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COMPARISON OF CYTOCHROMES IN MYCOBACTERIA GROWN IN VITRO AND IN VIVO

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ABSTRACT

KUSAKA, TAKASHI (National Institute for Leprosy Research, Tokyo, Japan), RYO SATO, AND KO SHOJI. Comparison of cytochromes in mycobacteria grown in vitro and in vivo. J. Bacteriol. **87**:1383-1388. 1964.—Spectrophotometric investigations were made on cell suspensions or particulate fractions of four species of mycobacteria which had been cultivated in vitro. The results obtained indicated the presence in *Mycobacterium smegmatis* of cytochromes of the *a*, b_1, and *c* types, as well as a CO-binding pigment similar to cytochrome *o;* in BCG and *M. avium*, cytochromes of the *a*, *b*, and *c* types were present; and, in *M. paratuberculosis*, cytochromes of the *a*, b_1, and *c* types were present. Although no clear interrelations were evident between the cytochrome patterns of these organisms and the ease with which they can be cultivated in vitro, it was found that the total reduced nicotinamide adenine dinucleotide oxidase activity of the particulate fraction was remarkably low in *M. paratuberculosis*, which can be grown in vitro only with great difficulty. The cells of BCG grown in vivo and those of *M. lepraemurium* isolated from leprous nodules of mice were found to be completely deficient in cytochrome pigments.

Hanks and Gray (1956) reported that there is a correlation between the oxidative metabolism of mycobacteria and the ease with which they can be cultivated in vitro. Thus, it was confirmed that, as the difficulty in cultivation in vitro increases from saprophytic to attenuated and finally to parasitic strains of mycobacteria, their respiratory activities diminish sharply. Segal and Bloch (1956), on the other hand, reported that the cells of *Mycobacterium tuberculosis* H37Rv grown in vivo respond to only a limited number of respiratory substrates, whereas those grown in vitro can oxidize a wide variety of organic compounds.

Since respiration in an organism is generally mediated by a mechanism involving cytochromes, it seemed of interest to determine whether there are any correlations between the cytochrome patterns and respiratory activities of various species of mycobacteria, cultivated both in vitro and in vivo, to understand the biochemical bases of the respiratory variations mentioned above. Although the presence of cytochromes in mycobacteria has been repeatedly demonstrated (Todd, 1949; Yamamura et al., 1955; Andrejew, Gernez-Rieux, and Tagguet, 1957; Bastarrachea and Goldman, 1961; Goldman et al., 1963), little is known of their variations.

In the present paper, we report a comparative study of cytochrome patterns of *M. smegmatis*, BCG, *M. avium*, and *M. paratuberculosis* cells grown in vitro. No cytochromes could be detected in BCG cells grown in vivo and in *M. lepraemurium* cells isolated from leprous nodules of mice.

MATERIALS AND METHODS

Cultivation and isolation of organisms. *M. smegmatis*, BCG cells, and an isonicotinic acid hydrazide (INH)-resistant strain of BCG cells were supplied by the Department of Tuberculosis, Research Institute for Microbial Diseases, Osaka University; *M. avium*, Flamingo strain, was supplied by the Institute for Infectious Diseases, Tokyo University; and *M. paratuberculosis* was supplied by the National Institute of Animal Health, Tokyo. *M. smegmatis*, BCG cells, the INH-resistant strain of BCG cells, and *M. paratuberculosis* were grown at 37 C on the surface of Sauton medium; they were harvested after cultivation for 5 days, 10 days, 10 days, and 2 months, respectively, and thoroughly washed with water. *M. avium*, Flamingo strain, was grown at 37 C in rocked tubes by the method of Aoyagi and Mizuno (1959) in a medium which contained asparagine, 4.0 g; KH_2PO_4, 0.5 g; citric acid, 2.0 g; ferric ammonium citrate, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; D-glucose, 20 g; Tween 80, 10 g; and distilled water to 1,000 ml; the pH

was adjusted to 7.2. The cells were harvested after 5 days and washed well with water.

