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Use of recombinant purified protein derivative (PPD) antigens as specific skin test for tuberculosis

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***Background & objectives*: Purified protein derivative (PPD) is currently the only available skin test reagent used worldwide for the diagnosis of tuberculosis (TB). The aim of this study was to develop a *Mycobacterium tuberculosis* specific skin test reagent, without false positive results due to Bacillus Calmette-Guerin (BCG) vaccination using recombinant antigens.**

***Methods*: Proteins in PPD IC-65 were analyzed by tandem mass spectrometry and compared to proteins in *M. tuberculosis* culture filtrate; 54 proteins were found in common. Top candidates MPT64, ESAT 6, and CFP 10 were overexpressed in *Escherichia coli* expression strains and purified as recombinant proteins. To formulate optimal immunodiagnostic PPD cocktails, the antigens were evaluated by skin testing guinea pigs sensitized with *M. tuberculosis* H37Rv and BCG.**

***Results*: For single antigens and a cocktail mixture of these antigens, best results were obtained using 3 µg/0.1 ml, equivalent to 105 TU (tuberculin units). Each animal was simultaneously tested with PPD IC-65, 2 TU/0.1 ml, as reference. Reactivity of the multi-antigen cocktail was greater than that of any single antigen. The skin test results were between 34.3 and 76.6 per cent the level of reactivity compared to that of the reference when single antigens were tested and 124 per cent the level of reactivity compared to the reference for the multi-antigen cocktail.**

***Interpretation & conclusions*: Our results showed that this specific cocktail could represent a potential candidate for a new skin diagnostic test for TB.**

Key words Purified protein derivative (PPD) - recombinant antigens - skin test - tuberculosis

Tuberculin (purified protein derivative, PPD) is currently the only available skin test reagent used for the diagnosis of tuberculosis (TB) or for detection of latent TB infection (LTBI). Although tuberculin skin test (TST) has a remarkable sensitivity and has been in use for five decades to identify people infected with *Mycobacterium tuberculosis*, it has a lower specificity to TB, when compared to other mycobacterial species in the *M. tuberculosis* complex, such as BCG[1,2]. Cross-reactions due to vaccination with BCG, an attenuated

strain of *M. bovis* or exposure to, or infection with environmental mycobacteria (EM) can also lead to false positive results. Romania has a high level of endemic TB, and in 2008 the notification rate for TB disease (prevalence of TB) was 105.9 cases per 100,000 people[3].

The TST with the current PPD (PPD IC-65) was introduced in 1965 in Romania and since then it has been utilized as an *in vivo* assay for *M. tuberculosis* infection surveillance and TB diagnosis. Recently published studies[4,5] showed that the efficacy of PPD IC-65 was equal to PPD RT23, the tuberculin most widely used globally for skin testing[6]. The development of an improved skin test reagent for *in vivo* diagnosis of TB with higher specificity and sensitivity, using recombinant antigens specific for *M. tuberculosis* and not found in *M. bovis* BCG or environmental mycobacteria, could represent a useful tool for an accurate diagnosis of TB and can aid in the prevention and control of the disease.

Protein antigens present in the culture filtrate of *M. tuberculosis* including 10 kDa culture filtrate protein (CFP10), 6 kDa early secretory antigen target (ESAT-6) and immunogenic protein MPT 64 (MPT64) were found to distinguish TB patients from BCG-vaccinated subjects and could induce strong immune responses to TB[7,8]. Thus, culture filtrate proteins constitute prime candidates as potential tools for a diagnostic skin test[9]. Most of these proteins, which have been characterized by gene cloning and nucleotide sequencing[10-12] are potent antigens[13]. A new tuberculin should contain many antigens, since cocktails of multiple antigens are needed to elicit strong delalyed type hypersensitivity (DTH) responses[13] and to cover the broad spectrum of antigen recognition by different individuals[14]. Colangeli *et al*[15] showed that cocktails of *M. tuberculosis* complex-specific antigens elicit DTH responses that distinguish TB infection from sensitization with nontuberculous mycobacteria.

