Autofluorescence of Mycobacteria as a Tool for Detection of M. tuberculosis

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The diagnosis of tuberculosis in developing countries still relies on direct sputum examination by light microscopy, a method that is easy to perform and that is widely applied. However, because of its poor sensitivity and requirement for significant labor and training, light microscopy examination detects the bacilli in only 45 to 60% of all people whose specimens are culture positive for Mycobacterium tuberculosis. Therefore, new diagnostic methods that would enable the detection of the undiagnosed infected population and allow the early commencement of antituberculosis treatment are needed. In this work, the potential use of mycobacterial cyan autofluorescence for the detection of M. tuberculosis was explored. The tubercle bacilli were easily visualized as brilliant fluorescent bacilli by microscopy and were easily tracked ex vivo during macrophage infection. Assays with seeded sputum and a 96-well microplate reader fluorimeter indicated that 104 bacilli ml−1 of sputum could be detected. Moreover, the use of microplates allowed the examination of only 200 μl of sputum per sample without a loss of sensitivity. Treatment with heat or decontaminating chemical agents did not interfere with the autofluorescence assay; on the contrary, they improved the level of bacterial detection. Autofluorescence for the detection of bacilli is rapid and easy to perform compared to other methodologies and can be performed with minimal training, making this method suitable for implementation in developing countries.

The diagnosis of tuberculosis (TB) in low- and middle-income countries, where more than 90% of TB cases occur, is mainly performed by microscopy examination of stained sputum smears for acid-fast bacilli (25). The International Standards for Tuberculosis Care considers the microscopic analysis of two or three specimens per patient to be a cornerstone for the diagnosis of TB (13). Although light microscopy is inexpensive, easy to perform, and highly specific in areas where there is a high prevalence of TB, it is relatively insensitive, requiring ≥104 bacilli ml−1 of sputum to achieve a positive result (15, 35). In addition, the procedure requires the observation of from 100 to 300 fields in order to obtain accurate results (31).

The sensitivity of microscopy is influenced by numerous factors, such as the prevalence and severity of the disease, the quality of specimen collection, the type of specimen, the method of processing (direct or concentrated, centrifugation, liquefaction), staining, and finally, the quality of the examination (20, 23, 26, 29). In well-trained hands, the test detects tubercle bacilli in 75% of all people who have active pulmonary TB, but the sensitivity may fall to 45 to 60%, depending on the training, eye, and motivation of the laboratory technician (1). The basis of conventional light microscopy was developed more than 100 years ago, and, at present, conventional light microscopy uses carbol-fuchsin Ziehl-Neelsen or Kinyoun acid-fast stains. Fluorescence microscopy with fluorochrome dyes such as auromine O or auramine-rhodamine is reputed to have higher degrees of sensitivity and specificity and is thus a more accurate test for the diagnosis of TB (16, 32). However, it is mainly performed in developed countries because it is more expensive than the conventional method, as it requires unstable and expensive fluorescent staining reagents, which increases the cost of the assay, thus hindering its widespread use in developing countries (32). Actions are required to diminish that disadvantage and to allow the wider applicability of fluorescence microscopy in developing countries.

Intrinsic fluorescence (autofluorescence) is a characteristic previously described in a few microorganisms that allows their detection without the need for fluorescence staining and has been used for the detection of bacterial cells (6). Here we report for the first time that the mycobacteria, including M. tuberculosis, emit autofluorescence in the cyan range of the visible spectrum. Autofluorescence was first explored with cultured microorganisms; then with experimentally infected macrophages, which were used to mock a natural intracellular infection; and finally, with sputum specimens spiked with M. tuberculosis and submitted to the decontamination processes routinely used in diagnostic laboratories.