The BCG cells grown in vivo were isolated from the nodules formed, due to infection with the organism, at the great omentum region of guinea pigs. The cells of *M. lepraemurium*, Hawaiian strain, were obtained from subcutaneous leprous nodules formed in mice which had been infected by the bacterium for 4 months. The isolation of the cells was carried out as described previously (Kusaka, 1958), except that digestion of remaining tissue residues was conducted by incubating the preparation at 4 C for 24 hr with a protease from *Streptomyces griseus* (0.1%), instead of trypsin.

Preparation of samples for spectrophotometry. The washed mycobacterial cells obtained as above were immediately lyophilized. A suitable quantity [usually 15 to 20 mg (dry weight) per ml] of the lyophilized cells was suspended homogeneously in a 1:1 (by volume) mixture of glycerol and 0.1 M phosphate buffer (pH 7.2) as described by Smith and White (1962). This was used as the "cell suspension."

Spectrophotometry and reduced nicotinamide adenine dinucleotide (NADH) oxidase assay were also conducted with the particulate fraction obtained from each type of cells by the following method. The lyophilized cells were ground in a mortar for 40 min at 4 C with approximately ten times their weight of quartz sand, and were suspended in 10 volumes of 0.1 M phosphate buffer (pH 7.2) containing 0.25 M sucrose. After removing the quartz sand and debris by centrifugation at 1,000 × *g* for 40 min, the supernatant fluid was centrifuged at 10,000 × *g* for 40 min. The precipitate thus obtained was relatively low in cytochrome content and was usually discarded.

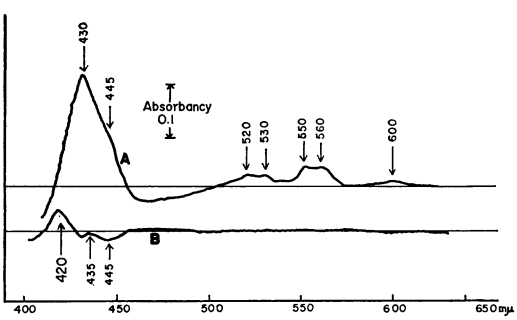

FIG. 1. *Difference spectra of particulate fraction from Mycobacterium smegmatis. Curve A, reduced minus oxidized; curve B, CO difference; 5.1 mg of protein per ml.*

The supernatant fluid was again centrifuged at 100,000 × *g* for 40 min, and the precipitate obtained, containing the majority of cytochromes, was washed with water and suspended in 0.1 M phosphate buffer (pH 7.2). This was designated as the "particulate fraction." Only a very small amount of cytochromes could be detected in the final supernatant.

Spectrophotometric demonstration of cytochromes. The sample to be tested was placed in both the sample and reference cells (optical path, 1 cm) of a Cary model 14 recording spectrophotometer. After recording the base line, the content of sample cell was treated with a few milligrams of solid sodium dithionite to reduce the cytochromes. When cell suspensions were used, full reduction of the pigments required 5 to 10 min because of the viscosity of glycerol in the medium. After complete reduction of the pigments, the difference spectrum (reduced minus oxidized) was measured. For detection of carbon monoxide-binding pigments, the contents of both sample and reference cells were fully reduced by dithionite, and then CO was carefully bubbled for 1 min into the sample cell. The spectral difference induced by CO was then recorded and called the CO-difference spectrum. All the spectrophotometric measurements were made at room temperature. The cytochrome pattern in each sample was estimated from the peaks and trough in the two types of difference spectra.

Other procedures. NADH oxidase activity of particulate sample was measured at room temperature by following the decrease in absorbancy at 340 mμ in a Cary spectrophotometer. The reaction mixture contained (in a total volume of 1.0 ml) 0.2 ml of sample, 80 μmoles of tris(hydroxymethyl)aminomethane (tris) buffer (pH 7.5), and 0.1 μmole of NADH purchased from Sigma Chemical Co., St. Louis, Mo. The reference cell contained all the components except NADH. The reaction was started by adding NADH. Protein was determined by the method of Lowry et al. (1951).