Three dominant antigens secreted by *M. tuberculosis* during its early and active growth phase, ESAT-6, CFP10, and MPT64, were present in PPD IC-65, and thus these may elicit strong, specific DTH responses. These proteins, known to induce strong immune responses to TB were found to distinguish TB patients from BCG-vaccinated subjects[16].

The development of a new tuberculin that will allow discrimination by a skin test of LTBI from vaccination with BCG without false positive results due to BCG vaccination, based on specific antigens will have to maintain the PPD sensitivity to emphasize TB infection. In the present study, we evaluated the diagnostic potential of intradermal recombinant mycobacterial antigens in guinea pigs as a pre-clinical study for human use.

Material & Methods

The study was performed between 2008-2010 at Mycobacterial Antigen and Enzymology & Applied Microbiology Laboratories, Cantacuzino Institute, Bucharest, Romania.

Bacterial strains and products: *M. tuberculosis* $H_{37}Rv$ was obtained from Pasteur Institute, Paris, France. *M. bovis* BCG Romanian substrain was initially obtained by Cantacuzino Institute from Pasteur Institute, kept and continuously utilized for BCG vaccine production in Romania. Mycobacteria were cultured on the surface of Sauton medium, reaching full growth after 6-8 wk at 37°C.

Escherichia coli BL15 expression strain was grown with standard liquid and solid media. PPD IC-65, master batch # 20, was prepared in Cantacuzino Institute, Bucharest, Romania. The internationally used 2 TU PPD RT23 dosage is considered equivalent to the 5 TU PPD-S formulation[17].

Digestion of the whole sample in solution: One mg of PPD IC-65 Master batch #20 was reduced in 6M guanidine HCl with dithiothretol (DTT), alkylated with iodacetamide, exchanged into 50 mM ammonium bicarbonate, dried, resuspended in 10 per cent ACN/ 0.2 M ammonium bicarbonate, and proteins digested with trypsin (1:50 E/S ratio). Digested PPD was analyzed by liquid chromatography-mass spectrometry (LC-MS) using a nano-spray liquid chromatography-linear trap quadrupole (LC-LTQ), data extracted using BioWorks (Thermo Finnigan, San Jose, CA), searched against the *M. tuberculosis* genome (GenBank accession number AL123456, 3991 entries) using Sequest (Thermo Finnigan, USA) and Mascot (Matrix Science, London, UK) and analysis validated using Scaffold (Proteome Software, Portland, OR) as described previously[18].

In solution digestion of the crude PPD: Digested PPD was analyzed by LC-MS using a nano-spray LC-LTQ and triplicate injections. Tandem mass spectra were extracted and charge state deconvoluted by Bioworks version 3.3 (Thermo Finnigan). All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, USA; version v.27, rev. 11) and X! Tandem (The GPM, thegpm.org; version 2007.01.01.1). Both search engines were set up to search the Mtb_V3 database

(GenBank accession number AL123456, 3991 entries) assuming digestion with trypsin, fragment ion mass tolerance of 1.00 Da, parent ion tolerance of 1.5 Da, and a maximum of 4 missed cleavages. Oxidations of methionine and iodoacetamide derivative of cysteine were specified in Sequest and X! Tandem as variable modifications.

Scaffold (version Scaffold_3_00_01, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if these could be established at greater than 95.0 per cent probability as specified by the Peptide Prophet algorithm[19]. Peptide identifications were also required to exceed specific database search engine thresholds. Sequest identifications required at least deltaCn scores of greater than 0.10 and XCorr scores of greater than 1.5, 2.2, 3.0 for single-, double-, and triple-charged peptides, X! Tandem identifications required at least - Log (Expect Scores) scores of greater than 2.0. Protein identifications were accepted if these could be established at greater than 99.0 per cent probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm[20]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

The GenBank accession number for the CFP-10, ESAT-6 and MPT-64 genes are: gi| 15611010 |ref| NP_218391.1|, gi |57117165 |ref| YP_178023.1| and gi |15609117 |ref| NP_216496.1|, respectively.

