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Immunogenic synthetic peptides against mycobacteria of potential immunodiagnostic and immunoprophylactic value

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Introduction

Tuberculosis continues to be a great public health problem throughout the world. 30.000.000. new cases are reported anually of whom 5.000.000 die per year.

In search for new immunoprophylactic methods to control this disease, a series of chemical and immunological studies of the Koch's bacilli, capable of inducing a protective immune response against the infection caused by the *Mycobacterium tuberculosis* have been developed.

The great chemical complexity of this microorganism is seen when analyzing the *Mycobacterium tuberculosis* sonicates on Coomassie-blue stained SDS-PAGE. A large number of protein bands of different sizes can be observed, with molecular weights ranging from 175KD (Kilodaltons) to peptides of 6.000 daltons.

Despite the numerous studies on the composition of the *M. tuberculosis* bacilli, little is known about the chemical structure of its proteins mainly due to the fact that only a few have been isolated and characterized in detail.

To understand the biology of *M. tuberculosis* our laboratory started working on the isolation and chemical and immunological characterization of a series of these molecules, centering its interest in the molecules capable of inducing an immunogenic specific response in human beings. This research is the purpose of this report.

Identification of immunogenic molecules of *M. tuberculosis*

Studies of the specific humoral immune response against the proteins of the *M. tuberculosis* sonicates developed by the Western blot technique, using sera from more than 400 patients with pulmonary tuberculosis in different stages of development of the disease, show the great antigenic complexity of the *Mycobacterium tuberculosis*.

When analyzing the reactivity of these sera, according to evolutionary patterns of the disease (Fig. 1), it was found that the individuals in the acute or active stage of tuberculosis presented antibodies against molecules in the range of 80KD to 50KD of molecular weight, thus suggesting that these proteins may not play a critical role in the defense against the infection.

When treatment has been started and the patients begin to recover the antibody levels against proteins between 80KD and 50KD rise, and the bands become very prominent. Simultaneously, in these sera appear antibodies against another group of proteins of variable

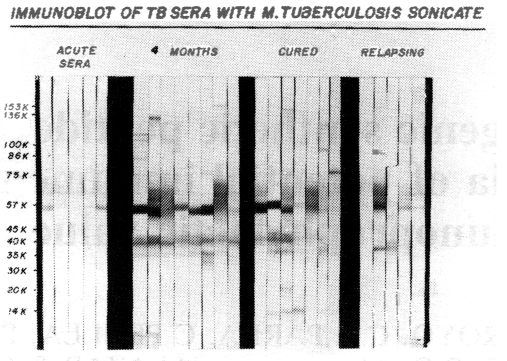

Fig. 1.

molecular weight, the most prominent being the ones of 43KD, 35KD and 30KD. Antibodies directed against proteins of 153KD, 140KD, 120KD, 115KD, 100KD and 86KD of molecular weight appear in some individuals in a variable pattern.

When a patient, after a year of treatment, according to all clinical, radiological and laboratory criteria is considered cured, it is found consistently that the antibody levels against proteins of 80KD to 50KD continue to be high, as well as those directed against the molecules of 43KD, 35KD and 30KD. In some patients antibodies against molecules of 155KD, 140KD, 100KD and 86KD are found without a consistent pattern.

Systematically, in a peculiar way, the sera of these cured patients show a high level of antibodies against the 13KD molecule.

The analysis of sera of some relapsing patients showed that antibodies against the molecules of 80KD to 50KD decrease considerably and that the antibodies against the proteins of 43KD, 33KD and 13KD totally disappear, while circumstantially showing in some patients antibodies against proteins of 155KD, 140KD, 120KD, 110KD, 86KD and 35KD.

The disappearance of the antibodies directed against the molecules of 43KD, 33KD and 130KD during relapse, suggest that antibodies against these proteins can be directly involved in the control process of the disease since they are apparent in recovering and cured patients but not in the relapses or the acute stages of the disease.

The search for these molecules in other mycobacterial sonicates by the Western blot technique, studied with the same tuberculosis patients' sera, showed that the proteins of 43KD, 33KD and 13KD identified in sonicates of the *M. tuberculosis* are not found in sonicates of BCG, Pasteur sub-strain (Fig. 2). The molecules of 144KD, 110KD, 86KD and 68KD are not found in the BCG, Pasteur sub-strain strongly suggesting that these molecules are selectively found or at least in a higher concentration level in *Mycobacterium tuberculosis*.