MATERIALS AND METHODS

Strains and culture conditions. The microbial strains used in this study are listed in Table 1. Species belonging to the Mycobacterium genus, some partially acid-fast actinomycetes, as well as some pathogens frequently detected in the sputum of patients with respiratory infections were selected. The mycobacteria and the nocardia species were grown in Middlebrook 7H medium (Difco) supplemented with 0.5% (vol/vol) glycerol, 10% (vol/vol) albumin-dextrate-catalase (Difco), and 0.05% (vol/vol) Tween 80 (Sigma, St. Louis, MO) with gentle

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agitation at 30°C or 37°C, according to the optimal growth temperature for the organism. Corynebacterium glutamicum was grown in Luria broth medium (Difco) with shaking at 37°C. Haemophilus influenzae and Streptococcus pneumoniae were grown in brain heart infusion broth (Himedia, India) at 37°C without agitation. Cultures of Paracoccidioides brasiliensis (yeast phase) were kindly provided by G. Niño-Vega (Mycology Laboratory, Instituto Venezolano de Investigaciones Científicas, Venezuela). For microscopic observation, the microorganisms were harvested and washed twice with phosphate-buffered saline (PBS) solution at pH 7.4. Aliquots of 20 to 40 µl were applied to slides that had been pretreated with polylysine, as described previously (3).

For macrophage infection, wild-type M. tuberculosis H37Rv and H37Rv transformants bearing pGFP22-4, a plasmid that expresses the green fluorescent protein (GFP) (30), were grown in a roller agitator under the conditions described above. Pathogenic strains were grown and manipulated inside a biosafety level 3 containment facility.

**Macrophase infection.** To test whether the infection of macrophages can be tracked by using cyan autofluorescence, THP-1 cells were infected with M. tuberculosis H37Rv. THP-1 cells were grown in RPMI 1640 (Gibco, BRL) supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (10,000 U ml⁻¹), and streptomycin (10,000 µg ml⁻¹) at 37°C in 5% CO₂. The cells were subcultured every 3 days and passaged at least once in fetal calf serum-supplemented antibiotic-free RPMI 1640 growth medium. Before infection, 2.5 × 10⁵ macrophages were differentiated in eight-well Nunc Lab-Tek chamber slides by the addition of 100 µM phorbol-12-myristate-13-acetate (Sigma) and 100 U ml⁻¹ of recombinant human gamma interferon (specific activity, 2 × 10⁵ U mg⁻¹; Abcam) for 24 h at 37°C in a 5% CO₂ atmosphere.

M. tuberculosis inocula were prepared by resuspending the bacteria in warm RPMI 1640 medium at a concentration of 2 × 10⁶ bacilli ml⁻¹, and the inocula were used to infect activated THP-1 cells at a ratio of 10:1 (mycobacteria to macrophages). At 3 h postinfection, the cells were washed extensively with PBS (pH 7.4) to remove the extracellular bacilli and fixed with 4% paraformaldehyde for 30 min at 37°C. Finally, infected macrophages from two wells were stained with 25% (wt/vol) fluorescein diacetate (FDA; Sigma) for microscopic observation.

**Fluorescence microscopy and image analysis.** The microorganisms were photographed with a cooled Orca 12-ERG digital charge-coupled-device camera (Hamamatsu Photonics) mounted on a Nikon E600 fluorescence microscope through a ×100 (numerical aperture [NA], 1.3) oil immersion objective. Images were taken, and the images were processed with MetaMorph software (Universal Imaging Corporation). The cells were first photographed in the difference interference contrast mode and were then photographed with a cyan GFP filter (excitation, 426 to 446 nm; emission, 460 to 500 nm) to capture mycobacterial autofluorescence. M. tuberculosis-infected macrophages grown in chamber slides were visualized with the same equipment through a ×40 (NA, 0.55) objective and photographed as described above for the detection of mycobacterial autofluorescence. A B2-A filter (excitation, 450 to 490 nm; emission, 515 nm) was used to document FDA staining and the GFP transformants.

**Confocal laser scanning microscopy.** Bacteria were grown until they reached the exponential phase of growth, and aliquots were twice washed with PBS. M. tuberculosis was heat inactivated at 80°C for 20 min and washed twice in PBS.
fluorescence microscope by using the excitation and barrier filter selected. The Nocardia sp. also emitted cyan autofluorescence (Fig. 1). The remaining microorganisms, including C. glutamicum, an organism closely related to mycobacteria, did not show cyan autofluorescence.

*M. tuberculosis* cells infecting macrophages were distinguished by green fluorescence when the bacteria expressed GFP, an easily detected fluorescent molecule, or after they were stained with FDA. However, the tubercle bacilli were also easily visualized inside infected macrophages as bacilli with cyan fluorescence (Fig. 2).