Bacterial strains, plasmids, growth conditions and DNA manipulations: General DNA manipulations were performed as previously described[21]. The *M. tuberculosis* H37Rv gene Rv3874, encoding CFP10, was amplified from chromosome DNA as template. The PCR product was inserted into the vector pET24a and the expression construct was transformed into *E. coli* BL15. The recombinant strains were grown in 2YT medium supplemented with kanamycin and chloramphenicol to an absorbance of 1.5 at 600 nm. Overexpression was induced with 1mM isopropyl-β-D-thiogalactoside for 3 h at 37°C. The cells were then harvested by centrifugation and served as source for protein purification.

Recombinant plasmids pMRLB.7 and pMRLB.12A containing *Rv3875* and *Rv1980c* genes respectively, obtained through the TB Vaccine Testing and Research Materials Contract (Colorado State University, NIH/NIAID Contract #HHSN266200400091C), were introduced into *E. coli* BL 15 to overexpress ESAT-6 and MPT64 proteins. The recombinant strains were grown in 2YT medium supplemented with 100 µg/ml ampicilline and 30 µg/ml chloramphenicol (Sigma, USA) to an absorbance of 0.5 at 600 nm, then overexpression was induced by isopropyl-β-D-thiogalactoside induction (0.25 mM for ESAT-6 and 0.5 mM for MPT64, final concentrations) for 12 h at 25°C (ESAT-6) or 4 h at 37°C (MPT64). The cells were then harvested by centrifugation and served as the source for protein purification.

Purification of mycobacterial proteins: The CFP10 protein was purified by ion exchange chromatography on DEAE-Sephacel, using an increasing salt gradient (0-0.5M NaCl), followed by size exclusion chromatography on AcA54. N-terminal His-tagged ESAT-6 and MPT64 proteins were purified by Nickel-nitriloacetic acid affinity chromatography using the QIA express system[22]. Protein concentration was measured according to Bradford[23]. SDS-PAGE was performed as described by Laemmli[24]. Purified proteins were conditioned in PBS and stored at -20°C before use.

DTH responses in guinea pigs: Twelve groups of six white guinea pigs (female : male ratio of 1:1) weighing approximately 300 g, obtained from Cantacuzino Institute Animal facility and maintained under specific pathogen free conditions, were tested. Four groups were sensitized with inactivated, dried *M. tuberculosis* H37Rv cells, mixed with incomplete Freund's adjuvant (0.4 mg/ml) by intramuscular (i.m.) injections in each of the four limbs, for a total of 0.5 ml /animal; 4 groups were similarly sensitized with live *M. bovis* BCG cells (0.4 mg/ml), and 4 groups were mock sensitized by intradermal (i.d.) injection of phosphate buffered saline.

The assay of potency by measuring DTH responses was performed according to European Pharmacopoeia (Eur. Ph.) 7.0[25] using inactivated *M. tuberculosis* cells. For sensitization, the guinea pigs were injected i.m. with a total of 0.5 ml suspension, divided between (each of) the four limbs. Five weeks after sensitization, animals were epilated on both flanks and three i.d. injections on left side were made with 3 µg/0.1 ml of each purified recombinant antigen (CFP10, ESAT-6, MPT64) and different cocktail mixtures (1 to 3 µg/0.1 ml per antigen) dissolved in phosphate buffered saline containing 0.05 per cent Tween and with the *p*H adjusted between 6.5 and 7.5. Each animal was also

injected on the right flank with 2 TU/ 0.1 ml of PPD to control for sensitization and for the specificity of the assay. Skin lesions were measured and recorded 24 h after antigen injection.

The length and the width of the lesions were measured and the area calculated. To ensure that the suffering of the animals is kept to a minimum, all manipulations were performed according to the study protocol approved by local Animal Ethics Committee (IRB #0002508). All animal manipulations were carried out by trained technicians with more than 5 years experience in animal experimentation.

