Effect of Zinc Deficiency on *Mycobacterium tuberculosis* var. *bovis* (BCG)

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Mycobacterium tuberculosis var. *bovis* (BCG) grown on Sauton medium normally forms a pellicle; in the absence of added Zn^{2+} , however, the pellicle sank during incubation and the yield was only about 20% of normal. The Zn^{2+} -starved bacteria were morphologically similar to normal bacteria and were still acid-fast at 7 d as well as 14 d. The Zn^{2+} -starved bacteria had slightly higher free lipid and phospholipid contents than normal; the content of hexoses was lower and proteins slightly lower. The deficient culture medium became opalescent and alkaline. Aspartate and ammonium ions accumulated. There was twice as much protein in deficient as in normal medium; moreover, a class of proteins precipitable at pH 4.5, which was hardly detectable in normal medium, was present in appreciable amounts in deficient medium. The content of aldehydes, measured with yeast alcohol dehydrogenase, was also doubled in deficient medium. Fractionation of acid-soluble aldehydes obtained from deficient medium after acid treatment of a bisulphite precipitate suggested the presence of several complex molecules bearing aldehyde groups. The need for Zn^{2+} in the medium may be explained by the presence in normal BCG of a Zn^{2+} -requiring NADP-dependent alcohol dehydrogenase activity whose affinity for aldehydes is especially high.

INTRODUCTION

Mycobacterium tuberculosis var. *bovis* (BCG) grows on the surface of Sauton medium and forms a yellow-grey folded pellicle which can be harvested for vaccine production. The medium remains limpid and becomes straw-yellow and slightly acid. Sometimes the culture grows poorly and forms a thin, pale, unfolded pellicle that gets wet and sinks, and the medium becomes opalescent and alkaline (up to pH 8.8); such cultures are not suitable for vaccine production. Drea (1956) has shown that the wetting is linked to the use of some batches of glycerol and that the addition of small quantities of Zn^{2+} is sufficient to ensure good cultures. By omitting Zn^{2+} it is possible to obtain wet sunken cultures by choice. We have done this in order to determine the metabolic lesion(s) caused by Zn^{2+} deprivation and its effect on the composition of the bacteria and on the products excreted into the culture medium.

METHODS

Culture of bacteria. *Mycobacterium tuberculosis* var. *bovis* (BCG), French strain 1173P2, was grown on the surface of Sauton medium containing 5 μM -zinc sulphate. Media with or without Zn^{2+} were inoculated with portions of the same culture and incubated at 37.5 °C. After 7 or 14 d, the cells were separated from the medium by filtration (in the presence of Zn^{2+}) or by decantation and centrifugation (in the absence of Zn^{2+}), and washed with phosphate-buffered saline (when used for enzyme preparation) or with physiological saline and then distilled water followed by freeze-drying (when used for chemical assays). Before analysis the culture media were centrifuged at 20 °C for 10 min at 6000 g and then for 45 min at 26 000 g. After the second centrifugation, the opalescence of the Zn^{2+} -deficient medium disappeared and a whitish pellet was obtained. This pellet was washed twice in saline. The clear normal medium showed no visible pellet.

Chemicals were 'p.a.' products from Merck; enzymes and cofactors were from Boehringer. Freshly distilled water and Pyrex or Duran 50 glassware were used for the preparation of culture media and solutions to avoid Zn^{2+} contamination.

Chemical analysis of bacteria. Bacteria from 14 d-old cultures were used in all analyses. Weights of freeze-dried bacteria were corrected for residual moisture estimated by the Karl Fisher method.

Total lipids were determined after acid hydrolysis (5 h at 100 °C in 6 M-HCl) and ether extraction as described by Kotani *et al.* (1959). Free lipids were extracted by the method of Bligh & Dyer (1959) using two successive extractions, each for 2 d, at room temperature and one extraction for 18 h at 37 °C (1.5 ml solvent per 10 mg dried bacteria) and determined as described by Kotani *et al.* (1959). Lipid phosphorus was determined by the method of Ames (1966).

Proteins were determined by the micro-Kjeldahl technique as described by Chibnall *et al.* (1943).

For hexose determination, freeze-dried bacteria were hydrolysed in sealed ampoules under nitrogen in 1 M- H_2SO_4 at 100 °C for 2 h and then hexose was determined with the anthrone reagent (Morris, 1948).

Enzyme assays. Preparation of cell-free extracts and the measurement of alcohol dehydrogenase activity were as described by De Bruyn *et al.* (1981). Glutamic-oxaloacetic transaminase (GOT) (EC 2.6.1.1) activity was measured as described by Karmen (1955) in 100 000 g supernatants.

Chemical analysis of culture media. Medium for 14 d-old cultures with or without added Zn^{2+} and samples of uninoculated medium (as controls) were analysed.

Ammonium ions were determined by the Nessler reagent (Charlot, 1961).

Free L-aspartate was determined with glutamic-oxaloacetic transaminase (Bergmeyer *et al.*, 1974).

Proteins were measured by the Coomassie blue method (Spector, 1978) with the Bio-Rad reagent.

