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REDUCTION OF TELLURITE AND DEESTERIFICATION OF FLUORESCEIN DIACETATE ARE NOT WELL CORRELATED WITH THE VIABILITY OF MYCOBACTERIA

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SUMMARY

Both *Mycobacterium leprae* and *M. lepraemurium* (MLM) were capable of reducing tellurium as tellurite ion (Te^{4+}) to elemental tellurium (Te), seen by electron microscopy as fine crystals within the bacterial cells. There appeared to be close correspondence between the capacity to reduce tellurite, bright green fluorescence after staining with fluorescein diacetate (FDA) and the ability of *M. smegmatis* to multiply in culture. Likewise, there appeared to be correspondence between tellurite reduction and fluorescence after FDA staining for MLM subjected to prolonged storage in the cold or to heating at 70°C. However, correspondence with tellurite-reduction or fluorescence after FDA staining was not observed when death of MLM occurred *in vivo*.

KEY-WORDS: *Mycobacterium leprae, Mycobacterium lepraemurium, Mycobacterium smegmatis,* Tellurite, Fluorescein, Viability.

INTRODUCTION

Following the recommendations of the World Health Organization Study Group on Chemotherapy of Leprosy for Control Programmes [**15**], which called for the employment of multi-drug regimens and pointed out the need

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for new drugs active against *Mycobacterium leprae*, the search for simple and rapid systems for drug-screening and for distinguishing between viable and non-viable *M. leprae* has become more urgent. The possibility that reduction of potassium tellurite might represent one such system was suggested by the demonstration by David *et al.* [1] that *M. leprae* and other *Mycobacteria* were capable of reducing Te^{4+}. Another system possibly suitable for drug-screening which has been reported to be capable of distinguishing between viable and non-viable *Mycobacteria* involves differential staining with fluorescein diacetate (FDA) and ethidium bromide (EB) [3-5, 11, 12].

Potassium tellurite, K_2TeO_3, has been used for many years as a selective inhibitor for isolation of *Corynebacteria*. Those organisms capable of multiplication on agar in the presence of TeO_3^{2-} and of reducing Te^{4+} to metallic Te produce gray-black colonies. Reduction of Te^{4+} is an active process which depends upon the presence in the organisms of intact reductase enzymes [10]; *Bacillus subtilis* has been shown to produce needle-like crystals of Te at sites of respiratory enzyme activity such as the cytoplasmic membrane [13].

Differential staining of *Mycobacteria* by FDA-EB depends upon deesterification of the non-fluorescent FDA by bacterial esterases to yield fluorescein, which emits a bright green fluorescence during excitation by blue light (λ 410-485 nm). The fact that fluorescein is a polar molecule, whereas the ester is non-polar, enhances the selectivity of the staining; FDA readily diffuses into the bacterial cell, whereas fluorescein diffuses only slowly out of cells possessing intact cell membranes. EB, which is permanently ionized, is excluded by viable cells, whereas it diffuses through the cell membranes of damaged or dead bacteria, binding to double-stranded nucleic acids to produce an orange fluorescence.

Thus, both the reduction of tellurite and the deesterification of FDA are active, enzymatically catalysed processes which are carried out by viable *Mycobacteria*, and may not be carried out by *Mycobacteria* incapable of multiplication. However, correspondence between viability, ability to reduce Te^{4+} and bright green fluorescence after staining with FDA-EB remains to be demonstrated.

MATERIALS AND METHODS

M. smegmatis was grown in Dubos broth supplemented with bovine serum albumin (BSA). To determine the proportions of viable *M. smegmatis*, serially diluted suspensions were inoculated on plates of brain-heart infusion agar. *M. leprae* of the strain

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BSA = bovine serum albumin.
CFU = colony-forming unit.
EB = ethidium bromide.
FDA = fluorescein diacetate.
MLM = *Mycobacterium lepraemurium*.
MPN = most probable number.
PBS = phosphate-buffered saline.

employed in this laboratory for many years, was harvested from the footpads and *M. lepraemurium* (MLM) of the Douglas strain from the spleens of infected CBA/Ca mice. To determine the proportion of viable MLM, CBA mice were inoculated in the hind footpads with serially diluted suspensions of the organisms and 4 months later, harvests were performed, the organisms counted [9] and the most probable number of viable organisms (MPN) calculated [2].