Results

PPD IC-65 Master batch 20 was digested, analyzed by LC-MS/MS and interrogated against the *M. tuberculosis* genome. This led to the identification of 132 proteins in PPD IC-65 (data not shown). Of these, 54 proteins were found to be common with those identified in the *M. tuberculosis* culture filtrate[26], 21 annotated as known function (Table I) and 33 as hypothetical, putative or possible proteins.

A search was initiated for DTH-active antigens expressed by *M. tuberculosis* but not by *M. bovis* BCG. CFP10, ESAT-6 and MPT64 were over-expressed and purified (Fig. 1). Corresponding spots were identified in different culture filtrates of *M. tuberculosis* H37Rv (Fig. 2). The secreted antigens were clearly visible in the two-week old culture filtrate.

To investigate the species specificity of the recombinant antigens, DTH reactions were measured in guinea pigs sensitized with live *M. bovis* BCG, with inactivated *M. tuberculosis* H37Rv and mock sensitized

Table I. Common proteins identified in PPD IC-65 and *M. tuberculosis* culture filtrate

#	Protein description	Accession number	Unique peptides*	*M. bovis* AF2122/97**	*M. bovis* BCG strain Pasteur 1173P2**
1	Molecular chaperone DnaK [MASS=66830]	gi|15607491|ref|NP_214864.1|	27	NP_854021 molecular chaperone DnaK	YP_976487 molecular chaperone DnaK
2	Co-chaperonin GroES [MASS=10804]	gi|15610554|ref|NP_217935.1|	17	NP_857092 co-chaperonin GroES	YP_979567 co-chaperonin GroES
3	Heat shock protein SPX (ALPHA-CRSTALLIN HOMOLOG) (14 kDa ANTIGEN) (HSP16.3) [MASS=16227]	gi|15609168|ref|NP_216547.1|	15	NP_855707 heat shock protein hspX	YP_978140 heat shock protein hspX
4	Immunogenic protein MPT64 (ANTIGENMPT64/MPB64) [MASS=24855]	gi|15609117|ref|NP_216496.1|	13	NP_855652 Immunogenic protein MPT64 (antigen MPT64/MPB64)	
5	10 KDA culture filtrate antigen ESXB (LHP) (CFP10) [MASS=10794]	gi|15611010|ref|NP_218391.1|	10	NP_857541 10 kDa culture filtrate antigen EsxB	
6	Alanine and proline rich secreted proteinAPA (fibronectin attachment protein) (Immunogenic protein MPT32) (Antigen MPT-32) (45-kDa glycoprotein) (45/47 kDa antigen) [MASS=32720]	gi|57116926|ref|YP_177849.1|	8	CAD94594 alanine and proline rich secreted protein apa	YP_977986 alanine and proline rich secreted protein apa
7	Glutamine synthetaseGLNA1 (glutamine synthase) (GS-I) [MASS=53570]	gi|15609357|ref|NP_216736.1|	7	NP_855893 glutamine synthetase GLNA1 (glutamine synthase) (GS-I)	YP_978326 glutamine synthetase glnA1
8	Periplasmic phosphate-binding lipoproteinPSTS1 (PBP-1) (PSTS1) [MASS=38243]	gi|57116801|ref|YP_177770.1|	6	NP_854616 periplasmic phosphate-binding lipoprotein PSTS1 (PBP-1) (PSTS1)	YP_977082 periplasmic phosphate-binding lipoprotein pstS1

Contd...