Immunologic studies of some *M. tuberculosis* isolated proteins

Based on this data the isolation of these proteins was carried out by different methods, obtaining milligram quantities of molecules of 13KD, 30KD, 33KD, 43KD, 50KD, 60KD, 67KD, 78KD, 86KD and 175KD against which antisera in rabbits were produced.

The exchange of reagents with Dr. M. Harboe from the University of Oslo, showed that some of these antisera had not been only recognizing molecules previously identified in the BCG reference system, but also that some molecules present in the *Mycobacterium tuberculosis* sonicates, had not been identified in the BCG reference system.

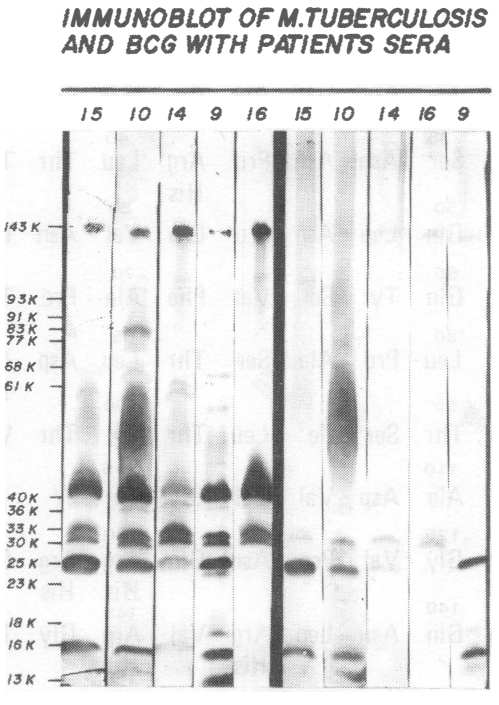

Fig. 2.

The molecule of 175KD corresponds to either the Beta antigen, *M. leprae* antigen or BCG-51; the protein 50KD corresponds to BCG-20; the molecule of 47KD is immunologically similar to the BCG-63; the molecule of 43KD present in high concentrations of *Mycobacterium tuberculosis* was not detected in the reference system of BCG the same as the molecules 86KD, 33KD and 13KD.

These data are in agreement with the information obtained from the patients' sera where the proteins 86KD, 43KD, 33KD and 13 KD of molecular weight, selectively recognized by the patients' sera as molecules present in high concentrations in the *Mycobacterium tuberculosis* sonicates were not found in the BCG Pasteur sub-strain sonicates.

Immunochemical studies of mycobacterial proteins

As a result of the collaborative study with Harboe's group, we received from them a series of identified and isolated proteins denominated MPB-70, MPB-80, MPT-59 and MPB-64 from which our group determined the amino acid sequences.

The complete amino acid sequence of MPB-70 was determined (Table 1), and it was found identical in the first 35 N terminal residues to the MPB-80. The N terminal amino acid sequences of the MPB-64, MPB-59 and MPT-59 molecules were also identified (Table 2).

The partial amino acid sequence from the molecules of 13KD, 33KD, 35KD, 40KD, 68KD, 77KD and 86KD were also obtained from the isolated proteins of *M. tuberculosis* (Table 3).

