Translationally Controlled Tumor Protein from *Madurella mycetomatis*, a Marker for Tumorous Mycetoma Progression

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About 40 years ago Abs against the fungus *Madurella mycetomatis* were first demonstrated to be present in eumycetoma patients, a disease characterized by tumorous swellings. To date nothing is known about the individual immunoreactive Ags present in this fungus. In the present study, we identify its first immunogenic Ag, a protein homologous to the translationally controlled tumor protein (TCTP), a well-conserved histamine release factor in a range of eukaryotes. The gene for this Ag was demonstrated to be present in two variants in *M. mycetomatis*, with 13% aa difference between the two proteins encoded. In vitro, TCTP was secreted into the culture medium. In vivo, it was found to be expressed on hyphae present in developing stages of the eumycetoma-characteristic black grain. Significant IgG and IgM immune responses, against the whole protein and selected *M. mycetomatis*-specific peptides, were determined. The Ab levels correlated with lesion size and disease duration. Overall, the patients with the largest lesions had the highest Ab level, which lowered with decreasing size of the lesion. After 6–15 years of disease duration the Ab levels were the highest. TCTP is the first well-characterized immunogenic Ag, simultaneously the first monomolecular vaccine candidate, identified for the fungus *M. mycetomatis*. *The Journal of Immunology,* 2006, 177: 1997–2005.

Madurella mycetomatis is a fungus capable of inducing eumycetoma in humans (Fig. 1) (1). Eumycetoma is a major mycological health problem of severe morbidity in tropical and subtropical areas (2). The disease is characterized by extensive tumor-like s.c. masses and discharge of fungal grains, blood, and pus and primarily affects the earning members of the society (1–3). Treatment of advanced cases usually implies amputation of the infected limb (4, 5). This disease has been reported in many countries including Sudan, Somalia, Senegal, Mauritania, Kenya, Niger, Nigeria, Ethiopia, Chad, Cameroon, Djibouti, India, Yemen, Mexico, Venezuela, Columbia, and Argentina (1, 3). In Sudan alone, within 2.5 years 1231 mycetoma cases were recorded (6, 7).

Almost nothing is known about the pathogenic routes used by this fungus. Both host and fungal factors involved in disease progression are essentially unknown. Identification of such factors is important for the development of prophylactic and therapeutic measures against eumycetoma. In addition, the diagnosis of *M. mycetomatis* as the primary cause of eumycetoma is troublesome. Recently developed PCR tests are useful but do not provide a simple tool for bedside use (8). There is an urgent need for the development of simple high speed and high-throughput tests for use in the endemic tropical regions. The usefulness of serological tests is underexplored and ELISA tests need to be developed.

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As early as in 1964 Mahgoub et al. (9, 10) was able to demonstrate that eumycetoma patients developed Abs against *M. mycetomatis*. Counterimmunoelectrophoresis, immunodiffusion, and ELISA were developed to detect Abs raised against different mycetoma causative agents, using crude culture extracts as Ag (11, 12). This did not identify the type of Abs produced or the nature of the Ags involved. It was not until the second half of the 1980s that it was experimentally demonstrated that the cytoplasm, organelles, and, predominantly, the cell wall of *M. mycetomatis* were antigenic (13). About the same time it was also determined that IgM and IgG were the dominant Igs resulting from mycetoma (14). In 1991, the first attempts were made to characterize the nature of the epitopes present in the crude extracts used for the initial experiments (15). Cytoplasmic proteins were extracted from several eumycetoma agents and, although the different *M. mycetomatis* isolates had very heterogeneous protein profiles by SDS-PAGE, the antigenic make-up was quite similar within the species (15). The precise nature of the immunodominant proteins was not determined in this study.

Molecular biology techniques were previously used for the identification of Ags and allergens from various fungal species such as *Aspergillus fumigatus* and *Fusarium culmorum* (16, 17). In analogy to these studies, we now searched a newly developed *M. mycetomatis* cDNA expression library for immunodominant Ags. Our aim was to identify Ags present on *M. mycetomatis*, which might be used in serodiagnostic tools or as vaccines, both therapeutic and prophylactic. We present the full characterization of the translationally controlled tumor protein (TCTP),[2] the first immunoreactive protein of *M. mycetomatis,* its histopathological features, and its use in immunological monitoring of disease progression.

Materials and Methods

M. mycetomatis *strains*

M. mycetomatis strain mm55, isolated from the lesion of a 22-year-old patient seen in the Mycetoma Research Centre, University of Khartoum,

[2] Abbreviation used in this paper: TCTP, translationally controlled tumor protein.