#	Protein description	Accession number	Unique peptides*	*M. bovis* AF2122/97**	*M. bovis* BCG strain Pasteur 1173P2**
9	Secreted antigen 85-A FBPA (mycolyl transferase 85A) (fibronectin-binding protein A) (ANTIGEN 85 COMPLEX A) [MASS=35686]	gi|15610940|ref|NP_218321.1|	6	NP_857471 secreted antigen 85-A FBPA (Mycolyl transferase 85A)	YP_979945 secreted antigen 85-A fbpA
10	Secreted antigen85-B FBPB (85B) (ANTIGEN 85 COMPLEX B) (mycolyl transferase 85B) (fibronectin-binding protein B) (extracellular alpha-antigen) [MASS=34581]	gi|15609023|ref|NP_216402.1|	6	NP_855570 secreted antigen 85-B fbpB (85B)	YP_978013 secreted antigen 85-B fbpB
11	Low molecular weight antigen CFP2 (low molecular weight protein antigen 2) (CFP-2) [MASS=16635]	gi|15609513|ref|NP_216892.1|	5	NP_856046 low molecular weight antigen CFP2	YP_978479 low molecular weight antigen cfp2
12	Secreted L-alanine dehydrogenase ALD (40 KDA antigen) (TB43) [MASS=38713]	gi|15609917|ref|NP_217296.1|	5	NP_856449 L-alanine dehydrogenase	YP_978884 secreted l-alanine dehydrogenase aldb
13	Catalase-peroxidase-peroxynitritaseT KATG [MASS=80604]	gi|15609045|ref|NP_216424.1|	5	NP_855594 catalase-peroxidase-peroxynitritase T KATG	YP_978037 catalase-peroxidase-peroxynitritase T katG
14	Immunogenic proteinMPT63 (ANTIGEN MPT63/MPB63) (16 kDa immunoproctective extracellular protein) [MASS=16514]	gi|15609063|ref|NP_216442.1|	4	NP_855611 immunogenic protein MPT63	YP_978054 immunogenic protein mpt63
15	6 KDA early secretory antigenic target ESXA_rec (ESAT-6) [MASS=9904]	gi|57117165|ref|YP_178023.1|	4	NP_857542 6 kDa early secretory antigenic target EsaT6 (EsaT-6)	
16	Thioredoxin TRXC (TRX) (MPT46) [MASS=12544]	gi|15611050|ref|NP_218431.1|	4	NP_857580 thioredoxin trxC (TRX) (MPT46)	YP_976123 thioredoxin trxC (TRX) (MPT46)
17	Thiol peroxidase [MASS=16896]	gi|15609069|ref|NP_216448.1|	3	NP_855617 thiol peroxidase	YP_978060 thiol peroxidase
18	Secreted antigen 85-C FBPC (85C) (antigen 85 complex C) (mycolyl transferase 85C) (fibronectin binding protein C)[MASS=36771]	gi|57116693|ref|YP_177694.1|	3	NP_853801 secreted antigen 85-C FBPC (85C)	YP_976265 secreted antigen 85-c fbpC (85C)
19	Heat shock protein 90 [MASS=72961]	gi|15609436|ref|NP_216815.1|	3	NP_855970 heat shock protein 90	YP_978404 heat shock protein 90
20	Iron-dependent repressor and activator IDER [MASS=25233]	gi|15609848|ref|NP_217227.1|	2	NP_856376 IRON-dependent repressor and activator IDER	YP_978810 Iron-dependent repressor and activator ideR
21	Soluble secreted antigenMPT53 precursor [MASS=18383]	gi|15610015|ref|NP_217394.1|	2	NP_856548 soluble secreted antigen mpb53	YP_978984 soluble secreted antigen mpb53

*Unique peptides = the number of unique peptides found in PPD IC-65 by LC-MS/MS

**Accession number and description of similar proteins related by sequence similarity (BLAST) score to the current protein from *M. tuberculosis*

Only three proteins (bold), from 54 identified in PPD IC65 and *M. tuberculosis* culture filtrate, have no counterpart in *M. bovis* BCG strains sequenced genomes