				5					10					15
Gly	Asp	Leu	Val	Gly	Pro	Gly	Val	Ala	Glu	Tyr	Ala	Ala	Ala	Asn
Pro	Thr	Gly	Glu	20 Ala	Ser	Val	Gln	Gly	25 Met	Ser	Gln	Asp	Pro	30 Val
Ala	Val	Ala	Ala	35 Ser	Asn	Asn	Pro	Arg His	40 Leu	Thr	Thr	Leu	Thr	45 Ala
Ala	Leu	Ser	Gly	50 Gln	Leu	Asn	Pro	Gln	55 Val	Asn	Leu	Val	Asn	60 Thr
Leu	Asn	His	Gly	65 Gln	Tyr	Ser	Val	Phe	70 Ala	Pro	Thr	Asn	Ala	75 Ala
Phe	Ser	Ser	Lys	80 Leu	Pro	Ala	Ser	Thr	85 Leu	Asp	Arg His	Leu	Thr	90 Asn
Ser	Ser	Leu	Leu	95 Thr	Ser	Ile	Leu	Thr	100 Tyr	Thr	Val	Val	Ala	105 Gly
Gln	Thr	Ser	Pro	110 Ala	Asp	Val	Val	Gly	115 Thr	//	//	Val	Ile	120 Gly
Arg His	Arg His	Pro	Gly	125 Gly	Val	Pro	Asn	Gln	130 Arg His	Arg His	Gly	Asp	//	135 //
Lys	Gly	Val	Asn	140 Gln	Asn	Leu	Arg His	Val	145 Arg His	Gly	Tyr	Pro	Gly	150 Arg His
Lys	Pro	Val	Ala											

Table 1. Amino acid sequence of MPB 70

Immunogenic synthetic peptides against mycobacteria

Based on these amino acid sequences and trying to identify epitopes of potential immunogenic value against *Mycobacterium tuberculosis* the chemical synthesis of a series of these peptides corresponding to amphilicity or alphahydrophilic areas of these molecules was carried out, obtaining them in a pure form according to their HPLC chromatograms (Table 4).

The antigenicity study of the synthetized peptides corresponding to an amphilitic area of the 13KD molecule showed that this peptide reacted in the Dot-Blot test, with sera of patients who had recovered from tuberculosis but not with sera of patients in the acute stages of the disease, similar to what happens with the complete protein. These data strongly suggest that this epitope can be involved in the protective immune response against *Mycobacterium tuberculosis.*

Studies on delayed type hypersensitivity (DTH) reactions showed the existence of specific reactivities against the synthetic peptides: the sythetic peptide of the 13KD molecule induced a selective DTH response to *Mycobacterium tuberculosis* not observed when the guinea pigs were immunized with other mycobacterial sonicates or lysates. Similar circumstances happened with the peptide corresponding to the MPT-64 molecule where no delayed hypersensitivity was observed in animals immunized with mycobacteria different from *Mycobacterium tuberculosis*. These data are in concordance with the selective expression of this protein in the Koch bacilli (Table 5).

Histopathology studies showed on the peptide innoculation site a classical delayed type hypersensitivity response with a marked infiltration of lymphoid and monocytic as well as

MPB-70	Gly	Asp	Leu	Val	Gly	Pro	Gly	Val	Ala	Glu	Tyr	Ala	Ala	Ala	Asn
18 KD	Pro	Thr	Gly	Glu	Ala	Ser	Val	Gln	Gly	Met	Ser	Gln	Asp	Pro	Val
MPB-80	Gly	Asp	Leu	Val	Gly	Pro	Gly	Val	Ala	Glu	Tyr	Ala	Ala	Ala	Asn
18 KD	Pro	Thr	Gly	Glu	Ala	Ser	Val	Gln	Gly	Met	Ser	Gln	Asp	Pro	Val
MPB-59	Phe	Ser	Arg His	Pro	Gly	Leu	Pro	Val	Glu	Tyr	Leu	Gln	Val	Pro	Ser
30 KD	Pro	Ser	Met	Gly	Gly	Cys	Ile	Lys	Val	Gln	Phe	Gln	Ser	Gly	Gly
MPB-64	Ala	Pro	Lys	Thr	Tyr	Arg His	Glu	Glu	Leu	Lys	Gly	Thr	Asp	Thr	Gly
23 KD	Gln	Ala	Tyr	Gln	Ile	Gln	Met	Ser	Asp	Pro	Ala	Tyr	Asn	Ile	Asn

