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Evidence for Three Separate Genes Encoding the Proteins of the Mycobacterial Antigen 85 Complex

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The secreted *Mycobacterium bovis* BCG antigen 85 complex, which is known to bind to human fibronectin, consists of three closely related cross-reacting antigens. Amino-terminal sequence analysis of the purified proteins showed distinct differences. Data are presented to show that the three components are produced by individual cells, which indicates that three separate genes are involved.

In crossed immunoelectrophoresis (CIE), the antigen 85 complex is a major precipitate in the *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* H37Rv patterns (3, 15, 18). It cross-reacts widely with different species of mycobacteria (7, 20).

Quantification of the components in culture fluids and bacterial sonic extracts shows that the proteins of the 85 antigen complex are actively secreted from the mycobacterial cells (2, 3, 6, 18, 19). This corresponds to the demonstration by Matsuo et al. (11) of a typical leader sequence upon cloning of the corresponding alpha-antigen gene. The current interest in this antigen complex was markedly enhanced by the demonstration of fibronectin binding to BCG85A and BCG85B (1). Antigen 85A (P32) (5) has also been recognized as an inducer of gamma-interferon production in peripheral blood mononuclear cells of patients with tuberculosis (9).

BCG Copenhagen substrain 1331 was kindly provided by Statens Seruminstitut, Copenhagen, Denmark. The other BCG strains, i.e., Tokyo substrain 172, Glaxo substrain 1077, Pasteur substrain 1173P2, Moreau, Tice, Sweden, and Russia, and *M. tuberculosis* H37Rv ATCC 27294 were grown in Japan as described elsewhere (8).

The polyclonal rabbit antiserum K1019A, which gave three precipitate lines with the antigen 85 complex in CIE, has been described previously (19). Polyvalent anti-BCG immunoglobulin (lot 063; DAKO Immunoglobulins, Copenhagen, Denmark) was used in CIE to control the purity of isolated protein fractions. CIE and fused rocket immunoelectrophoresis were performed as described in detail previously (3, 19).

BCG85A and BCG85B were purified with hydroxyapatite by DEAE-Sephacel and phenyl-Sepharose chromatography (19). The DEAE column was eluted with a gradient instead of stepwise. To obtain sufficient purity for the sequence determination, BCG85A and BCG85B were subjected to anion-exchange chromatography on MonoQ HR 5/5 (Pharmacia, Uppsala, Sweden) equilibrated with 30 mM Tris hydrochloride (pH 8.7) eluting with a gradient of 0 to 0.3 M NaCl. BCG85B was further subjected to gel filtration on Superose 12 (Pharmacia) and hydrophobic interaction chromatography on phenyl-Superose HR 5/5 (Pharmacia) which was equilibrated with 50 mM phosphate buffer (pH 6.8) and eluted with decreasing molarity. Finally, an additional step on MonoQ was performed. Purification of BCG85C involved the same steps as were used for the purification of BCG85A. Other fractions were recovered by fused rocket immunoelectrophoresis with the K1019A antiserum as an indicator for selection of fractions.

Amino-terminal sequences were determined by automatic Edman degradation by an Applied Biosystems 477A gas-phase sequenator with an on-line 120A PTH amino acid analyzer.

To delineate the relationship between the different components of the 85 complex and to avoid any difficulties due to DNA polymorphism in BCG (4), we purified the BCG85A, BCG85B, and BCG85C components from the same batch of BCG Copenhagen culture fluid. It was found that the amino-terminal sequence of BCG85B (Fig. 1) was identical to the sequence deduced by Matsuo et al. (11) from the BCG alpha-antigen gene. By two-dimensional polyacrylamide gel electrophoresis (12), MPB59 (antigen 85B) and the native alpha-antigen had the same position, while MPB44 (antigen 85A) had a distinctly different localization (data not shown). Alanine appeared for the first time in the BCG85A sequence at position 31. Leucine appeared at positions 36 and 11, but the distance between these two residues precludes leftover as the basis for this identification. The differences between alanine and asparagine and between leucine and valine are very marked in the identification step. At position 31, the substitution would require two base changes in the corresponding codon. In the BCG85C sequence, there was a single exchange at position 16. Again, the first occurrence of alanine is at position 31, and the chemical distinction between alanine and proline is technically very clear. Exchanges of proline with alanine and asparagine with alanine are known to have a marked influence on the tertiary structure of the polypeptide chain.

