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Article

Comparison of Two Rapid Colorimetric Methods for Determining Resistance of *Mycobacterium tuberculosis* to Rifampin, Isoniazid, and Streptomycin in Liquid Medium

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Abstract The usefulness of two colorimetric methods for the determination of the susceptibility or resistance of *Mycobacterium tuberculosis* to rifampin, streptomycin, and isoniazid in liquid medium based on the reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was investigated. The agar proportion method was used as the reference method. Results obtained indicate that the sensitivity of the XTT reduction assay for the detection of rifampin resistance was comparable to that observed, and previously described, for the MTT assay. However, the reduction of XTT yields a water-soluble formazan that can be easily quantified without performing additional steps such as addition of lysing buffer and solubilization. Furthermore, the colorimetric assays, based on the reduction of XTT and MTT for the detection of isoniazid and streptomycin resistance in *Mycobacterium tuberculosis*, were standardized. The inhibition of MTT and XTT reduction after treatment with rifampin, streptomycin, or isoniazid was directly proportional to the reduction in the number of viable bacteria, and a strain of *Mycobacterium tuberculosis* could be reported as susceptible or resistant to rifampin, streptomycin, or isoniazid after 3, 6, or 8 days, respectively. The XTT and MTT reduction assays are rapid, reliable, and affordable and do not require the use of radioisotopes. Moreover, they can be performed with common laboratory equipment.

Introduction

The resurgence of tuberculosis in recent decades is associated with the emergence of multidrug-resistant strains of *Mycobacterium tuberculosis*, both in HIV-infected [1] and immunocompetent populations. Primary and acquired drug resistance of *Mycobacterium tuberculosis* to antimicrobial agents may occur. Primary resistance to one or more antimycobacterial drugs was found to be as high as 9% in strains isolated from patients who were never previously treated [2] and is estimated to be around 40% in some developing countries [3]. Acquired resistance usually develops as a consequence of a patient's failure to complete an adequate course of chemotherapy [4] or because inappropriate regimens are prescribed by providers [5]. Therefore, rapid methods to determine susceptibility of isolates of *Mycobacterium tuberculosis* to antimicrobial agents are needed.

The proportion method, which employs semisynthetic solid media, requires a 3-week period of incubation before an isolate can be reported as susceptible or resistant. The Bactec radiometric susceptibility method produces susceptibility results in 7–10 days but requires heavy investment in equipment, is costly to perform, and requires the use of radioisotopes. The application of molecular methods for the identification and charac-

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terization of genes that confer resistance in *Mycobacterium tuberculosis* to primary antimycobacterial agents such as isoniazid [6, 7] or rifampin [8] is not always possible, especially in developing countries, because of the equipment required and the specialized skills needed to perform such methods. Furthermore, rapid methods to identify new and more potent antimycobacterial agents are also necessary.

Recently, promising alternative methods for determining drug resistance and MICs of antimicrobial agents for yeast, *Mycobacterium tuberculosis*, and *Mycobacterium avium* have been proposed. These methods utilize the oxidation-reduction colorimetric indicator Alamar blue (Alamar Biosciences, USA) [9, 10] or the reduction by metabolically active cells of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan [11, 12]. Furthermore, 2,3,5-triphenyltetrazolium chloride (TTC) has been proposed as an indicator of animal cell [13] and bacterial growth [14–16]. A new tetrazolium salt, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazoliumhydroxide (XTT), which, after reduction, yields a water-soluble formazan, has been recently proposed for use in a drug susceptibility assay for yeast [17–19]. As a consequence of its water-solubility, the XTT reduction assay can be easily quantified without performing additional steps such as centrifugation of cells, removal of medium, use of organic solvent, and sonication.

Until now, the XTT reduction assay was not studied for the evaluation of the growth of *Mycobacterium tuberculosis* in liquid medium. In this study we assessed the ability of the XTT assay to detect the viability of standard strains of *Mycobacterium tuberculosis* after exposure to rifampin, streptomycin, and isoniazid in comparison with the MTT and TTC assays. Furthermore, the MTT reduction assay for the detection of isoniazid and streptomycin resistance in *Mycobacterium tuberculosis* was standardized.