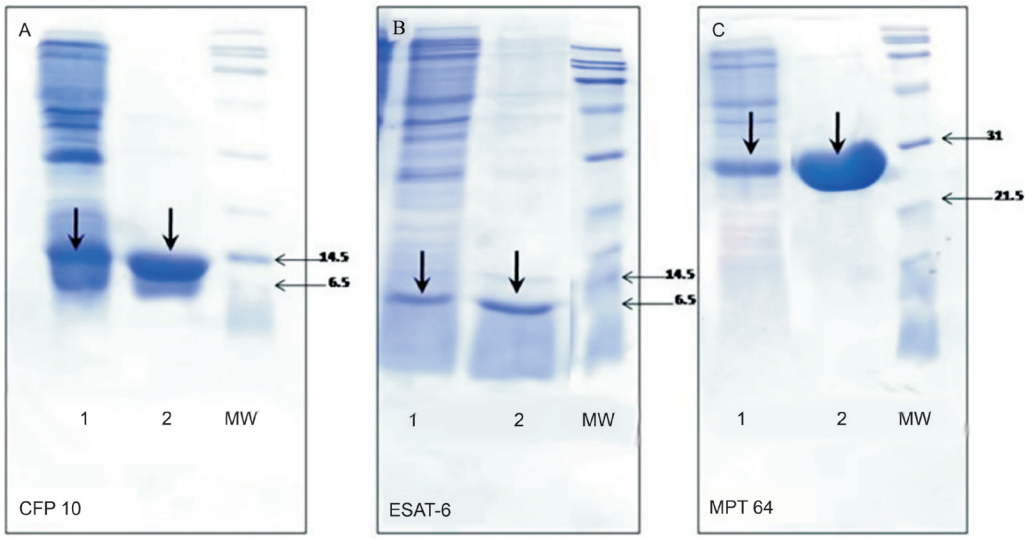

Fig. 1. Purification of CFP-10 **(A)**, ESAT-6 **(B)** and MPT-64 **(C)** recombinant proteins. BE, brute extract; El, purified protein. Molecular markers are indicated in kilodaltons to the right of each panel. The arrows show each specific antigen position.

with PBS. Skin reactivity to CFP10, ESAT-6 and MPT64 was zero in BCG sensitized guinea pigs (Fig. 3, blue bars) and positive in animals sensitized with *M. tuberculosis* H37Rv (Fig. 3, green bars). The DTH responses induced by PPD 2 TU/0.1 ml was similar in BCG and *M. tuberculosis* H37Rv sensitized animals. The mock sensitized animals gave no DTH resposes to any of the antigens or to PPD (yellow bars).

In order to formulate optimal specific immunodiagnostic cocktails, the recombinant antigens (individually or as a mixture) were evaluated by skin testing several guinea pigs groups sensitized with *M. tuberculosis* H37Rv and BCG, as controls. For single antigens, good results were obtained using 3 µg/0.1 ml of each recombinant protein, equivalent to 105 TU/0.1ml; while for the mixture, 1 µg of each recombinant antigen /0.1 ml, gave the highest reaction. Each animal was simultaneously tested with PPD IC-65, 2 TU/0.1 ml, as reference. The BCG controls did not react with any of these antigens, but elicited skin test responses with the reference. Reactivity of multi-antigen cocktails was greater than that of any single antigen, when compared to the reference: 34.3-76.6 per cent for single antigen tests and 124 per cent for cocktail (Table II).

Discussion

The accurate diagnosis of TB infection by skin testing requires a new tuberculin, consisting of defined protein antigens that are unique to *M. tuberculosis.* A new tuberculin should contain many antigens, since cocktails of multiple antigens are needed to elicit strong DTH responses[13,26] and to cover the broad spectrum of antigen recognition by different individuals, typical of TB. Lyashchenko *et al*[13] formulated cocktails of two to eight specific antigens of *M. tuberculosis* complex purified from recombinant *E. coli*, which were evaluated by skin testing in guinea pigs sensitized with *M. bovis* BCG. The mapping of the DTH-inducing epitope of secreted protein MPT64 from *M. tuberculosis* has been performed since 1995[10]. More recently, a double blind randomized phase 1 study was reported assessing the safety of intradermal-recombinant ESAT 6 compared to tuberculin and determining the human dose[16] and the use of recombinant ESAT 6 in a skin test in human volunteers[27].