**Fluorescence emission spectra.** In order to gain insight into the nature of the intrinsic mycobacterial autofluorescence, the emission spectra of four *Mycobacterium* species were determined by confocal laser scanner microscopy. The results are shown in Fig. 3. The mycobacteria exhibited an emission maximum at 475 nm when they were excited with a laser at a wavelength of 405 nm. No fluorescence emission was detected when the mycobacteria were excited with a laser at a wavelength of 488 nm.

**Influences of temperature and pH on autofluorescence of *M. tuberculosis*.** In order to determine the influences that standard diagnostic procedures could have on *M. tuberculosis* autofluorescence, the fluorescence emissions from heat-treated cultures as well as from *M. tuberculosis* cells resuspended in buffer solutions with different pH values were determined. The level of *M. tuberculosis* cyan fluorescence increased fourfold after incubation for 20 min at 80°C (Fig. 4A). The autofluorescence of *M. tuberculosis* did not vary over a pH range of from 6 to 9 in phosphate and borate buffers, whereas in borate buffer adjusted to pH 10, the cyan autofluorescence of the tubercle bacilli increased eightfold (Fig. 4B).

The minimum amount of bacilli required for autofluorescence detection by fluorimetry was determined by using exponential-phase *M. tuberculosis* cultures and a 96-well microplate format. For untreated cells, $3 \times 10^6$ bacilli per well (corresponding to $2 \times 10^7$ CFU ml$^{-1}$) were required to clearly detect cyan autofluorescence. The limit of detection dropped to $4 \times 10^4$ bacilli per well ($2.7 \times 10^5$ CFU ml$^{-1}$) when the same cultures were heat inactivated (at 80°C for 20 min). Therefore, the heat treatment substantially increased the emission of fluorescence, making possible the detection of approximately 75-fold fewer bacilli.

**Influence of standard methods of sputum decontamination on *M. tuberculosis* autofluorescence.** The cyan autofluorescence of the seeded sputa was detected with all clarifying agents except NaOCl. This molecule showed a high level of intrinsic fluorescence that interfered with that of *M. tuberculosis*, which, as a consequence, was undetectable. When chemical reagents other than NaOCl were considered, the levels of the autofluorescence at room temperature were similar regardless of the liquefying agent used. However, drastic differences were found when seeded sputa were incubated at 80°C for 20 min (Fig. 5). The autofluorescence increased about 10 times
when the seeded sputa were heat inactivated and treated with NaOH or NALC-NaOH. In the presence of DTT, the autofluorescence showed a moderate increase when the bacteria were heated. On the contrary, heat inactivation in the presence of other reagents, such as dimethyl benzyl lauryl ammonium bromide or SDS-NaOH, hindered the detection of the autofluorescence, probably due to the lysis of the bacilli under these conditions (Fig. 5).

Detection of *M. tuberculosis* in sputum by using autofluorescence. In order to examine the potential use of the mycobacterial autofluorescence for the diagnosis of TB, we determined the detectable range of tubercle bacilli from sputum specimens by the use of autofluorescence and a 96-well microplate format. Known concentrations of *M. tuberculosis* H37Rv cells that had previously been heat inactivated were seeded in sterilized sputum, and an equal volume of NaOH or NALC-NaOH was added to mimic the usual manipulation of the samples that occurs during microbiological diagnostic procedures.

The autofluorescence of *M. tuberculosis* was detected with all

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**FIG. 2.** Visualization of *M. tuberculosis* inside macrophages. THP-1 cells were infected with *M. tuberculosis* H37Rv (stained with FDA) or *M. tuberculosis* bearing plasmid pGFP22-4 and visualized by fluorescence microscopy with a B2-A filter (green fluorescence) or a cyan GFP filter (cyan fluorescence). With both filters, *M. tuberculosis* was observed as green as well as cyan bacilli inside the infected macrophages.