Materials and Methods

Reagents and Antimicrobial Agents. 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,3,5-triphenyltetrazolium chloride (TTC) were obtained from Sigma Chemical, USA. XTT was dissolved in phosphate-buffered saline at a concentration of 1.111 mg/ml at room temperature. The solution was filtered through a 0.22 μm filter (Millipore, USA), aliquoted, and stored at –70 °C until needed. MTT and TTC were dissolved in phosphate-buffered saline at 5 mg/ml. Solutions were then sterilized by filtration and stored at 4 °C in dark vials until use. Phenazine methyl sulfate (PMS; Sigma), an electron-coupling agent necessary for reduction of XTT [20], was prepared as a stock solution of 1 mg/ml, filter sterilized, and kept at 4 °C until use. Lysing buffer was obtained by dissolving 20% (w/v) sodium dodecyl sulfate in 50% N_1N-dimethylformamide [21]. The pH was then adjusted at 4.7. Isonicotinic acid hydrazide (isoniazid) and rifampin (Sigma) were dissolved in dimethylsulfoxide at a concentration of 10 mg/ml. To avoid interference by the solvent [22], the maximum concentration of dimethylsulfoxide employed in the experiments was 0.05%. Streptomycin sulfate (Sigma) was dissolved in H_2O at a concentration of 4 mg/ml as streptomycin, filter sterilized, and stored at –20 °C until use.

Bacterial Strains and Cultures. Mycobacterium tuberculosis H37Rv (ATCC 27294), rifampin-resistant *Mycobacterium tuberculosis* (ATCC 35838), isoniazid-resistant *Mycobacterium tuberculosis* (ATCC 35822), and streptomycin-resistant *Mycobacterium tuberculosis* (ATCC 35820) were included in this study. They were grown on slants of Löwenstein-Jensen medium (bioMérieux, France) by incubation at 37 °C for 3–4 weeks. Culture suspensions were prepared by subculturing the mycobacteria in Middlebrook 7H9 broth (Difco Laboratories, USA) supplemented with 10% OADC (oleic acid-albumin-dextrose-catalase) enrichment (Difco) and 0.05% Tween 80. Suspensions were shaken and briefly sonicated for 10–15 s [23] in a bath-type ultrasonicator (output power 80 W) and then diluted in 7H9 broth to 10^7 bacteria/ml using the McFarland standard. Suspensions were incubated in an incubator shaker for 48–72 h at 37 °C and then further diluted in the same medium to obtain final concentrations of mycobacteria of 1×10^7 and 1×10^5 cells/ml in the susceptibility test tubes.

Since inhalation of aerosols is the most likely route of infection by *Mycobacterium tuberculosis*, great care must be used to minimize aerosol production; all manipulations of cultures should take place inside an exhaust protective cabinet that has been properly installed and tested. The cultures of *Mycobacterium tuberculosis*, which fall into hazard group 3 in the report of the Advisory Committee on Dangerous Pathogens, were manipulated in a class 3 safety cabinet, and procedures to prevent laboratory-acquired infection were followed according to Fallon and Pether [24].

Determination of Mycobacterium tuberculosis Growth, and Standardization of Inoculum Size. The analytical sensitivity of the XTT, MTT, and TTC tests in assessing the growth of *Mycobacterium tuberculosis* was evaluated. Flasks containing 50 ml of 7H9 broth were inoculated with 1×10^5 or 1×10^7 bacteria/ml of *Mycobacterium tuberculosis* H37Rv ATCC 27294, obtained as described above and incubated at 37 °C in an incubator-shaker for 2–30 days. After each time point, 40 μl of the bacterial suspension was inoculated into each well of a flat-bottomed microtiter plate (Becton Dickinson, USA) and processed for XTT, MTT, and TTC assays as described below. In order to determine the relationship between the XTT, MTT, and TTC reduction and the amount of live bacilli, colony-forming units (cfu) were counted after each time point. Seven serial tenfold dilutions were made by transferring 0.5 ml of each bacterial suspension to 4.5 ml of 7H9 broth, and 10 μl of each dilution was plated onto 7H10 agar. After 21–25 days of incubation at 37 °C, colonies were counted and numbers of cfu per milliliter were calculated. The growth of the bacteria was also determined by measuring the optical density (OD) of the suspensions by a spectrophotometer (Pharmacia Biotech, UK) at a wavelength of 620 nm.