RESULTS

Cytochrome patterns in mycobacteria grown in vitro. Figure 1 shows the difference spectra of the particulate fraction of *M. smegmatis* grown on Sauton medium. The reduced minus oxidized difference spectrum showed a distinct Soret peak at 430 mμ with a shoulder at 445 mμ. In the α-

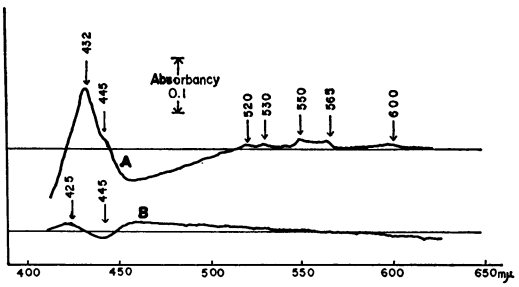

FIG. 2. *Difference spectra of particulate fraction from BCG cells grown in vitro. Curve A, reduced minus oxidized; curve B; CO difference; 2.0 mg of protein per ml.*

and β-band region, peaks at 560, 550, and 520 to 530 mμ were evident, but the α-band of an *a*-type cytochrome expected at 605 mμ was hardly visible. However, these peaks and shoulder suggested the occurrence in this organism of cytochromes of the *a* (α, 605?; Soret, 445 mμ), b_1 (α, 560; β, 530?; Soret, 430 mμ), and *c* (α, 550; β, 520 mμ?) types; the Soret peak of the *c*-type cytochrome was unseen because of the intense Soret peak of the *b*-type pigment. Figure 1 indicates further that the CO-difference spectrum had two peaks at 420 and 435 mμ and a trough at 445 mμ. Of these, the trough at 445 mμ and the peak at 435 mμ could be ascribed to the CO binding by the *a*-type cytochrome, whereas the peak at 420 mμ suggested the presence of a new CO-binding pigment similar to the one, sometimes called cytochrome *o*, reported in *Staphylococcus albus*, *Proteus vulgaris*, etc. (Change, 1953; Smith, 1954; Castor and Chance, 1959).

In Fig. 2 are illustrated the two types of spectra of the particulate fraction of BCG cells grown in vitro. The reduced minus oxidized difference spectrum of this sample was similar to that of *M. smegmatis*, except that the Soret and α peaks due to a *b*-type component were shifted in the former to longer wavelengths by a few millimicrons (Soret, 432; α, 565 mμ), and the α-band of the *a*-type pigment around 600 mμ was somewhat more pronounced. It was also evident from the CO-difference spectrum that the *a*-type pigment is the only CO-binding component in this organism. It may be tentatively concluded that BCG cells contain cytochromes of the *a*, *b* (but not b_1), and *c* types, but lacks the *o*-type CO-binding pigment. In agreement with Andrejew et al. (1957), the difference spectra obtained with the particulate fraction of an INH-resistant strain of BCG were practically identical with those of the sensitive strain.

The difference spectra of the particulate fraction of *M. avium*, Flamingo strain, are shown in Fig. 3. These spectra were identical, at least qualitatively, with those of BCG cells, suggesting the presence of cytochromes of the *a*, *b*, and *c* types. Finally, Fig. 4 shows the difference spectra of the cell suspension of *M. paratuberculosis*. These spectra resembled those of *M. smegmatis* in that they had peaks ascribable to the b_1-type (rather than *b*-type) pigment, but differed in that no evidence was available for the presence of *o*-type CO-binding cytochrome.

The cytochrome patterns of the four species of mycobacteria deduced from Fig. 1 to 4 are summarized in Table 1.

NADH oxidase activites and yields of particulate fraction. Table 2 shows the NADH oxidase activities of particulate samples obtained from BCG, *M. avium*, and *M. paratuberculosis* cells,

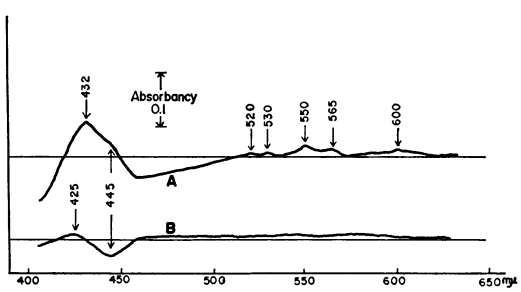

FIG. 3. *Difference spectra of particulate fraction from Mycobacterium avium. Curve A, reduced minus oxidized; curve B, CO difference; 4.2 mg of protein per ml.*

