Effect of Zinc Deficiency on the Appearance of Two Immunodominant Protein Antigens (32 kDa and 65 kDa) in Culture Filtrates of Mycobacteria

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After growth of six strains of mycobacteria on Sauton medium in the absence of added Zn^{2+} , cell yields were lowered, to between 22% and 67% of the yields obtained when Zn^{2+} (5 μM) was added. Two immunodominant proteins, named P_{64} and P_{32} (antigens of 62–65 kDa and 29–33 kDa, respectively) were abundant in culture filtrates after growth of mycobacteria. P_{64} was present at elevated concentrations (showing a 9- to 16-fold increase as a percentage of the total protein released) after Zn^{2+} -deficient growth of five of the six strains studied; in *Mycobacterium tuberculosis* it represented 25% of all released proteins. However, little P_{64} was detected in culture filtrates of *M. fortuitum* and of *M. phlei* grown under Zn^{2+} deficiency, and in the latter there was no increase of P_{64} during Zn^{2+} deficiency.

INTRODUCTION

By growing *Mycobacterium bovis* BCG on Zn^{2+} deficient medium, two immunodominant proteins, P_{64} (65 kDa or antigen 82 in the reference system of Closs *et al.*, 1980) and P_{32} (antigen 85A in the same reference system) could be readily obtained; they represented respectively about 20% and 7% of the excreted proteins. Thus such culture filtrates were conveniently used as starting material for the purification of these antigens (De Bruyn *et al.*, 1987*a, b*), which present interesting immunological properties (Thole *et al.*, 1987; Kaufmann *et al.*, 1987; Young *et al.*, 1987; Wiker *et al.*, 1986; Huygen *et al.*, 1988; Turneer *et al.*, 1988). Protein P_{64} is thought to correspond to the *Escherichia coli* GroE1 protein that allows *E. coli* to grow at elevated temperatures. It is a common antigen (Thole *et al.*, 1988), involved in response to shock (Shinnick *et al.*, 1988), and in mycobacteria it may be associated with the T-cell response to some of its epitopes in rheumatoid arthritis (Van Eden *et al.*, 1988).

While the physiological role of the P_{32} protein in mycobacteria remains unknown, it may be specific for mycobacteria, as tuberculous patients give significantly higher serological responses to it than do healthy controls (Huygen *et al.*, 1988; Turneer *et al.*, 1988). More interestingly, the possible identification of this antigen as the fibronectin-binding protein from *Mycobacterium tuberculosis* could help in understanding the immunopathology of the disease (Abou-Zeid *et al.*, 1988). The purpose of this work was to determine whether the observations previously made only on BCG (De Bruyn *et al.*, 1981) could be extended to other mycobacteria, perhaps generally.

METHODS

Bacterial strains. These are presented in Table 1.

Culture of bacteria. The mycobacterial strains were grown on the surface of Sauton medium. As the medium was prepared with distilled water (specific resistivity $10^6 \Omega \times \text{cm}$) zinc sulphate was added to a final concentration of 5 μM (normal Sauton medium). When Zn^{2+} -deficient medium was needed, zinc sulphate was omitted. Media with or without added Zn^{2+} were inoculated with equal volumes of the same culture and incubated at 37.5 °C.

Table 1. *Strains of Mycobacterium used*

Strain	Species	Relevant properties	Origin
An5	<i>M. bovis</i>	Reference strain	ATCC
H37Ra	<i>M. tuberculosis</i>	Reference strain	ATCC
42	<i>M. avium</i>	Clinical isolate	L. Demoulin Brahy*
190	<i>M. kansasii</i>	Clinical isolate	L. Demoulin Brahy*
141	<i>M. fortuitum</i>	Clinical isolate	L. Demoulin Brahy*
AM76	<i>M. phlei</i>	Environmental isolate	L. Demoulin Brahy*

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Preparation of culture media for analysis. After culture for 7 d (*Mycobacterium phlei*), 14 d (*M. bovis*, *M. avium*, *M. kansasii* and *M. fortuitum*) or 25 d (*M. tuberculosis*), media from normal and Zn²⁺-deficient cultures were collected by suction and sterilized by filtration (Millex GV filter, 0.22 µm porosity, Millipore) prior to analysis. Time of harvesting was defined for each strain according to preliminary experiments taking account of their relative growth rate. Where necessary, the culture media were concentrated in an Amicon stirred cell equipped with a PM10 membrane.