To develop a better skin test reagent, without false positive results due to BCG vaccination, all the proteins present in a PPD IC-65 concentrated harvest were analysed by tandem mass spectrometry. The three top proteins present in both PPD IC-65 and *M. tuberculosis* culture filtrate: CFP10/ Rv3874, ESAT-6/ Rv3875 and MPT64/ Rv1980c were considered. These unique antigens, secreted by *M. tuberculosis* during its early and active growth phase, and absent from *M. bovis* BCG, Pasteur 1173P2 and Tokyo 172 strains, and also from *M. bovis* AF2122/97 strain could elicit strong, specific DTH responses in guinea pigs.

In our study all three recombinant antigens, known to be immunodominant secreted antigens, elicited

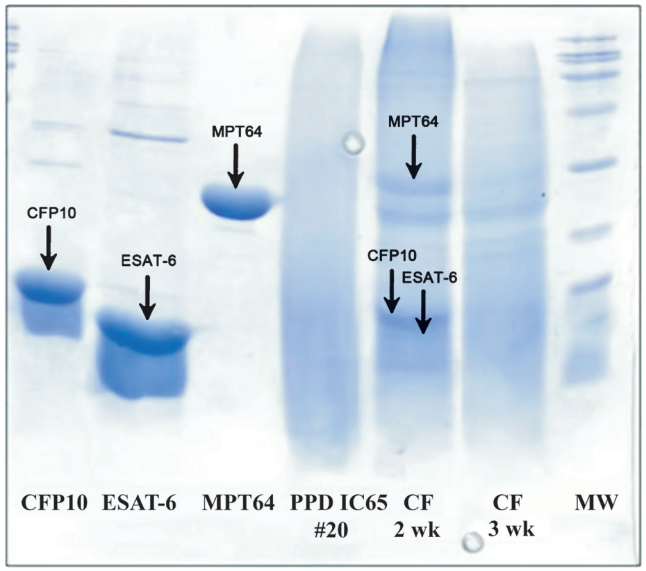

Fig. 2. Presence of CFP-10, ESAT-6, and MPT-64 in two and eight week old, unheated culture filtrates of respectively, *M. tuberculosis* H37Rv, PPD IC-65 Master batch #20 and MW (SDS PAGE 15%).

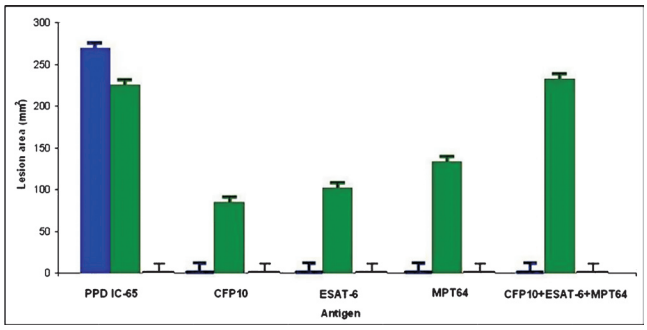

Fig. 3. Immunological activity and specificity of recombinant antigens CFP-10, ESAT-6 and MPT-64. DTH responses to selected recombinant antigens were compared to PPD IC- 65 in guinea pigs immunized with living *M. bovis* BCG (blue bars), inactivated *M. tuberculosis* H37Rv (green bars) and PBS (yellow bars). Twelve groups of six guinea pigs were sensitized and skin tested 5 wk after sensitized by intradermal injection of 2 TU/0.1 ml of PPD and 3 µg / 0.1 ml of purified recombinant antigens and a cocktail mixture (1 µg / 0.1 ml of each). Results are expressed as the mean lesion area (in millimeters^2), measured 24 h after antigen injection in each group of animals.

specific DTH responses only in *M. tuberculosis* H37Rv sensitized guinea pigs and not in BCG sensitized animals. In all of the animal studies, intradermal CFP10, ESAT-6, MPT64 and their cocktail mixture were safe. All doses were tolerated well and induced specific skin test responses. In the future studies, the biopsy of the skin test site needs to be done, as confirmatory to detect the presence of mononuclear infiltrates.