2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) Reduction Assay. Forty microliters of bacterial suspension was transferred to each well of a flat-bottomed 96-well plate; to each well was added 10 μl of a solution obtained by combining 100 μl of the stock solution of PMS 1 mg/ml with 900 μl of the stock solution of XTT 1.111 mg/ml (final concentrations: XTT, 200 μg/ml; PMS 20 μg/ml). Quadruplicate wells were used for each of the experimental conditions. Plates were incubated for 3 h at 37 °C, and adsorption at 490 nm was determined with a 96-well plate reader (Reader 510; Organon Teknika, Belgium) [19]. Wells containing 7H9-OADC and XTT-PMS mixture served as controls.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,3,5-triphenyltetrazolium chloride (TTC) Reduction

Assays. Forty microliters of bacterial suspension was transferred to each well of a flat-bottomed 96-well microtiter plate. Ten microliters of the MTT 5 mg/ml or TTC 5 mg/ml solutions was then added to each well (final concentration 1 mg/ml) and plates were incubated at 37 °C for 4 h. Fifty microliters of the lysing buffer was then added to each well. Plates were further incubated overnight at 37 °C, and absorbance was measured with an automatic enzyme-linked immunosorbent assay reader at wavelengths of 570 nm for the MTT reduction assay and 490 nm for the TTC reduction assay. Wells containing 7H9-OADC, MTT, or TTC and lysing buffer served as controls. Quadruplicate wells were used for each of the experimental conditions.

Determination of Rifampin Resistance. Tubes containing rifampin 2 μg/ml in 5 ml of 7H9 broth supplemented with 10% OADC and drug-free control tubes containing 5 ml of 7H9 broth were inoculated with the same volume of a suspension of *Mycobacterium tuberculosis* H37Rv ATCC 27294 or rifampin-resistant *Mycobacterium tuberculosis* ATCC 35838 obtained as described above. The final concentration of rifampin in the test tubes was 1 μg/ml, and the final concentration of mycobacteria was 1×10^7 cfu/ml. To establish the reliability of the method in determining the resistance to rifampin, tubes containing rifampin 1 μg/ml and 1×10^7 cfu/ml of isoniazid-resistant *Mycobacterium tuberculosis* ATCC 35822 or streptomycin-resistant *Mycobacterium tuberculosis* ATCC 35820 were also employed.

Determination of Streptomycin Resistance. Tubes containing streptomycin 4 μg/ml in 5 ml of 7H9 broth supplemented with 10% OADC and control tubes containing 5 ml of 7H9 broth were inoculated with the same volume of a suspension of *Mycobacterium tuberculosis* H37Rv ATCC 27294 or streptomycin-resistant *Mycobacterium tuberculosis* ATCC 35820 obtained as described above. The final concentration of streptomycin in the test tubes was 2 μg/ml, and the final concentration of mycobacteria was 1×10^7 cfu/ml. To establish the reliability of the method in determining the resistance to streptomycin, tubes containing streptomycin 2 μg/ml and 1×10^7 cfu/ml of isoniazid-resistant *Mycobacterium tuberculosis* ATCC 35822 or rifampin-resistant *Mycobacterium tuberculosis* ATCC 35838 were also employed.

Determination of Isoniazid Resistance. Tubes containing 5 ml of 7H9 broth with isoniazid 2 μg/ml supplemented with 10% OADC and drug-free control tubes containing 5 ml of 7H9 broth were inoculated with the same volume of a suspension of *Mycobacterium tuberculosis* H37Rv ATCC 27294 or isoniazid-resistant *Mycobacterium tuberculosis* ATCC 35822 obtained as described above. The final concentration of isoniazid in the test tubes was 1 μg/ml, and the final concentration of mycobacteria was 1×10^7 or 1×10^5 cfu/ml To establish the specificity of the method in determining the resistance to isoniazid, tubes containing isoniazid 1 μg/ml and 1×10^7 or 1×10^5 cfu/ml of rifampin-resistant *Mycobacterium tuberculosis* ATCC 35838 or streptomycin-resistant *Mycobacterium tuberculosis* ATCC 35820 were also employed.