Aldehydes were measured using yeast alcohol dehydrogenase (EC 1.1.1.1) (Holzer & Goedde, 1963). Alternatively, in preliminary assays, aldehydes and methylketones were isolated from media by precipitation with 30 % sodium bisulphite (final concn) in the presence of 0.01 M-EDTA and 5 % methanol. After 18 h at 4 °C, the precipitates were sedimented and washed three times with 30 % sodium bisulphite and once with ethanol at 4 °C. Treatment of these precipitates with 0.1 M-HCl gave acid-soluble and acid-insoluble fractions which were separated by centrifugation. The acid-soluble material was either extracted with ether and concentrated to dryness or lyophilized. Dry material was dissolved in 0.05 M-ammonium acetate buffer (pH 6.5) and subjected to chromatography on a Sephadex G-15 column (2.6 × 50 cm) at 4 °C. Fractions containing aldehydes were first detected with Schiff reagent: 1 vol. Schiff reagent was added to 2 vol. samples of each fraction and allowed to react for several hours at room temperature. Schiff-positive fractions corresponding to peaks were pooled and analysed for carbonyl content with 2,4-dinitrophenylhydrazine as described by Daron & Gunsalus (1962) except that the assay mixtures were incubated for 30 min at 70 °C in sodium dodecyl sulphate (SDS; 0.1 %, w/v). Pooled fractions were also tested as substrates in an enzymic assay with yeast alcohol dehydrogenase and BCG 100 000 g supernatant extract. The acid-insoluble fraction of 0.1 M-HCl-treated bisulphite precipitate was analysed by SDS-polyacrylamide gel electrophoresis (10 to 20 %, w/v, acrylamide gradient) as described by Laemmli (1970).

Addition of acetic acid to precooled culture media to lower the pH to 4.5 gave precipitates. After 18 h at 4 °C, these precipitates were harvested by centrifugation and washed successively with 0.01 M-acetic acid/NaOH buffer (pH 4.5) and ethanol. This material was also analysed by SDS-polyacrylamide gel electrophoresis.

RESULTS

Effect of Zn^{2+} deficiency on growth and yields of BCG. Without addition of Zn^{2+} , cultures sank and the medium became opalescent and alkaline (pH 8.3); the growth yield (measured as bacterial weight and total protein) was 20 to 25 % of that obtained in the presence of 5 μM - Zn^{2+} , which we considered to be normal growth conditions. A Zn^{2+} concentration of 2.5 μM was sufficient to obtain pellicle culture, acidification of the medium (pH 6.44) and 93 % of normal protein yield. A Zn^{2+} concentration of 100 μM gave 112 % of the normal protein yield and a pH of 6.26. When Zn^{2+} (5 μM) was added after 7 d growth in deficient medium, the total protein yield after 14 d was 37 % higher than without the addition of Zn^{2+} ; nevertheless the culture still sank.

The Zn^{2+} -starved bacteria were morphologically similar to normal bacteria and were still acid-fast at 7 d as well as 14 d.

Changes in composition of BCG grown in Zn^{2+} -deficient medium. The content of some components of normal and Zn^{2+} -starved bacteria are given in Table 1. Analysis of two different culture batches for total lipids did not show any significant difference between normal and Zn^{2+} -starved bacteria (about 31 % in each case). The Zn^{2+} -starved bacteria had

Table 1. *Effects of Zn²⁺ deficiency on composition of BCG cells and culture medium*

Bacteria were grown on Sauton medium with or without added Zn²⁺ and harvested after 14 d. Analyses were performed as described in Methods. The values are based on at least four individual determinations; all the differences were significant at $P < 0.01$. Phospholipid is expressed as lipid phosphorus, hexose as D-glucose and aldehydes as glycolaldehyde.

	Concn ($\mu\text{g ml}^{-1}$) in culture medium		Concn (% w/w) in lyophilized bacteria	
	With Zn ²⁺	Without Zn ²⁺	With Zn ²⁺	Without Zn ²⁺
Free lipids	—	—	23	28
Phospholipids	—	—	0.16	0.20
Proteins	44	93	50	45
Hexose	774	457	13	9
Aldehydes	10	18	—	—
Aspartic acid	76	928	—	—
Ammonium*	-109	+310	—	—

* Difference from uninoculated Sauton medium incubated at 37.5 °C.

slightly higher free lipid and phospholipid contents. Protein and hexose contents were lowered.

A soluble NADP-dependent alcohol dehydrogenase activity with high affinity for aldehydes was found to be deficient in Zn²⁺-starved bacteria. In extracts from bacteria grown on deficient media the specific activity of this enzyme was 15% of its value in normal culture cells after 7 d or 14 d growth.

We also looked for an effect of Zn²⁺ deficiency on the activity of glutamic-oxaloacetic transaminase, which is responsible for L-glutamate biosynthesis in BCG growing on Sauton medium using asparagine as nitrogen source. The activity of the transaminase was lowered by Zn²⁺ deficiency to 45 to 50% of the activity of normal cultures of the same age (7 or 14 d-old).