**FIG. 3.** Emission spectra of *Mycobacterium* species recorded at an excitation wavelength of 405 nm.

**FIG. 4.** Effect of heat treatment (A) and pH (B) on the cyan autofluorescence of *M. tuberculosis*. The fluorescence emissions from cultures at room temperature (RT) or that had previously been heat inactivated for 20 min (80°C) were calculated once the fluorescence of the broth medium (A) or the buffer used to resuspend the bacteria (B) was subtracted. PPB, potassium phosphate buffer; SBB, sodium borate buffer; 6 to 10, pHs 6 to 10. The bars and the error bars show the means and the standard deviations, respectively, of at least three separate determinations.
the concentrations of bacilli assayed, but overnight sedimentation rendered the best results. A better correlation between the fluorescence emissions and the bacillus concentration was observed when NALC-NaOH was used. Overnight sedimentation improved the rate of detection of the autofluorescence of seeded sputum liquefied with NALC-NaOH 1.4 times (Fig. 6).

**DISCUSSION**

The diagnosis of TB is primarily based on clinical features, histopathology, the identification of acid-fast bacilli, and the isolation of *M. tuberculosis* from the clinical specimens. Cultivation of the tubercle bacilli is the most sensitive method for the detection of *M. tuberculosis* and can detect 100 bacilli per ml of sputum (18); unfortunately, it can take several weeks to yield results. However, the routine implementation of the identification of *M. tuberculosis* by culture is very limited, especially in low-income countries.

WHO strongly recommends the use of the sputum smear examination as the primary tool for case detection and the diagnosis of TB as a pivotal element of the directly observed therapy strategy. Sputum smear examination is the preferred screening test for the detection of *M. tuberculosis* in developing countries, since this technique is simple and inexpensive and allows the detection of infectious cases. However, WHO (http://www.who.int/tb/features_archive/global_plan_to_stop_tb/) has also recognized the significant limitations of standard direct sputum smear microscopy. The requirement of time-consuming slide examination by light microscopy of two or three sputum samples and the training needed for the application of the technique imply a heavy workload on already overstretched health care systems in the developing world. The use of fluorescence microscopy decreases the run time for sample examination and shortens the time required to obtain results. Unfortunately, the cost derived from the requirement for unstable and expensive fluorescent reagents, such as auramine-rhodamine, is hindering the use of fluorescence microscopy in developing countries (32).

In this work we explored the ability of *M. tuberculosis* to autofluoresce in order to improve the detection of the tubercle bacilli in clinical samples. Very few studies on the use of autofluorescence for the detection of bacteria have been reported (2, 14). Some bacteria exhibit spontaneous emission spectra following excitation at specific wavelengths. The energy of the photons emitted depends on the intrinsic fluorophores retained, such as protein tryptophans, nucleic acids, and coenzymes (19), or on the production of other fluorescent substances (4, 21). This work shows for the first time that *M. tuberculosis* can emit a cyan autofluorescence which can be measured with sensitivity and speed comparable to that of standard light microscopy.
M. tuberculosis can easily be detected by its intrinsic autofluorescence in the cyan emission spectrum as a brilliant fluorescent bacillus in vitro as well as in vivo during macrophage infection (Fig. 1 and 2). In fact, the cyan fluorescence of M. tuberculosis cells was as brilliant as that of M. tuberculosis cells expressing cytoplasmic GFP or FDA-stained bacilli (Fig. 2).

Confocal laser scanning microscopy demonstrated that the mycobacteria exhibited a unique emission maximum at 475 nm when they were excited at 405 nm (Fig. 3), suggesting that the autofluorescence of mycobacteria is possibly due to the fluorescence emitted by the coenzyme F420, or its biosynthetic intermediate, FO (5). F420/FO shows a bright blue-green fluorescence with a maximum of about 470 nm when cells are excited with a laser at a wavelength of 405 nm (11, 12). F420 is a two-electron transfer coenzyme which has 7,8-didemethyl-8-hydroxy-5-deazaflavin, a phospholactyl moiety, and several numbers (two to six) of glutamate residues. FO contains a deazaflavin ring and ribityl sugar but lacks the phospholactyl group and glutamate moieties (5). Coenzyme F420 is present in some members of three distantly related groups of prokaryotes: archaea, aerobic actinomycetes, and cyanobacteria (28). The presence of F420/FO has previously been reported in Mycobacterium avium, Mycobacterium smegmatis, Mycobacterium fortuitum, Mycobacterium hovis BCG, Mycobacterium phlei, and M. tuberculosis (5, 9, 17, 24).