Tubes were incubated at 37 °C in an incubator-shaker for 2–12 days. After each time point, 40 μl aliquots from drug-containing suspensions and from control tubes were distributed in wells on microtiter plates and processed as described for the XTT and the MTT reduction assays. Colony-forming units were counted at each time point to confirm the correlation between the reduction of XTT and MTT and the number of viable cells.

Presentation of Data. Spectrophotometric readings of the XTT, MTT, and TTC assays are presented as relative optical density units (RODU) derived by dividing the optical density (OD) of drug-containing cultures by the OD of untreated cultures [12]. The susceptibility or resistance was reported according to Abbate et al. [25], with some modifications. A strain was defined as susceptible to the drug when the RODU, determined after a suitable period of incubation, was ≤ 0.2 and the OD, after the same time, was lower than the OD value on the second day. A strain was reported as resistant when the RODU, determined as described above, was >0.5 and the OD, after the same time, was equal to or higher than the OD value on the second day. The period of incubation after which a strain can be reported as susceptible or resistant to each of the several drugs tested was determined.

Results

Establishment of the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 2,3,5-triphenyltetrazolium chloride (TTC) Assays. To establish the range of bacterial concentrations in which the reduction of XTT, MTT, and TTC is proportional to the number of viable bacteria, 1×10^5 or 1×10^7 cells/ml of *Mycobacterium tuberculosis* H37Rv ATCC 27294 were grown in 7H9 broth supplemented with 10% OADC for 2–30 days at 37 °C. After each time point, the suspension was processed for the XTT, MTT, and TTC assays and colony-forming units were counted as described above. Figure 1 indicates that there was a correlation between the reduction of tetrazolium salts and the number of viable cells. However, absorbance of formazan obtained from the reduction of TTC was much lower than the absorbance obtained when XTT or MTT was used. The relationship between absorbance of formazan obtained from the reduction of XTT or MTT and the number of viable cells was observed for bacterial concentrations that ranged between 1×10^6 and 5×10^9 cells/ml, and the relationship was stronger for concentrations between 2×10^6 and 1×10^9 cells/ml. Figure 1 also shows that the values of absorbance of formazan dye resulting from the reduction of MTT and XTT were

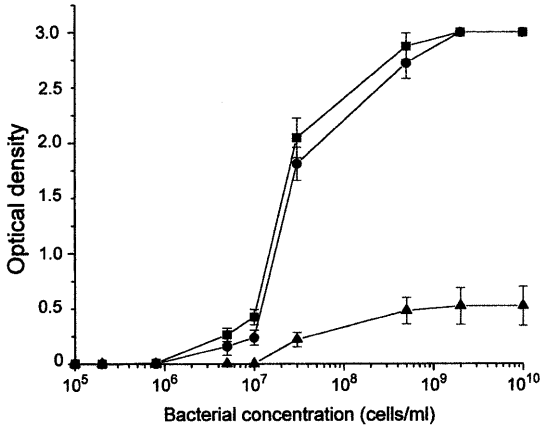

Figure 1 Reductions of XTT (●) and MTT (■) by *Mycobacterium tuberculosis* H37Rv ATCC 27294 are comparable. The absorbance of formazan dye demonstrates an excellent correlation between the intensity of the colorimetric reactions and the number of viable cells for bacterial concentrations ranging between 2×10^6 and 1×10^9 cells/ml. Reduction of TTC (▲) gives signals too weak to be useful in drug assays. The data represent the means for four replicates of three separate experiments

comparable. Employing inocula of 1×10^7 cells/ml of *Mycobacterium tuberculosis*, the colorimetric reactions were positive after 2 days of incubation for both MTT and XTT.

Effect of Rifampin on 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Reduction by Mycobacterium tuberculosis H37Rv and Rifampin-Resistant Mycobacterium tuberculosis. Figure 2 shows that the rifampin-sensitive strain of *Mycobacterium tuberculosis* H37Rv showed almost complete inhibition of the ability to reduce XTT and MTT after 3 days of incubation when rifampin was used at a concentration of 1 μg/ml (<0.2 RODU). At this drug concentration, the RODU values obtained with the rifampin-resistant strain were about 1, even after 12 days of incubation. The RODU values obtained with the streptomycin- or isoniazid-resistant strains of *Mycobacterium tuberculosis* and *Mycobacterium tuberculosis* H37Rv were comparable (data not shown).

