Production of Multivalent Fluorescent Antisera for Identification of Organisms in the *Mycobacterium avium-Mycobacterium intracellulare* Complex

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Antisera to ten strains of mycobacteria in the *Mycobacterium avium-Mycobacterium intracellulare* group were obtained by injecting rabbits with ultraviolet light-killed cells. The antisera were conjugated with fluorescein isothiocyanate and used in the direct fluorescent antibody test. Individual antisera reacted specifically with the mycobacterial serotype used to produce them. The antisera were then combined in two multivalent pools. Each multivalent pool reacted specifically with its corresponding antigens. The multivalent antisera were thus found to provide a rapid identification method for the mycobacteria studied.

The application of fluorescent antibody (FA) methods for the identification of mycobacteria has shown considerable promise (1, 2, 4, 5). However, the occurrence of many serotypes, especially in group II photochromogens, group III non-photochromogens, and group IV rapid growers, can present problems. If individual antisera are used the number of tests will be large. Furthermore, when working with direct sputum smears, the amount of material may be limited. This study was undertaken to determine if multivalent antisera could be prepared to the more commonly occurring *Mycobacterium avium-Mycobacterium intracellulare* organisms.

**MATERIALS AND METHODS**

Culture materials used in this study were provided by the United States-Japan Cooperative Medical Science Program of the National Institute of Allergy and Infectious Diseases and obtained from the Trudeau Institute, Saranac Lake, N.Y. Species, strain, and Trudeau Mycobacterial Culture Collection number of strains used in this study are listed in Table 1.

A detailed procedure for producing antisera by injecting ultraviolet (UV) light-killed cells into rabbits has been previously described (6). Preparation of inoculum, injection schedule, fractionation of antisera, method of labeling, and preparation of slides were described.

The method consists of growing the mycobacterial cells in Middlebrook 7H9 broth, washing them, and adjusting the inoculum to an optical density of 0.2 at 525 nm. The cells were placed in an open petri dish and killed with UV light of 253.7 nm. In this experiment, three New Zealand white rabbits (5 to 6 lb. each; ca. 2.27 to 2.72 kg) were injected intravenously with each mycobacterial strain used. The rabbits were injected weekly with increasing doses of 0.1, 0.3, 0.6, and 1.0 ml of inoculum. One week after the last injection the rabbits were exsanguinated and the sera of the three rabbits injected with the same inoculum were pooled.

The gamma globulin was extracted with one 50% ammonium sulfate fractionation, followed by three 33.3% extractions. The precipitated gamma globulin obtained in the final extraction was dissolved in 5 ml of phosphate-buffered saline (PBS) and dialyzed against PBS until the ammonium sulfate was removed.

The gamma globulin fractions were tagged with fluorescein isothiocyanate (FITC, isomer 1, BBL) by the method of Kawamura (3). The amount of FITC used was 1/100 of the amount of protein. The conjugation reaction was run at pH 8.5 at 7 to 9 C for 4 h with gentle stirring. After completion of the reaction, the free fluorescein was removed from the conjugated protein by gel filtration through a column of Sepha-

**Table 1. Strains of mycobacteria used**

| Trudeau culture no. | Species       | Strain   |
|---------------------|---------------|----------|
| 701                 | *M. avium*    | Sheard   |
| 706                 | *M. avium*    | McKee #1 |
| 1403                | *M. intracellulare* | Boone    |
| 1406                | *M. intracellulare* | Yandle   |
| 1463                | *M. intracellulare* | Sparrow 185 |
| 1468                | *M. intracellulare* | S.J.B.   |
| 1469                | *M. intracellulare* | Darden   |
| 1472                | *M. intracellulare* | AI2314-67 |
| 1476                | *M. intracellulare* | Runyon P-49 |
| 1479                | *M. intracellulare* | 17584-286 |
dex G-50 (Pharmacia Fine Chemicals). All conjugates were stored in the dark at 2 to 4°C with 0.1% sodium azide added as a preservative. Fluorescein-to-protein molecular ratios (F:P) were determined in a UV-visible spectrophotometer. All F:P ratios fell between 1:1 and 2:1.