Incubation with K_2TeO_3 was carried out for 24 h in a medium consisting of 5 mM glucose in distilled water, to which the tellurite had been added at a final concentration of 0.2 % (w/v); the pH of this medium was 7.2. Suspensions of *M. leprae* were incubated at 33°C and those of the other organisms at 37°C. After incubation in the tellurite-containing medium, 5-µl portions of the bacterial suspensions were layered on collodion- and carbon-coated grids; after 1 min, the excess suspension was blotted with filter paper and the grids were left to dry at room temperature. In the case of *M. leprae,* grids were fixed in formalin vapour for 5 min. Without any further treatment, the grids were examined in a «Phillips 300» transmission electron microscope at 60 kV; at least 200 individual organisms were examined for the presence of intracellular Te crystals. Smears of the same organisms prepared on Reich counting slides were stained by exposure to a 0.25 mg % (w/v) solution of FDA in phosphate-buffered saline (PBS) for 30 min, and then to a 0.4 mg % (w/v) solution of EB in PBS for 1 min [4], after which at least 200 organisms were examined by means of an epifluorescence microscope, employing «Nikon» excitation filter IF 410-485, and scored according to the colour and intensity of their fluorescence. Those organisms emitting a bright green fluorescence were considered to possess esterase activity, whereas those emitting an orange fluorescence lacked this activity.

RESULTS

M. leprae were found to be capable of reducing Te^{4+}; in two experiments, 11 and 12 % of the organisms were found to contain crystals of Te. In other experiments, MLM were also shown to be capable of reducing Te^{4+}. Typical electron micrographs of *M. leprae* and MLM containing Te are shown in figures 1 and 2; Te appears as fine crystals within the organisms. Rarely were more than one or two crystals found in a single organism.

To demonstrate that reduction of Te^{4+} did not result from the activity of host enzymes coating the organisms, MLM that had been killed by immersion in water at 70°C for 30 min were inoculated intravenously into mice and recovered two weeks later by harvest from the spleens; the harvested organisms did not reduce Te^{4+}. In a second experiment, MLM that had been incubated at 37°C for 8 weeks, after which time no organism (among 200 examined) was observed to reduce Te^{4+}, were inoculated intraperitoneally into mice and recovered two weeks later by harvest from the spleens; in this experiment also, the harvested MLM did not reduce Te^{4+}. Thus, it appeared that the tellurite-reductase activity of *M. leprae* and MLM is a property of the host-grown organisms themselves, and does not result from coating of the organisms with host enzyme.

A number of experiments were carried out with *M. smegmatis* and MLM, in which a correlation was sought between tellurite-reductase activity, staining by FDA-EB and the ability of the organisms to multiply — *in vitro* in the

FIG. 1. — *Electron micrograph of* M. leprae *harvested from mouse footpad, incubated with tellurite and subsequently placed on a collodion- and carbon-coated grid.*

Two needle-like Te crystals (×49,000) are seen.

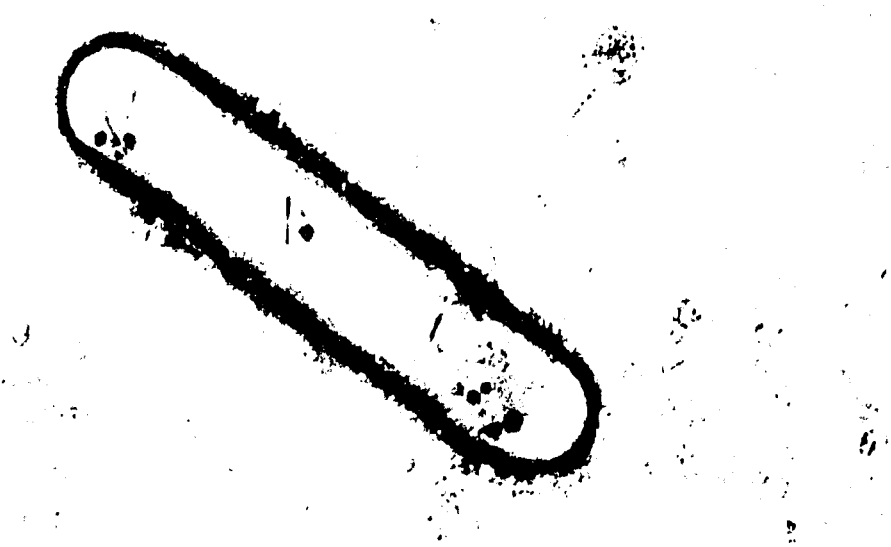

FIG. 2. — *Electron micrograph of MLM harvested from a mouse spleen, incubated with tellurite and placed on a grid.*

Three Te crystals (×25,500) are seen.

TABLE I. — **Effect of exposure to NaOH on *M. smegmatis*.**

Duration of exposure to NaOH (min)	Proportion of viable organisms as CFU	Proportion of organisms containing Te crystals	Proportion of organisms yielding bright green fluorescence
0	0.51	0.66	0.14
7	< 0.0001	< 0.01	0.006
15	< 0.0001	< 0.01	< 0.003
30	< 0.0001	< 0.01	0.007
60	< 0.0001	< 0.01	< 0.006

case of *M. smegmatis* and *in vivo* in the case of MLM. As shown in table I, treatment of *M. smegmatis* with NaOH for as little as 7 min both abolished staining with FDA and reduction of Te^{4+} and rendered the organisms incapable of multiplying in culture.