The cyan autofluorescence of the M. tuberculosis cultures was appreciably increased by high temperature and a basic pH (Fig. 4), perhaps due to spectral changes in F420 under these conditions (27).

The role of F420 in Mycobacterium species is poorly understood. M. tuberculosis encodes four glucose-6-phosphate dehydrogenases, two of which (fgd-1 and fgd-2) require F420 as a cofactor (8). The loss of F420 biosynthesis or the inability to reduce this cofactor by Fgd-1 results in resistance to nitroimidazoles (2,1-b) oxazine (PA-824), a drug that shows activity against the tubercle bacilli under both aerobic and anaerobic conditions (22, 34). In addition, it has been suggested that reduced F420 has a role in the low-redox-potential electron transfer reactions that would be associated with the survival of M. tuberculosis under anaerobic conditions (7).

When M. tuberculosis cells were observed under the fluorescence microscope, the autofluorescence decayed quickly, but micrographs could be made (Fig. 1 and 2). The decay of the autofluorescence may result from the photoreduction of F420 as was observed in methanogenic bacteria (10, 11, 12). However, fading of the autofluorescence was not detected when the emitted light was detected by spectroscopy, probably due to the lower-power lamp used in the fluorimeter.

Although the autofluorescence of mycobacteria is shared by Nocardia spp., it was not observed in other frequent respiratory pathogens, such as S. pneumoniae and H. influenzae. Bacteria in the genus Nocardia are closely related to mycobacteria; they also synthesize the coenzyme F420 (9), and like mycobacteria, they have a cell envelope with high lipid content, which also allows the cell to be stained by carbol fuchsin Ziehl-Neelsen. Thus, the application of autofluorescence to the identification of M. tuberculosis is likely to present the same limitations in specificity as microscopy of acid-fast bacilli.

Some chemical reagents, such as NaOH, NALC-NaOH, and NaOCl, are currently used to liquefy and decontaminate the sputum, which is further concentrated by sedimentation or centrifugation to increase the sensitivity of detection of the bacilli (33). Since sputum samples are the most common clinical specimens analyzed for the diagnosis of TB and because clarification is strictly required to handle sputa, we sought to examine the effects of these and other reagents on the mycobacterial autofluorescence in seeded sputa. We found that the fluorescence emission of the seeded sputa liquefied with NaOH or NALC-NaOH and treated with heat was considerably higher that that when any other decontaminating agent was used. We speculate that the basic pH of these liquefaction agents avoids the reduction of F420/FO and, together with heat treatment, increases the natural autofluorescence of M. tuberculosis in the seeded sputum samples.

Our preliminary results showed that under the assay conditions used, at least 7.5 x 10⁴ tubercle bacilli per ml of sputum could be detected by autofluorescence (Fig. 6). Hobby et al. (15) related the bacillus microscopy results to the mycobacterial concentrations in the sputum of patients with TB. They established that sputum samples with microscopy scores of 3+ have from 1.08 x 10⁶ to 4.02 x 10⁵ bacilli/ml⁻¹, while sputum samples with scores of 2+ have from 2.4 x 10⁴ to 1.41 x 10⁵ bacilli ml⁻¹. Hence, the results of this work suggest that the autofluorescence of M. tuberculosis could have a sensitivity similar to that of light microscopy examination, but with the advantage that it avoids the staining step in the procedure.

Fluorimetry in a 96-well plate format might become a user-friendly tool for the screening of sputum samples for M. tuberculosis, thus enabling the simultaneous examination of multiple samples. It has the added advantages that it employs a lower sample volume, is easy to perform by any laboratory technician, and is fast. In addition, the results are easily interpretable with simple training. All these characteristics make the use of cyan autofluorescence a potential method for the detection of mycobacteria, including M. tuberculosis.

In order to determine the applicability of the autofluorescence method in the field, the analysis of sputum samples from TB patients from diverse regions of Venezuela is in progress. The application of autofluorescence will improve the detection of tubercle bacilli.

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