Composition of medium after growth of BCG under normal and Zn²⁺-deficient conditions. Free aspartate accumulated in Zn²⁺-deficient growth medium in very high amounts (about 12-fold higher than normal) together with ammonium ions (Table 1); this confirms previous observations made on spontaneously sunken BCG cultures (Bance, 1942).

The amount of aldehydes measured with yeast alcohol dehydrogenase was about twice as high in the deficient medium as in the normal medium. Fractionation on Sephadex G-15 of the acid-soluble ether-extractable (as interface material) aldehydes originating from deficient medium gave a few major peaks with K_{AV} values ranging from 0.2 to 3. Four fractions with K_{AV} values of 0.5, 1.1, 1.3 and 1.8, respectively, were tested as substrates for yeast alcohol dehydrogenase and BCG 100 000 g supernatant extract. Only the fraction with a K_{AV} value of 1.1 gave a reaction with yeast alcohol dehydrogenase, in good correlation with the carbonyl content determined chemically. All four fractions tested gave NADPH oxidation with BCG 100 000 g supernatant but NADPH oxidation was much higher than would have been expected on the basis of the carbonyl content determined chemically. Acid-soluble ether-extractable aldehydes were less abundant in normal medium than in low-Zn²⁺ medium.

Total proteins were at least twice as abundant in Zn²⁺-deficient medium as in normal medium. The fraction of these proteins precipitated at pH 4.5 was much higher in deficient medium than in normal medium; it represented up to 8% of the protein content of normal medium but up to 67% of the protein content of the deficient medium. SDS-polyacrylamide gel electrophoresis of equal amounts of proteins precipitated at pH 4.5 from normal and deficient media showed a very different pattern; in particular a component of approximately 66 000 molecular weight was scarcely detectable in the normal medium, but accumulated in the deficient culture medium. This component was already abundant at 7 d (Fig. 1).

Fig. 1. SDS-polyacrylamide gels after electrophoresis of proteins precipitated at pH 4.5 from media after 7 d growth (a) or 14 d growth (b). BCG was grown in Sauton medium with or without added Zn^{2+} , and after sedimenting the bacteria, acid-precipitable proteins were isolated from the supernatant medium as described in Methods. A, proteins (7.5 μ g) from normal medium, i.e. with added Zn^{2+} . B, proteins (7.5 μ g) from Zn^{2+} -deficient medium. C, markers: 1, bovine serum albumin (mol. wt 68 000); 2, ovalbumin (45 000); 3, *E. coli* asparaginase (33 000); 4, tobacco mosaic virus (17 000); 5, *E. coli* lysozyme (14 400); 6, insulin (7 000). The arrows indicate the 66 000 molecular weight component accumulated in Zn^{2+} -deficient medium.

SDS-polyacrylamide gel electrophoresis of the acid-insoluble fraction of HCl-treated bisulphite precipitate gave an identical pattern to the one obtained for pH 4.5-precipitable proteins.

The pellet obtained after centrifugation of medium from a Zn^{2+} -deficient culture for 45 min at 26 000 *g* consisted largely of polysaccharides rich in hexose (0.6 to 0.8 μ g ml⁻¹ of the original medium); no glucosamine or protein could be detected. No such pellet was formed in normal culture medium.

DISCUSSION

We have observed (unpublished results) that after homogenization of comparable amounts of normal and Zn^{2+} -starved bacteria by the standard procedure used for BCG vaccine production using steel balls, followed by centrifugation, the supernatant obtained from normal bacteria is pale yellow and turbid while the supernatant from Zn^{2+} -starved bacteria is whitish and only half as turbid; this suggested that loosely attached surface components were lacking in Zn^{2+} -starved bacteria. Furthermore, the observed liberation of polysaccharides in Zn^{2+} -deficient opalescent medium and the slightly higher content of free lipids and phospholipids of the Zn^{2+} -starved bacteria (Table 1) suggested an alteration of the cell wall. However, the Zn^{2+} -starved bacteria were still acid-fast after 7 and 14 d.

Although only slight differences in the contents of several bacterial components were found, the values reported were consistent for several independent cultures. Aldehyde accumulation and consequent possible deficiency in some esterifiable alcohols (possibly on hydrophilic

molecules) could be responsible for the slightly higher free lipid content (possibly free fatty acid) of Zn^{2+} -starved bacteria.

The accumulation of aldehydes may be explained by the decrease in activity of a soluble NADP-dependent alcohol dehydrogenase with high affinity for aldehydes. Preliminary results suggested that substances bearing aldehyde groups found in deficient media were substrates for this alcohol dehydrogenase activity.

The amount of protein was slightly lower in Zn^{2+} -starved bacteria and an increased excretion of proteins into the medium was observed; furthermore, a class of proteins precipitable at pH 4.5, which was hardly detectable in normal medium, was present in large amounts in the deficient medium. The immunological activities of the pH 4.5-precipitable proteins liberated into the medium in deficient cultures (De Bruyn *et al.*, 1980) reinforce our interest in pursuing these studies which could also help our understanding of BCG cell wall synthesis.

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