The central question is whether the three precipitate lines of the antigen 85 complex reflect heterogeneity of the bacterial population in the culture or a situation in which individual bacterial cells synthesize and secrete the three different components. In all of the eight different substrains of BCG and in *M. tuberculosis* H37Rv, we observed the same pattern, i.e., three distinct precipitate lines, in this complex. The culture fluid of BCG Pasteur substrain 1173P2 was obtained by secondary culture of a clone obtained by colony picking from a primary culture (Fig. 2) (13). If the three components were derived from different cells, the BCG Pasteur clone must have changed during culture, again

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	1								10								20
Alpha-gene	F	S	R	P	G	L	P	V	E	Y	L	Q	V	P	S	P	S	M	G	R
BCG85B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BCG85A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BCG85C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-

	21								30								40				45
Alpha-gene	D	I	K	V	Q	F	Q	S	G	G	N	N	S	P	A	V	Y	L	L	D	G	L	R	A	Q
BCG85B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BCG85A	-	-	-	-	-	-	-	-	-	A	-	-	-	-	L	-	-	-							
BCG85C	-	-	-	-	-	-																			

FIG. 1. N-terminal amino acid sequences of BCG85A, BCG85B, and BCG85C compared with the sequence deduced from the alpha-antigen gene (11). –, Identical residue.

producing the three antigens from several mutated cells. This is highly unlikely. Thus, the consistent occurrence of the three components in all of these culture fluids strongly indicates that BCG85A, BCG85B, and BCG85C are synthesized by individual bacterial cells.

The individual components have distinct immunological properties, even though the components are very similar in molecular weight. This observation favors the view that the three components are synthesized independently rather than being the result of postsynthetic modifications or enzymatic degradation.

The N-terminal sequence described previously in our studies on the MPB59 protein (20) differs at positions 20 and 21 from the sequences described here. The explanation for this difference has not been definitely established. The previous finding of glycine at position 20 might be due to a technical error with the leftover from the previous step in view of the low signal provided by arginine. However, the finding of cysteine instead of aspartic acid at position 21 cannot be explained in this way.

Preliminary evidence from restriction mapping has indicated two genes for the 30,000- and 31,000-kilodalton antigens of *M. tuberculosis* that correspond to the antigen 85 complex (17; C. Abou-Zeid, personal communication). Our observations on the structural differences between BCG85A, BCG85B, and BCG85C could be explained by the occurrence of three corresponding structural genes (arising from a single original gene) which multiplied and diversified. Gene duplication in eucaryotes is known. It allows for sufficient synthesis of proteins in great demand during regular physiological processes. A similar mechanism has been demonstrated for synthesis of rRNA in procaryotes (10). In mycobacteria, the number of gene copies for rRNA is surprisingly low and related to bacterial growth. *Mycobacterium leprae* and other slow-growing species have one set and rapid-growing species have two sets of genes encoding rRNA (16). The 85 complex is obviously of a different kind but might well be required in relatively large amounts for optimal growth of mycobacteria.

In *M. tuberculosis* H37Rv, antigen 85 constitutes more than 30% of the total protein of the secreted antigens. The physiological significance of this observation has not been established but may be related to the demonstration of antigen 85 serving as a fibronectin receptor (1, 14), since fibronectin may be of particular significance for phagocytosis of mycobacteria and thus for the ability of these organisms to survive intracellularly in the host in vivo.

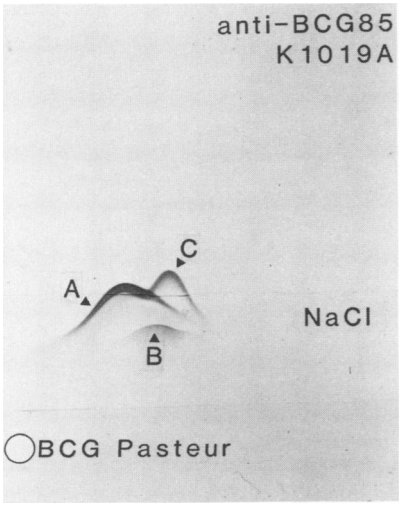

FIG. 2. The circular antigen well contained 5 μl of BCG Pasteur culture fluid at a concentration of 3 mg/ml, the intermediate gel contained only buffer, and the top gel contained 200 μl of rabbit anti-BCG85 antiserum (K1019A), which precipitated the three constituent components of the BCG85 complex, i.e., BCG85A (A), BCG85B (B), and BCG85C (C). The plate was stained with Coomassie brilliant blue R-250.

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ADDENDUM IN PROOF

Borremans et al. have sequenced a gene encoding a 32-kilodalton protein of *M. tuberculosis* (M. Borremans, L. de Wit, G. Volckaert, J. Ooms, J. De Bruyn, K. Huygen, J.-P. van Vooren, M. Stelandre, R. Verhofstadt, and J. Content, Infect. Immun. **57**:3123-3130, 1989). The sequence corresponds to our amino-terminal sequence of BCG85A with the specific exchanges in positions 31 and 36. The molecular masses calculated from the deduced sequences of the alpha-antigen gene and the 32-kilodalton-protein gene show the same difference as those the purified native BCG85A and BCG85B antigens give in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19). We conclude that two of the three genes we propose in this paper have now been identified.

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