In a second experiment, portions of suspensions of *M. smegmatis* and MLM were immersed in a 70°C water bath for different durations. After heating, portions of the suspensions were incubated with tellurite; in addition, portions of the suspension of *M. smegmatis* were diluted and stained with FDA-EB or inoculated onto plates of brain-heart infusion agar which were then incubated at 37°C. As shown in table II, good correlation was found between reduction of Te^{4+}, fluorescence after staining with FDA-EB and multiplication of *M. smegmatis in vitro,* all of which decreased progressively with increasing duration of exposure to heat. The proportion of MLM containing crystals of Te decreased similarly.

In another experiment, a suspension of MLM was stored at 4°C and, at intervals, a portion of the suspension was taken for study of Te^{4+}-reduction

TABLE II. — **Effect of exposure to heat on *M. smegmatis* and MLM.**

Duration of exposure to heat (min)	Proportion of viable *M. smegmatis* as CFU	Proportion of *M. smegmatis* containing Te crystals	Proportion of *M. smegmatis* yielding green fluorescence	Proportion of MLM containing Te crystals
0	0.83	0.80	0.72	0.67
2	0.67	0.52	0.54	0.51
4	0.031	0.046	0.063	0.22
8	0.0006	< 0.01	< 0.005	0.027
16	< 0.0001	< 0.01	< 0.01	< 0.01

TABLE III. — **Effect of storage at 4°C on enzyme activities of MLM.**

Duration of storage (days)	Proportion of organisms containing Te crystals	Proportion of organisms yielding green fluorescence
0	0.61	0.97
7	0.53	0.80
14	0.46	0.71
28	0.37	0.66

and fluorescence after incubation with FDA-EB. As shown in table III, the proportion of organisms found to contain crystals of Te decreased during the course of the experiment, as did the proportion of organisms demonstrating bright green fluorescence after incubation with FDA-EB. Thus, treatment of *M. smegmatis* and MLM with heat or NaOH *in vitro*, or prolonged storage of the organisms at 4°C, appeared to reduce in parallel the ability of the organisms to reduce Te^{4+} and to yield bright green fluorescence after staining with FDA-EB and, with respect to *M. smegmatis*, the viability of the organisms.

On the other hand, experiments with MLM, in which death of the organisms occurred *in vivo* and in which, of necessity, the proportion of viable organisms was measured *in vivo*, showed that organisms that were incapable of multiplication nevertheless appeared to be capable of reducing Te^{4+} and hydrolysing FDA (see table IV). Finally, as shown in table V, in an experiment in which

TABLE IV. — **Reduction of Te^{4+} by MLM *in vitro*.**

Experiment number	Proportion of organisms containing Te crystals	Proportion of viable organisms (*)
1	0.36	0.48
2	0.23	0.48
3	0.21	0.26
4	0.31	0.18
5	0.20	0.062
6	0.12	0.028
7	0.19	0.028
8	0.17	0.021
9	0.38	0.0026
10	0.32	0.0025

(*) Determined from the MPN.

TABLE V. — **Fluorescence of MLM after staining with FDA-EB *in vitro*.**

Time from inoculation (days)	Nb of MLM per spleen ($\times 10^8$)	Proportion of viable organisms (*)	Proportion of organisms yielding green fluorescence
70	0.50	1.0	0.88
90	2.0	0.19	0.51
128	170	1.0	0.10
158	4,600	0.008	0.62
196	3,400	0.004	0.91

(*) Determined from the MPN.

MLM were harvested at intervals from the spleens of mice that had been inoculated intraperitoneally with 10^7 organisms 70 to 196 days earlier [7], although the proportions of brightly fluorescing MLM and those determined to be capable of multiplying in the mouse footpad appeared to decrease in a roughly parallel fashion, there was not close correspondence between esterase activity and viability of the organisms.