Preparation of cell extracts. The pellicles were harvested after 25 d culture for *Mycobacterium tuberculosis* and after 7 d for *Mycobacterium phlei*. Extracts of the washed bacilli were prepared in 50 mM-KH₂PO₄/NaOH buffer pH 7.3 by passage through a French pressure cell, as previously described (De Bruyn *et al.*, 1987a, b).

Dry weight determination. The organisms from heat-killed cultures (121 °C for 30 min) were harvested by centrifugation. After three washes in distilled water, the cells were dried to constant weight at 70 °C.

Analysis of culture media and cell extracts. Chemicals were p.a. products from Merck; enzymes and cofactors were from Boehringer. Proteins were estimated by the Coomassie blue method (Spector, 1978). Aldehydes were determined using yeast alcohol dehydrogenase as described previously (De Bruyn *et al.*, 1981).

Protein analysis by polyacrylamide gel electrophoresis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Laemmli (1970) in 13% (w/v) acrylamide gels. The gels were stained with Coomassie Brilliant Blue R-250 and, for quantitative analysis of P₃₂, scanned at 595 nm with a Beckman DU8 spectrophotometer.

The bands immunologically related to an antigen were identified by immunoblotting (see below). Quantitative analysis of P₆₄ was not performed, as this antigen was partially degraded.

Immunoblotting techniques. (a) *Western blots.* After completion of SDS-PAGE the proteins were transferred to nitrocellulose sheets by the method of Towbin *et al.* (1979). Rabbit sera raised against BCG P₃₂ and P₆₄ were prepared as described previously (De Bruyn *et al.*, 1987a, b). Peroxidase-labelled anti-rabbit immunoglobulin was from Bio-Rad and used at a dilution of 1/3000.

(b) *Dot-blotting assay.* Equal volumes (100 µl) of serial twofold dilutions of the two purified BCG antigens and of culture filtrates, adjusted initially to 10 µg and 100 µg protein ml⁻¹, respectively, were applied to nitrocellulose (Bio-Rad) using a Bio Dot apparatus. The nitrocellulose was transferred to a plastic bag and incubated in 5% (w/v) BSA in Tris-buffered saline (TBS) pH 7.5 for 1 h. It was then incubated for 2 h with rabbit antisera raised against BCG P₃₂ or BCG P₆₄ and diluted respectively 1/500 and 1/1000 in TBS containing 1% (w/v) BSA and 0.05% Tween 20. After incubation, the nitrocellulose was washed twice in the same buffer, then incubated with peroxidase-conjugated anti-rabbit immunoglobulins at the same final dilution as in the Western blot. A reflectance densitometer (Bio-Rad) was used to quantify the immunoperoxidase stain on the immunoblots. A calibration curve was set up for each BCG purified protein. In the linear portion of the calibration curve, the variation between assays was less than 5% for all dilutions tested. All samples were run in parallel on the same nitrocellulose sheet.

RESULTS AND DISCUSSION

Yield and composition of the medium after growth of the different mycobacterial strains under normal and Zn²⁺-deficient conditions

All the mycobacteria tested showed some common responses to Zn²⁺-deficient conditions: appearance of the pellicle generally smooth instead of wrinkled, less pigmentation, low cellular yield (22% to 67% of normal), and both opalescence and alkalinization of the culture medium – the pH increased significantly ($P < 0.01$) by 0.4 to 2.1 units. Aldehydes in the medium increased 5- to 11-fold (expressed per mg dry weight of bacteria) in four out of six strains; they decreased in *M. fortuitum*, while for *M. phlei* their appearance in the medium was not affected by Zn²⁺.