Effect of Streptomycin on 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Reduction by Mycobacterium tuberculosis H37Rv and Streptomycin-Resistant Mycobacterium tuberculosis. Inhibition of reduction of XTT and MTT by *Mycobacterium tuberculosis* H37Rv was observed after 3 and 4 days of incubation (0.25–0.35 RODU for both XTT and MTT) when streptomycin 2 μg/l was employed, but a value of <0.2 RODU was detected only after 6 days of incubation. However, when streptomycin-resistant *Mycobacterium tuberculosis* ATCC 35820 was employed, the RODU values were between 0.9 and 1, even after 12 days of incubation (Figure 3). No differences were observed on the reduction of XTT and MTT by *Mycobacterium tuberculosis* H37Rv or by the isoniazid- or streptomycin-resistant strains in the presence of streptomycin 2 μg/ml (data not shown).

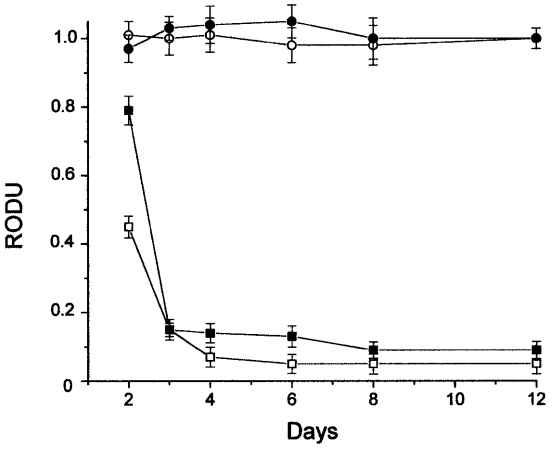

Figure 2 Reduction of XTT by *Mycobacterium tuberculosis* H37Rv ATCC 27294 (■) and rifampin-resistant *Mycobacterium tuberculosis* ATCC 35838 (●) in the presence of rifampin (1 μg/ml). After 3 days of incubation RODU values of <0.2 and 1 were determined for rifampin-sensitive and rifampin-resistant *Mycobacterium tuberculosis*, respectively. The RODU values obtained by reduction of MTT by *Mycobacterium tuberculosis* H37Rv ATCC 27294 (□) and rifampin-resistant *Mycobacterium tuberculosis* ATCC 35838 (○) are comparable. The data represent the means for four replicates of four separate experiments

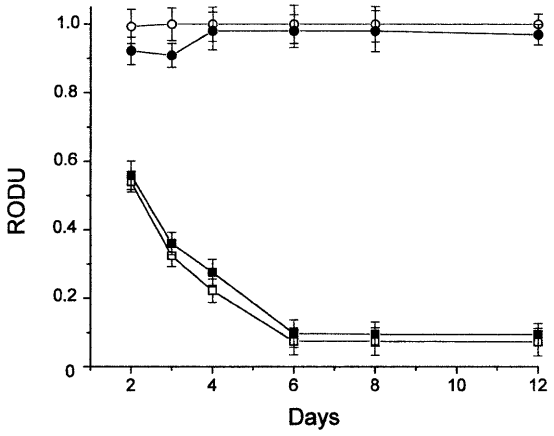

Figure 3 Reduction of XTT by *Mycobacterium tuberculosis* H37Rv ATCC 27294 (■) and streptomycin-resistant *Mycobacterium tuberculosis* ATCC 35820 (●) in the presence of streptomycin (2 μg/ml). Six days of incubation are needed to obtain RODU values of <0.2 and 1 for streptomycin-sensitive and streptomycin-resistant *Mycobacterium tuberculosis*, respectively. The RODU values obtained by reduction of MTT by *Mycobacterium tuberculosis* H37Rv ATCC 27294 (□) and streptomycin-resistant *Mycobacterium tuberculosis* ATCC 35820 (○) are comparable. The data represent the means for four replicates of four separate experiments