Table 2. N-terminals of some BCG isolated proteins

MTP-13	Ala	Lys	Val	Asn	Ile	Lys	Pro	Glu Leu	Arg	Asp	Lys	Ile	Leu	Val	Gln
13 KD	Arg	Asn	Glu	Ala	Arg His	Val	Glu	Ala	Glu	Glu	Glu	Val	Ala	Ala	Ala
MTP-59	Phe	Ser	Arg His	Pro	Gly	Leu	Pro	Val	Glu	Tyr	Leu	Gln	Val	Pro	Ser
30 KD	Pro	Ser	Met	Gly	Gly	Cys	Ile	Lys	Val	Gln	Phe	Gln	Ser	Gly	Gly
MTP-33	Trp	Ser	Ser	Leu	Glu	Glu	Gly	Thr	Leu	Thr	Thr	Arg His	Ala	Tyr	Glu
33 KD	Leu	Arg His	Gly	Glu											
MTP-35	Trp	Val	Glu	Val	Arg His	Gln	Val	Trp	Val	Trp	Arg His	Leu	Arg His	Trp	Arg His
35 KD	Arg His	Thr	Glu	Leu	Asn	Trp	Leu	Val	Arp	Arg His	Leu	Arg His	Arg His	Ser	Leu
MTP-43 43 KD	Ser	Pro	Trp	Ile	Leu	Lys	Gly	Lys	Ala	Lys					
MTP-68 68 KD	Trp	Met	Thr	Met	Thr	–	–	–	Cys	–	–	Cys			
MTP-77	Gly	Lys	Arg His	Ile	Ala	Tyr	Asp	Arg	Glu	Ala	Ala	Ala	Ala	Ala	Leu
77 KD	Leu	Leu	Leu												

Table 3. N-terminals of some M. tuberculosis *isolated proteins*

some plasma cells. This demonstrates that the synthetic peptides corresponding to a molecular sequence of amono acids pertaining selectively to *M. tuberculosis* or other mycobacteria can be used in a near future as potentially useful reagents for immunodiagnosis since they permit the detection of antibodies or specific immunologic cellular response for each of the mycobacteria under study.

Acknowledgements
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Peptide Nr.	Molecule	Residues	Sequence															
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SM 8	MTP 13	1 - 9	Ala	Lys	Val	Gln	Ile	Lys	Pro	Glu	Arg							
SM 9	MPB 70	19 - 28	Glu	Ala	His	Val	Glu	Ala	Glu	Glu	His	Val						
SM 14	MPB 64	1-16	Ala	Pro	Leu	Thr	Tyr	Arg	Glu	Glu	Leu	Lys	Gly	Gly	Asp	Thr	Gly	Gln
SM 15	MPB 59	19-30	Gly	Gly	Cys	Ile	Lys	Val	Gln	Phe	Gln	His	Gly	Gly				
SM 67	MTP 33	1-12	Trp	Ser	Ser	Leu	Glu	Glu	Gly	Thr	Leu	Thr	Thr	Arg				
SM 68	MTP 35	10-24	Trp	Arg	Leu	Arg	Trp	Arg	Arg	Thr	Glu	Leu	Asn	Trp	Leu	Val	Arg	
SM 69	MTP 40	1-10	Ser	Pro	Trp	Ile	Leu	Lys	Gly	Lys	Ala	Lys						
SM 70	MTP 35	25-37	Arg	leu	Arg	Arg	Ser	Leu	Leu	Leu	Arg	Arg	Thr	Trp	Trp			
SM 71	MTP 33	7-19	Gly	Thr	Leu	Thr	Thr	Arg	Ala	Tyr	Glu	Leu	Arg	Gly	Glu			
SM 72	MTP 13	8-22	Leu	Arg	Asp	Lys	Ile	Leu	Val	Gln	Arg	Asn	Glu	Ala	Arg	Val		

Table 4. Peptides synthetized for DTH studies

	Micrograms in 0.1 ml	M. tub. H37 Rv n = 7	BCG Pasteur n = 5	M. kansasii n = 5
SM 8 MTP 13 K	50	4 ± 1	0	0
	100	6 ± 3	0	0
	250	7 ± 3	0	0
SM 9 MPB 70 K	50	0	0	0
	100	0	0	0
	250	0	0	0
SM 14 MPB 64 K	50	0	0	0
	100	0	0	2.5 ± 3
	250	6 ± 4	3	4 ± 3
SM 15 MPB 59 K	50	3 ± 2	0	0
	100	7 ± 3	0	2
	250	10 ± 3	0	5.1 ± 2
		4/7		

Table 5. DTH of some synthetic peptides