Under the growth conditions used, the pellicles were still floating at the time of harvesting, and the bacteria were acid-fast.

Zn²⁺ deficiency resulted in a 1.5- to 28-fold increase in the amount of protein found in the medium, and it is this response which we have investigated further, particularly that of the proteins apparently related to the BCG P₃₂ and P₆₄ antigens. In Zn²⁺-deficient conditions, release of P₆₄ by *M. bovis* (strain An5), *M. tuberculosis* (strain H37Ra), *M. avium* and *M. kansasii* was markedly stimulated.

Presence of P₃₂ and P₆₄ in normal and Zn²⁺-deficient media

The electrophoretic patterns of proteins originating from normal culture media were always very different from those originating from Zn²⁺-deficient media, except in the case of *M. phlei*. Proteins of the same or very similar mobility to P₃₂ were found in both normal and Zn²⁺-deficient media with all the species studied (Fig. 1*a*: I, II, III). The amounts of antigens immunologically related to the BCG P₃₂ (Fig. 1*b*: I, II, III) in normal media, estimated by gel scanning and expressed as percentages of the total protein content of the respective normal culture medium, were 11% for *M. bovis* An5, 27% for *M. tuberculosis* H37Ra, 66% for *M. kansasii* and 52% for *M. avium*. In both normal and Zn²⁺-deficient culture media of the mycobacteria studied, proteins with identical or very similar electrophoretic mobilities to P₃₂ reacted with rabbit polyclonal anti-P₃₂ serum (Fig. 1*b*: I, II, III).

Using dot-blotting techniques the highest concentrations of P₃₂ and related antigens in normal media were found for *M. kansasii*, *M. avium* and *M. tuberculosis*, where these antigens represented 19%, 18% and 16%, respectively, of the proteins released (Table 2). The differences between the amounts of this antigen estimated in normal culture media by gel scanning (results shown above) and by dot-blotting assays (Table 2) suggest that the proteins related to the BCG P₃₂ are not identical in the different strains, and that these proteins from *M. kansasii* and *M. avium* (where gel scanning appears to be more sensitive than dot-blotting) share fewer epitopes with the BCG P₃₂ antigen than do those from *M. bovis* An5 and *M. tuberculosis*. The percentage of proteins immunologically related to P₃₂ was slightly lower in Zn²⁺-deficient media than in normal media (Table 2), as previously observed for BCG (De Bruyn *et al.*, 1987*b*).

In soluble cell extracts of *M. tuberculosis* H37Ra and *M. phlei*, the relative amounts of P₃₂ found were 2.5% and 1%, respectively, compared with 16% and 4% in the corresponding culture filtrates.

The presence of a protein of the same or very similar mobility to P₆₄ was seen in all the Zn²⁺-deficient culture filtrates except that originating from *M. phlei* (Fig. 1*a*: III, lane E; Fig. 1*c*: III, lane E).

Cross-reactive antigens of same and/or smaller molecular mass than P₆₄ (the sensitivity of this antigen to proteolytic action was reported earlier in *M. leprae*: Gillis *et al.*, 1985) were present in each Zn²⁺-deficient culture filtrate, except that of *M. phlei* (Fig. 1*a*: I, II, III; Fig. 1*c*: I, II, III). (In *M. phlei*, P₆₄ could only be demonstrated in 10 d normal culture filtrate – data not shown.) By dot-blotting assay, the highest concentrations of P₆₄ in Zn²⁺-deficient culture medium were found for *M. tuberculosis* H37Ra, *M. avium* and *M. kansasii*, while very little P₆₄ was found for *M. fortuitum* and *M. phlei* (Table 2). Generally, little P₆₄ was found in culture filtrates of mycobacteria grown in normal media. The amount of P₆₄ in culture filtrates appeared to reflect the amount in cells, as in extracts of *M. tuberculosis* and *M. phlei* P₆₄ comprised 11% and 1.5%, respectively (determined by dot-blotting), of the total protein.