Effect of Isoniazid on 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Reduction by Mycobacterium tuberculosis H37Rv and Isoniazid-Resistant Mycobacterium tuberculosis ATCC 35822. Figure 4 shows the RODU values obtained after 12 days of incubation of 1×10^7 cells/ml of *Mycobacterium tuberculosis* H37Rv and isoniazid-resistant *Mycobacterium tuberculosis* in the presence of isoniazid 1 μg/ml. Isoniazid-resistant *Mycobacterium tuberculosis* reduced both XTT and MTT (0.9–1 RODU), and the inhibition of reduction by *Mycobacterium tuberculosis* H37Rv was observed after 4 days of incubation (0.7 and 0.6 RODU employing XTT and MTT, respectively). However, RODU values lower than 0.2, which, according to Mshana [12], indicate a strain's susceptibility, were not obtained even after 12 days of incubation. When inocula were lowered to 1×10^5 cells/ml and *Mycobacterium tuberculosis* H37Rv and isoniazid-resistant *Mycobacterium tuberculosis* were incubated in the presence of isoniazid 1 μg/ml, values of <0.2 RODU and 0.9–1 RODU, respectively, were determined after 8 days (Figure 5). The

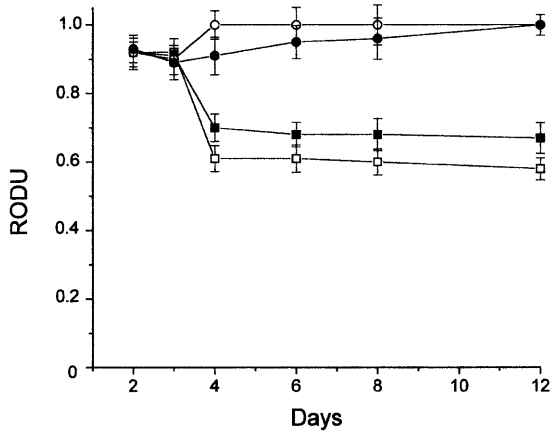

Figure 4 Reduction of XTT by *Mycobacterium tuberculosis* H37Rv ATCC 27294 (■) and isoniazid-resistant *Mycobacterium tuberculosis* ATCC 35822 (●) in the presence of isoniazid (1 μg/ml) employing an inoculum of 1×10^7 cells/ml. RODU values of 0.7 and 1 were determined after 8 days for isoniazid-resistant *Mycobacterium tuberculosis* ATCC 35822 and *Mycobacterium tuberculosis* H37Rv ATCC 27294, respectively, and isoniazid-resistance was not detectable on the basis of RODUs, even though an inhibition of reduction of XTT by the standard isoniazid-sensitive strain is clearly demonstrated. The RODU values obtained by reduction of MTT by *Mycobacterium tuberculosis* H37Rv ATCC 27294 (□) and isoniazid-resistant *Mycobacterium tuberculosis* ATCC 35822 (○) are comparable. The data represent the means for four replicates of four separate experiments

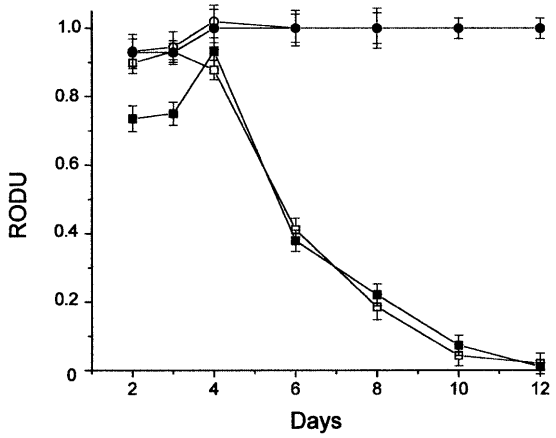

Figure 5 Reduction of XTT by *Mycobacterium tuberculosis* H37Rv ATCC 27294 (■) and isoniazid-resistant *Mycobacterium tuberculosis* ATCC 35822 (●) in the presence of isoniazid (1 μg/ml) employing inocula of 1×10^5 cells/ml. An RODU of < 0.2 was determined after 8 days for the standard isoniazid-sensitive strain *Mycobacterium tuberculosis* H37Rv ATCC 27294, while the reduction of XTT by isoniazid-resistant *Mycobacterium tuberculosis* ATCC 35822 was not observed, even after 12 days of incubation (RODU = 1). The RODU values obtained by reduction of MTT by *Mycobacterium tuberculosis* H37Rv ATCC 27294 (□) and isoniazid-resistant *Mycobacterium tuberculosis* ATCC 35822 (○) are comparable with the RODU obtained by the reduction of XTT. The data represent the means for four replicates of five separate experiments

inhibition of reduction of XTT and MTT by rifampin- and streptomycin-resistant *Mycobacterium tuberculosis* after 8 days of incubation in the presence of isoniazid 1 μg/ml was comparable to the inhibition shown by *Mycobacterium tuberculosis* H37Rv (data not shown).