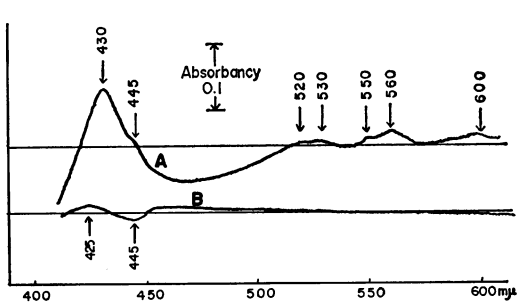

FIG. 4. *Difference spectra of cell suspension of Mycobacterium paratuberculosis. Curve A, reduced minus oxidized; curve B. CO difference; 20 mg of cells (dry weight) per ml.*

TABLE 1. *Cytochrome bands in difference spectra of mycobacteria grown in vitro**

Organism	Sample used	Reduced minus oxidized spectrum										CO difference spectrum		
		a		*b*			b_1			*c*†		*a*		*o*‡
		α	Soret	α	β	Soret	α	β	Soret	α	β	Peak	Trough	Peak
Mycobacterium smegmatis	Particles	600?	445	—	—	—	560	530?	430	550	520?	435?	445	420
BCG cells	Particles and cell suspension	600?	445	565	530?	432	—	—	—	550	520?	425	445	—
M. avium	Particles	605?	445	565	530?	432	—	—	—	550	520?	425	445	—
M. paratuberculosis	Cell suspension	600	445?	—	—	—	560	530?	430	550	520?	?	445	—

* Figures indicate the wavelength in millimicrons.

† The Soret peaks of *c*-type cytochrome were covered by the intense Soret band of *b* (or b_1)-type cytochrome.

‡ Not completely identical with cytochrome *o*.

TABLE 2. *NADH oxidative activity and yield of particulate fractions obtained from mycobacteria grown in vitro*

Organism	Specific NADH oxidase* activity	Yield of particulate fraction†	Total NADH oxidase activity‡
BCG cells	50.3	3.3	1,659.9
M. avium	9.1	3.4	309.4
M. paratuberculosis	14.1	0.12	16.9

* Expressed as mμmoles of NADH per min per mg of particulate protein.

† Expressed as percentage of particulate protein in total dry cell weight.

‡ Expressed as mμmoles of NADH per min per g of dry cell weight.

as well as the recoveries of protein in the particulate fractions. The specific activity of NADH oxidase of the particles derived from BCG cells was considerably higher than that of *M. avium* and *M. paratuberculosis*. No significant differences were observed between the latter two samples. However, the yield of particulate fraction from *M. paratuberculosis* was remarkably lower than that from BCG and *M. avium*. Consequently, the total NADH oxidase activity per gram of cellular weight (calculated on the assumption that the oxidase activity resides only on the particulate fraction) of *M. paratuberculosis* was estimated to be only 1 and 5% of those of BCG and M. *avium*, respectively.

Cytochrome patterns in mycobacteria grown in vivo. Figure 5 shows the reduced minus oxidized difference spectra of suspensions of BCG cells grown in vitro, BCG cells grown in vivo, and *M. lepraemurium* isolated from leprous nodules in mice. The spectrum of BCG cells grown in vitro was almost the same as that of the particulate fraction obtained from these cells; it indicated the presence of cytochromes of

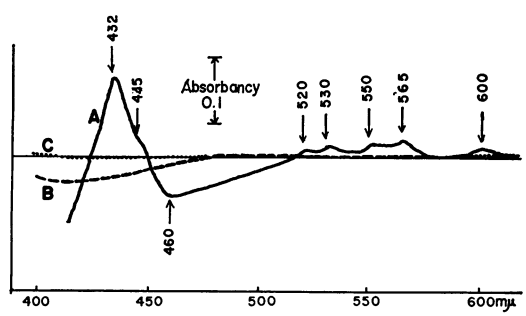

FIG. 5. *Reduced minus oxidized difference spectra of cell suspensions of BCG cells grown in vitro and in vivo, as well as Mycobacterium lepraemurium. Curve A, BCG cells in vitro, 15 mg (dry weight) of cells per ml; curve B, BCG cells in vivo, 20 mg (dry weight) of cells per ml; curve C, M. lepraemurium, 20 mg (dry weight) of cells per ml.*