An immune response to ESAT-6 was reported in individuals infected with *M. kansasii* and *M. marinum*, which are, however, relatively rare clinical entities[28]. Therefore, we intend to test the recombinant antigens and their mixture on guinea pigs sensitized with these environmental mycobacteria in order to confirm their specificity.

Our results supported earlier data[13] that the cocktail mixture of the recombinant antigens induced a stronger DTH response in sensitized animals than each of the single antigens. A recent study, using a mixture of seven recombinant proteins (ESAT-6, CFP10, TB10.3, TB10.4, MTSP11, MPT70, and MPT83), evaluated the DTH reactions in *Cavia porcellus* in comparison with a standard PPD. When applied together at a concentration of each recombinant protein of 0.04 mg/ml, the intradermoreaction of the mixture in *C. porcellus* was significantly higher than that obtained by standard PPD[29].

Because of immunological specificity of CFP10, ESAT-6 and MPT64 for the *M. tuberculosis* complex further evaluation of these antigens or their cocktail should be done, as a reagent for TB-specific immunodiagnostic assays in *ex vivo* and *in vivo* human studies. We plan to test the recombinant proteins mixture, in comparison to a commercial PPD (PPD IC-65 as reference) on experimental groups of children with active TB and children who were BCG vaccinated at birth (with a post-vaccinal scar >3 mm) who have not been in contact with TB infected people. The test results will also be compared to those obtained by performing commercial blood assays, which detect interferon-γ (IFN-γ) release[30].

The goal is to elicit a cellular response of the host to TB that will help us differentiate between TB infection and a successful vaccination. With a specificity compared to that of cytokine-derived assays such as the IFN-γ release assay, but not requiring venous puncture, the new PPD recombinant reagent will probably have a lower price and could represent a more affordable diagnostic test for high TB incidence in low income countries. Our data have demonstrated that in the case of a population with high TB incidence, as in Romania, the sensitivity of TST proved to be higher than IFN-γ release assay, even with BCG vaccinated people[4].

Recently, a first–in–man open clinical trial of recombinant *ESAT 6* and *CFP10* antigens in a TB specific skin test reagent was reported[31], which opens

Table II. Potency assays results

Test no.	Recomb. antigen	Protein conc. µg/ 0.1 ml	*M.tuberculosis* H37Rv immunization %	*M.bovis* BCG immunization %	Mock PBS inoculation %
			PPD-r potency (control area/ probe area) mean lesion area/animal	PPD-r potency (control area/ probe area)mean lesion area/ animal	PPD-r potency (control area/ probe area) mean lesion area/ animal
1	CFP10	3	34.9	0	0
			Guinea pigs reactivity	Guinea pigs reactivity	Guinea pigs reactivity
			(no.):	(no.):	(no.):
			6/6 control 244	6/6 control :255	0/6 control: 0
			6/6 Probe 85	0/6 Probe : 0	0/6 Probe: 0
2	ESAT 6	3	34.3	0	0
			Guinea pigs reactivity	Guinea pigs reactivity	Guinea pigs reactivity
			(no.):	(no.):	(no.):
			6/6 control:297	6/6 control: 290	0/6 control: 0
			6/6 Probe: 102	0/6 Probe: 0	0/6 Probe: 0
3	MPT 64	3	76.6	0	0
			Guinea pigs reactivity	Guinea pigs reactivity	Guinea pigs reactivity
			(no.):	(no.):	(no.):
			6/6 control :174	6/6 control:214	0/6 control: 0
			6/6 Probe:133	0/6 Probe: 0	0/6 Probe: 0
4	CFP10+	1 +	124	0	0
	ESAT6+	1 +	Guinea pigs reactivity	Guinea pigs reactivity	Guinea pigs reactivity
	MPT 64	1	(no.):	(no.):	(no.):
			6/6 control:187	6/6 control:253	0/6 control: 0
			6/6 Probe:232	0/6 Probe: 0	0/6 Probe: 0

the road for the use of recombinant antigens as an improved TST.

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