The protein P₆₄ released into the Zn²⁺-deficient culture media is antigenically related to a similarly-sized, ubiquitous bacterial common antigen (Young *et al.*, 1987; Thole *et al.*, 1987). These proteins with highly conserved sequences are thought to belong to the heat-shock protein family. By two different approaches Shinnick *et al.* (1988) recently demonstrated an increased synthesis of the mycobacterial 65 kDa protein during growth at a higher temperature. In the course of the present study, proteins from filtrates of 7 d cultures of BCG (French strain 1173P2) shifted from 37.5 °C to 41.5 °C and incubated at this temperature for 32 h were analysed by SDS-PAGE. The electrophoretic pattern obtained was very similar to that obtained with filtrates of Zn²⁺-deficient cultures. After the temperature shock, P₆₄ was excreted in large amounts. As a

Fig. 1. Protein and antigen content of culture filtrates of mycobacteria grown in normal and Zn^{2+} -deficient media. (a) SDS-PAGE analysis of proteins, stained with Coomassie Blue R-250; (b) reaction with rabbit anti- P_{32} serum after transfer from SDS-PAGE to nitrocellulose; (c) reaction with rabbit anti- P_{64} serum after transfer from SDS-PAGE to nitrocellulose. All lanes A contained molecular mass standards: phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa). All lanes D contained purified P_{64} and P_{32} . I: *M. bovis* An5 with Zn^{2+} (lanes B) and without Zn^{2+} (lanes C); *M. tuberculosis* H37Ra without Zn^{2+} (lanes E) and with Zn^{2+} (lanes F). II: *M. kansasii* with Zn^{2+} (lanes B) and without Zn^{2+} (lanes C); *M. avium* 42 without Zn^{2+} (lanes E) and with Zn^{2+} (lanes F). III: *M. fortuitum* with Zn^{2+} (lanes B) and without Zn^{2+} (lanes C); *M. phlei* without Zn^{2+} (lanes E) and with Zn^{2+} (lanes F). All mycobacterial samples contained 5 μg of protein except those used for transfer and reaction with anti- P_{32} serum, which contained 0.4 μg of protein.

Table 2. Amounts of the antigens P_{32} and P_{64} in culture filtrates of mycobacteria grown in normal and Zn^{2+} -deficient media

The amounts of the antigens [μg (mg total proteins) $^{-1}$] were estimated by a dot-blotting assay, using anti-BCG P_{32} or P_{64} , done in duplicate on culture media obtained from two independent experiments. The values reported represent means $\pm$ SD.

Antigen ... Filtrate ...	P_{32}		P_{64}	
	With Zn^{2+}	Without Zn^{2+}	With Zn^{2+}	Without Zn^{2+}
<i>M. bovis</i> AN5	140 $\pm$ 25	128 $\pm$ 12	12 $\pm$ 3	110 $\pm$ 15
<i>M. tuberculosis</i> H37Ra	155 $\pm$ 20	105 $\pm$ 15	16 $\pm$ 5	252 $\pm$ 34
<i>M. avium</i> 42	175 $\pm$ 17	115 $\pm$ 20	15 $\pm$ 3	185 $\pm$ 20
<i>M. kansasii</i> 190	190 $\pm$ 20	118 $\pm$ 15	12 $\pm$ 3	180 $\pm$ 25
<i>M. fortuitum</i> 141	48 $\pm$ 7	27 $\pm$ 5	10 $\pm$ 1	25 $\pm$ 2
<i>M. phlei</i> AM76	41 $\pm$ 6	30 $\pm$ 8	15 $\pm$ 3	18 $\pm$ 6

percentage of total proteins it was increased 8- to 10-fold as compared with control cultures, while the proportion of P_{32} remained unchanged (data not shown). Comparison of the proteins released into BCG culture media in response to Zn^{2+} deficiency or to a shift of growth temperature suggests that the release of P_{64} is not a specific consequence of Zn^{2+} deficiency but is a more general response to environmental stress conditions.

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