the *a*, *b*, and *c* types, and the distinct trough around 460 mμ suggested the occurrence of flavins. In contrast to this spectrum, the difference spectra of both BCG cells grown in vivo and *M. lepraemurium* quite unexpectedly showed no detectable peaks and troughs in the region from 400 to 650 mμ. The absence of cytochrome bands in these spectra might suggest that the cytochromes in these cells are maintained in the reduced form even after thorough washing of the cells. This possibility could, however, be ruled out by measuring the difference spectra of the particulate fractions obtained from these cells; no cytochromes could again be detected in these particles which had been washed free from endogenous reductants. As another possibility, it might be feared that the cytochromes originally present in these cells had been lost by the protease treatment employed for their isolation from tissues. However, this was also very unlikely because the cells isolated by this method have been shown to be practically intact (Kusaka, Yamanouchi, and Shoji, *in preparation*). Moreover, prolonged treatment of BCG cells grown in vitro with protease did not affect their cytochrome spectrum. It may, therefore, be concluded that the BCG cells grown in vivo as well as those of *M. lepraemurium* are practically devoid of cytochromes and possibly also of flavins.

DISCUSSION

All the strains of mycobacteria tested contained a set of cytochrome pigments when they were grown in vitro. This is in accordance with the well-known aerobic nature of this group of bacteria. However, the cytochromes present in individual species differ somewhat from one another. Although all the strains possess an *a*-type and a *c*-type pigment in common, the *b*-type pigment found in BCG and *M. avium* cells is replaced by a b_1-type pigment in *M. smegmatis* and *M. paratuberculosis*. Further, *M. smegmatis* is the only strain which contains a CO-binding pigment similar to cytochrome *o* (Chance, 1953; Smith, 1954; Castor and Chance, 1959).

It is rather difficult to correlate these cytochrome patterns to the cultivation characteristics of the organisms. The presence of the *o*-type CO-binding pigment in *M. smegmatis* might be related to the ease of cultivation in vitro of this organism, but direct evidence for this possibility is still lacking. It is also difficult at present to assess the cytochrome content in each organism, but a rough estimation suggests that there is no remarkable difference in the amount of cytochromes in the particulate fractions obtained from the four organisms. Other reasons than the mere cytochrome content must, therefore, be looked for to explain the differences in respiratory activity and cultivation characteristics. In this connection, the data shown in Table 2 are of interest in that they suggest that the particulate system equipped with cytochromes and NADH oxidase is only poorly developed in the cell of *M. paratuberculosis*, a strain most difficult to cultivate in vitro.

The complete absence of cytochromes from the BCG cells grown in vivo and *M. lepraemurium* does not seem to be due to artifact phenomena as already discussed. Instead, it seems likely that these cells growing in the interior of the host cells are actually incapable of synthesizing the respiratory pigments. Since BCG grown in vitro contains cytochromes, this finding suggests that a profound change occurs in respiratory mechanisms when the organism enters into parasitic life. It would, therefore, be of interest to study the energy metabolism in these cells and its relation to that of infected animal tissues to understand the nature of tubercular infection. As already mentioned, Segal and Bloch (1956) reported that the cells of a virulent strain of *M. tuberculosis* grown in vivo show only a very limited oxidative activity. Studies are in progress in our laboratories to determine whether cytochromes are also lacking in the tubercle bacterium grown in vivo.

Although the effects of environmental factors during growth on the cytochrome content in bacteria were reviewed in detail by Smith (1961), no cases have yet been known in which cytochromes disappear completely. The complete loss of cytochromes in BCG cells grown in vivo might be accounted for as an adaptation to the low oxygen tension in the host tissues. However, it has been reported that the cytochrome content in such bacteria as *Pseudomonas fluorescens* increases, rather than decreases, when grown under a poor supply of oxygen (Lenhoff, Nicholas, and Kaplan, 1956). In conclusion, the mechanism by which BCG cells lose the ability to synthesize cytochromes is still to be explored.

The finding that *M. lepraemurium* is also deficient in cytochromes is of special interest in view of the fact that this organism has never been cultivated in vitro. It may be suggested that the energy metabolism in this organism is similar to that of BCG cells grown in vivo. Therefore, studies of the respiratory mechanisms of BCG cells grown in vivo would throw light on the basic biochemical feature of leprosy bacteria.

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