* cultures, but fortunately most of those isolates give stable cell suspensions and can be serologically identified by MATS.

The reproducibility of the MAIT was confirmed by testing 110 *M. avium* and 13 *M. intracellulare* isolates without any variation of results in triplicate tests. The comparability of the MAIT and MATS methods was confirmed by complete agreement between the MAIT method conducted in triplicate and the MATS method conducted in duplicate on the same 10 *M. avium* and 10 *M. intracellulare* isolates giving stable cell suspensions.

Animal pathogenicity tests were included in this evaluation for purposes of comparing virulence for chickens with serotype and not to prove the validity of the MAIT method. The perfect correlation of serological and animal pathogenicity tests was not expected. The statistical analysis of these results was simplified because all serological and animal pathoge-

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**Table 1. Distribution of mycobacterial agglutination-inhibition test reactions**

| Test cultures used to absorb Av1As/Av2As | Extent of reactiona | Agglutination of Av1Ag with absorbed Av1As/Av2As (%) | Agglutination of Av2Ag with absorbed Av1As/Av2As (%) |
|-----------------------------------------|---------------------|--------------------------------------------------|--------------------------------------------------|
| *Mycobacterium avium* serotype 1 (total 81) | N 100.0b | 0.0 | 0.0 |
|                                          | 1 0.0 | 0.0 | 0.0 |
|                                          | 2 0.0 | 3.7 | 0.0 |
|                                          | 3 0.0 | 0.0 | 0.0 |
|                                          | 4 0.0 | 96.3 | 0.0 |
| *M. avium* serotype 2 (total 29)         | N 0.0 | 100.0b | 0.0 |
|                                          | 1 17.3 | 0.0 | 0.0 |
|                                          | 2 58.6 | 0.0 | 0.0 |
|                                          | 3 24.1 | 0.0 | 0.0 |
|                                          | 4 0.0b | 0.0 | 0.0 |
| *M. intracellulare* (total 13)           | N 0.0 | 0.0 | 0.0 |
|                                          | 1 0.0 | 0.0 | 0.0 |
|                                          | 2 0.0 | 0.0 | 0.0 |
|                                          | 3 0.0 | 0.0 | 0.0 |
|                                          | 4 100.0b | 100.0b | 0.0 |

a See Materials and Methods section.
b One-hundred percent of reactions should be at these points when conditions are ideal.
nicity test results were in agreement with the theoretically predicted results. When the tables for confidence limits were consulted, the lower 95% confidence limit was found to be approximately 0.975 for the 110 M. avium isolates. This means that one can predict (with 95% confidence) that the true proportion of M. avium isolates which will infect chickens will be at least 97.5%. This limit is reduced as the number of trials is decreased. For the 13 M. intracellulare isolates, the lower 95% confidence limit for the proportion of agreement was 0.794. This means that one can predict (with 95% confidence) that the true proportion of M. intracellulare isolates which will fail to infect chickens will be at least 79.4%.

Results of similar experiments at this laboratory were compatible with those of Yoder (9), Engbaek (1), and Schaefer (4) in which serological identification of M. avium was in close agreement, but the serological identification of M. intracellulare was occasionally in disagreement, with the results of tests for virulence in chickens. They reported that M. avium isolates generally caused tuberculosis in chickens but there were numerous exceptions to the latter.

The incidence of serotype 1 among the 100 unstable M. avium isolates, tested by the MAIT method, was approximately three times that of serotype 2. Conversely, the incidence of serotype 2 exceeds that of serotype 1 among stable M. avium isolates identified at this and other laboratories using the MATs. We believe that the MAIT method will enable investigators to determine the true prevalence of serotype 1 among M. avium isolates from human and animal sources. It also provides an effective method for differentiating unstable M. avium from unstable M. intracellulare isolates without the use of costly and time-consuming animal pathogenicity tests.

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