Antigen slides were prepared by growing the mycobacterial strains in Middlebrook 7H9 broth, diluting 1:10 in sterile water, and placing a drop on each etched ring of a FA slide. The slides were then heat-fixed at 65°C for 2 h. To stain a slide the conjugate was spread over the antigen smear and incubated in a moist chamber for 30 min at 37°C. Slides were then washed for 5 min in deionized water, air-dried, and mounted with Difco FA mounting fluid and a cover slip. Slides were examined on a large Zeiss fluorescence microscope using a BG-3 exciter filter and a 500-nm barrier filter.

RESULTS

The ten conjugates were titered against the mycobacterial strain used to produce them. The penultimate dilutions were determined and used in checking cross-reactivity and in preparing the multivalent antisera pools. The penultimate dilution of each conjugate is indicated in Table 2, which shows the results of cross-reactivity testing. All conjugates proved to be specific for the homologous antigen with the exception of conjugate 706 which cross-reacted with 1403 and 1479 cells.

Two multivalent antisera pools were then prepared. Pool A contained conjugates 1406, 1479, 1472, 1403, and 701. Pool B contained conjugates 1469, 1476, 1468, and 1463. The pools were prepared so that each conjugate was at its penultimate dilution in the complete pool. PBS was used as a final diluent.

The two pools were reacted with the 10 individual antigens. These pools proved to be specific for the corresponding antigens (Fig. 1 and 2). Results of the multivalent testing are shown in Table 3.

As a further check on the specificity of the pools, they were tested against other mycobacteria not in the M. avium-M. intracellulare group. These included M. tuberculosis, M. kansasii, M. marinum, M. scrofulaceum, M. gordonae, M. smegmatis, and M. fortuitum. None of these cells were stained with either of the multivalent pools. M. terrae, which had

| Penultimate dilutions | 1/8 | 1/4 | 1/4 | 1/4 | 1/4 | 1/4 | 1/4 | 1/4 | 1/4 |
|-----------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Conjugates            | 701 | 706 | 1403| 1406| 1463| 1468| 1469| 1472| 1476| 1479|
| **Cells**             |     |     |     |     |     |     |     |     |     |     |
| 701                   | +   | -   | -   | -   | -   | -   | -   | -   | -   |    |
| 706                   | -   | +   | -   | -   | -   | -   | -   | -   | -   |    |
| 1403                  | -   | +   | +   | -   | -   | -   | -   | -   | -   |    |
| 1406                  | -   | -   | -   | -   | +   | -   | -   | -   | -   |    |
| 1463                  | -   | -   | -   | -   | -   | +   | -   | -   | -   |    |
| 1468                  | -   | -   | -   | -   | -   | -   | +   | -   | -   |    |
| 1469                  | -   | -   | -   | -   | -   | -   | -   | +   | -   |    |
| 1472                  | -   | -   | -   | -   | -   | -   | -   | -   | +   |    |
| 1476                  | -   | -   | -   | -   | -   | -   | -   | -   | -   | +   |
| 1479                  | -   | -   | -   | -   | -   | -   | -   | -   | -   | +   |
The frequency of various serotypes of *M. avium*-*M. intracellulare* organisms was explored by Schaefer (6). The 10 most frequently occurring serotypes were used in this study. These 10 serotypes account for 92% of 315 *M. avium*-*M. intracellulare* organisms isolated from humans. Thus, the use of two multivalent pools could provide a rapid means of identification for the vast majority of *M. avium*-*M. intracellulare* organisms found in humans.

**DISCUSSION**

The only individual conjugate showing cross-reactivity was 706. It should be noted that the titer of this conjugate was low. The production of a higher titer might well have eliminated these cross-reactions. Because of the low titer and subsequent dilution problems in preparing multivalent antisera, plus the cross-reactivity, conjugate 706 was not included in either of the pools.

### Table 3. Reactions of multivalent antisera

| Antigens | Pool A | Pool B |
|----------|--------|--------|
| A 1406   | +      | -      |
| 1479     | +      | -      |
| 1472     | +      | -      |
| 1403     | +      | -      |
| 701      |        | -      |
| B 1469   |        | +      |
| 1476     |        | +      |
| 1468     |        | +      |
| 1463     |        | +      |

previously been shown to bind nonspecifically with fluorescent conjugates, was again stained by both pools (5).

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