DISCUSSION

The studies reported herein confirm the demonstration by David *et al.* [1] of the presence of Te crystals in *M. leprae* after incubation with K_2TeO_3. It is interesting to compare the finding by David and his coworkers of Te crystals in only 0.1 % of the organisms examined with our demonstration of crystals in 10 and 11 % of two suspensions of *M. leprae*. David *et al.* employed *M. leprae* derived from liver tissues of infected armadillos; although the tissues had not been irradiated, the organisms had been frozen at −20°C and treated with proteolytic enzymes, both procedures which may impair the viability of *M. leprae*. On the other hand, our studies employed suspensions of the organisms freshly harvested during the logarithmic phase from the footpads of infected mice; such suspensions might be expected to contain much larger proportions of viable organisms [14]. In addition, because David *et al.* [1] examined ultra-thin sections of the organisms, they «saw» a much smaller volume of organisms than we did in the course of our examination of whole organisms layered on grids. Finally, our incubation medium contained 5 mM glucose, which may well have served as a source of energy, permitting greater tellurite-reductase activity.

Our additional studies involving MLM and *M. smegmatis* reveal an unclear picture of the relationship between the presence of tellurite reductase and esterase activities and viability of the organisms. During heating at 70°C, the proportion of *M. smegmatis* containing Te crystals and that exhibiting bright

green fluorescence after staining with FDA-EB decreased parallel to the proportion of viable organisms; the proportion of MLM containing crystals decreased in a similar fashion, suggesting some degree of correspondence between viability and tellurite reduction. On the other hand, Te crystals were found in 12 to 38 % of MLM, both in those harvested from the spleens of mice early after systemic infection when the great majority of the organisms might be expected to be viable, and in those harvested late, when the majority of the MLM have been shown to be dead [7, 8]. Similarly, in suspensions of MLM in which virtually all of the organisms were found to be viable, from 10 to 88 % of the organisms demonstrated bright green fluorescence. And, in a number of suspensions containing fewer than 10 % viable MLM, the per cent of organisms exhibiting bright green fluorescence ranged from 1.5 to 98. Thus, at least in the case of death of MLM *in vivo,* the presence of tellurite-reductase and esterase activities did not appear to correspond to the ability of the organisms to multiply *in vivo;* this suggests that tellurite-reductase and esterase activities decay at rates different from those of other enzymes upon which the ability to multiply depends.

Tsukiyama *et al.* [12] have recently reported correlation between the proportion of MLM found to emit green fluorescence after exposure to heat for various periods of time and the viable proportion, as measured by the time required for the appearance of a grossly evident subcutaneous lesion in half of the mice inoculated with a standard number of organisms. The results of these workers also suggest a difference in enzyme activity between MLM which were killed by exposure to heat and those which died *in vivo.*

Seydel and his colleagues [6] have demonstrated synthesis of folate by extracts of *M. leprae* which had been recovered from frozen armadillo tissues, subjected to 2.5 Mrad of gamma irradiation and lyophilized. The preservation of certain enzymatic activities after the organisms have lost their ability to multiply has obvious implications for the employment of tellurite-reductase or any other enzyme activity as a means of screening drugs for antimycobacterial activity. Organisms which have been rendered incapable of multiplication *in vivo* as a result of drug action may nevertheless exhibit certain enzyme activities at the same time as the activities of other enzymes are lost. Moreover, the sensitivity of various enzyme activities may vary from drug to drug. Thus, there may not exist a single *in vitro* screen suitable for drugs active against *M. leprae.* Rather, it may be necessary to employ a battery of such screening methods and to establish the suitability of each technique as a screen for the activity of each class of drug.

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RÉSUMÉ

ÉVALUATION DE LA RÉDUCTION DU TELLURITE ET DE LA COLORATION PAR LE DIACÉTATE DE FLUORESCÉINE COMME TEST RAPIDE DE VIABILITÉ DES MYCOBACTÉRIES

Mycobacterium leprae, M. lepraemurium, et *M. smegmatis* sont capables de réduire le tellurite de potassium, ce qui résulte en un dépôt cristallin à l'intérieur de la cellule bactérienne, dépôt visible en microscopie électronique. Les même bactéries peuvent désestérifier le diacétate de fluorescéine activement et ainsi libérer la fluorescéine, qui reste préférentiellement dans les bactéries vivantes et les rend ainsi fluorescentes. *In vitro,* les traitements physiques (température), chimiques (alkalinisation) et la conservation par le froid réduisent ces 2 activités chez *M. smegmatis* d'une façon proportionnelle à sa viabilité mesurée en culture (nombre d'unités bactériennes formant colonie). Par contre, lorsque ces deux activités enzymatiques sont recherchées chez *M. lepraemurium* récolté à différents stades au cours de l'infection de la souris et dont la viabilité est mesurée par passage chez la souris, il n'y a pas de correlation entre viabilité et activité enzymatique. La valeur respective de ces deux techniques potentiellement utiles pour tester rapidement la sensibilité de *M. leprae* aux antibiotiques reste à démontrer.

MOTS-CLÉS: Tellurite, Fluorescéine, *Mycobacterium leprae, Mycobacterium lepraemurium, Mycobacterium smegmatis,* Viabilité.

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