Discussion

Several colorimetric methods have been proposed as rapid methods for detection of resistance to antimycobacterial drugs in isolates of *Mycobacterium tuberculosis*. The MTT reduction assay proposed, for the detection of rifampin-resistant *Mycobacterium tuberculosis* [12] and for determining MICs for *Mycobacterium avium-Mycobacterium intracellulare* complex [11], is particularly interesting. However, until now the MTT assay was not standardized as a method to detect the resistance of *Mycobacterium tuberculosis* to other antimycobacterial drugs such as isoniazid and streptomycin. The use of XTT, a newer tetrazolium salt, has been proposed for assessment of fungal cell damage [19] and for antifungal susceptibility testing of yeast isolates [17, 10], but it was never studied as a method for determining the growth of *Mycobacterium tuberculosis*. We found that the range of bacterial concentrations at which the reduction of tetrazolium salt is proportional to the number of viable cells is comparable to those obtained with the MTT and XTT assays. In particular, the linear relationship between the absorbance of the resultant formazan dye and the bacterial concentration was stronger when the concentration of *Mycobacterium tuberculosis* ranged between 2×10^6 and 1×10^9 cells/ml. These results are consistent with the findings of other investigators [12].

The sensitivity of the TTC reduction assay in determining the growth of *Mycobacterium tuberculosis* was not comparable with that of the MTT and XTT assays, indicating that TTC is not suitable for evaluating drug resistance. Even though the main disadvantage of the test is that it cannot be used to determine the proportion of resistant mutants, a calculation for which other methods can be employed, results obtained in this study clearly indicate that the reduction of tetrazolium salts can be used as a rapid and inexpensive method for assessing the susceptibility of *Mycobacterium tuberculosis* not only to rifampin but also to streptomycin and isoniazid. However, different periods of incubation and different inocula for the several antimycobacterial drugs are required to report a strain as sensitive or resistant. In fact, we found that 3 days and 6 days for rifampin and streptomycin, respectively, are required with an inoculum of 1×10^7 cells/ml of *Mycobacterium tuberculosis*, but 10 days are needed in the case of isoniazid. These differences are dependent on the inoculum size employed in the assays and on the mechanism of action of different drugs. For instance, rifampin and streptomycin allow the use of a relatively high inoculum (1×10^7 cells/ml), and this fact bears to deter-

mine the resistance after 3 and 6 days of incubation. In the case of isoniazid, the use of inocula as high as 1×10^7 cells/ml will still not enable susceptibility or resistance to be determined because the formazan production is high despite the inhibition of growth of *Mycobacterium tuberculosis* H37Rv, as shown by the calculation of cfu/ml. This might be attributable to the mechanism of action of isoniazid, which requires longer exposure time.

Difficulties in determining the resistance to isoniazid by colorimetric reduction assay were also encountered by other investigators, who therefore proposed the assessment of catalase activity as a rapid method for the identification of multidrug-resistant strains of *Mycobacterium tuberculosis* in conjunction with the MTT reduction method for determining resistance to isoniazid and rifampin, respectively [12]. We found that, by employing lower inocula of 1×10^5 cells/ml, the determination of susceptibility or resistance is achievable with both XTT and MTT assays, but a longer period of incubation is required to allow mycobacteria to reach a concentration at which the colorimetric reactions are positive.

Further studies with a substantial number of clinical isolates are needed to confirm the applicability of the colorimetric method for determining clinical susceptibility or resistance to antituberculous drugs. However, this report clearly demonstrates that results obtained with the XTT reduction assay are comparable with those obtained with the MTT assay. Furthermore, it must be emphasized that the reduction of MTT yields a water-insoluble formazan that requires additional steps, such as addition of extraction buffer and further incubation overnight, to be quantified by measurement of OD. Reduction of XTT yields a water-soluble formazan product and eliminates the need for a solubilization step before spectrophotometric analysis.

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