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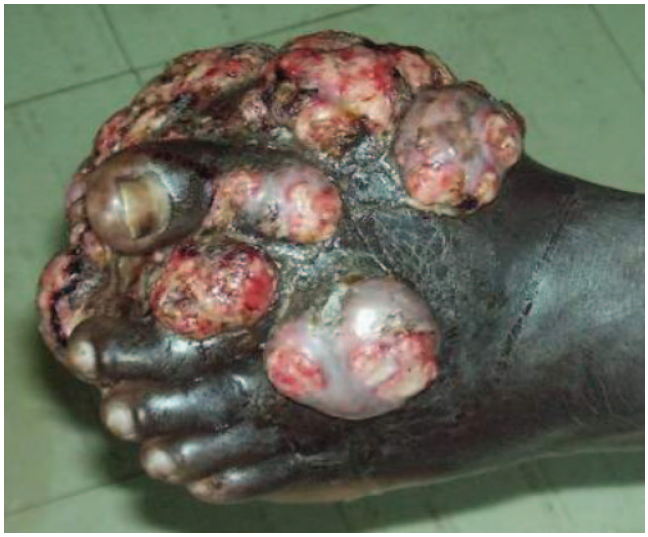

FIGURE 1. An advanced case of eumycetoma caused by *Madurella mycetomatis* of the foot.

was used in this study. This strain was isolated by direct culture of the black grains obtained by a deep biopsy and identified by morphology, PCR-RFLP, and sequencing of the internal transcribed spacer region (8). This strain is part of a well-defined strain collection deriving from Sudanese patients and is considered the type strain in phylogenetic and antifungal susceptibility testing as well (18–21). The strain was maintained on Sabouraud Dextrose Agar (Difco Laboratories) at 37°C. Passage to fresh medium was done on a monthly basis. Of this same collection four strains, strains mm30, mm45, mm46, and mm83, were used for comparative sequencing studies.

Sera and tissues

Forty-eight human sera were obtained from patients in various stages of mycetoma development (Mycetoma Research Centre, University of Khartoum). For each patient age, sex, disease duration, relative size of the lesion, and site of infection were recorded. The definition of small, moderate, and large lesions were based on comparisons among mycetoma patient lesions. More accurate measurements were not possible, since some mycetoma lesions are diffuse, have a mass, and have ill-defined margins. As a control population, random sera from 39 healthy Sudanese individuals from the same region were collected. As a true negative control, sera from six healthy Dutch individuals were taken. Mice sera were obtained by infecting female BALB/c mice i.p. with 140 mg of sonicated wet weight mycelia of the strain mentioned above according to the previously published protocol (21). The sera from 10 infected mice were pooled and used in the experiments described here. Furthermore, grains and surrounding tissue derived from infected mice were obtained and preserved in buffered formalin. Permission for animal experiments was granted by the Animal Ethics Committee of Erasmus MC. Polyclonal TCTP Abs were raised commercially in rabbits by Eurogentec. In short, rabbits were immunized by injecting 100 μg of recombinant TCTP, and after 2, 4, and 8 wk the immunization was boosted using 100 μg per boost. Serum was taken before immunization, 1 mo after immunization and 3 mo after immunization.

Construction of the cDNA library

The *M. mycetomatis* mm55 strain was cultured for 4 wk on Sabouraud Dextrose Agar (Difco Laboratories) at 37°C. The colony was excised from the agar, frozen in liquid nitrogen, and ground in a porcelain mortar. The resulting pulp was kept frozen in liquid nitrogen until the RLT buffer from the RNeasy Maxi system (Qiagen) was added. The sample was incubated for 3 min at 56°C to fully disrupt the cell walls. Total RNA was isolated using the bacterial protocol from the RNeasy Maxi system (Qiagen). After total RNA isolation, traces of DNA were removed with DNase I (Ambion) according to the manufacturer's indications. Poly(A)$^+$ mRNA was purified by binding it to Oligotex particles using the Oligotex mRNA spin protocol (Qiagen). The purified poly(A)$^+$ mRNA was used to synthesize double-stranded cDNA with the Universal RiboClone cDNA Synthesis System (Promega Benelux BV). Finally, the double-stranded cDNA was ligated into the λgt11/*Eco*RI/CIAP-treated vector (Stratagene Europe). The quality of the library was established by adding 5-bromo-4-chloro-3-indolyl β-D-galactoside (Promega) in a final concentration of 2 mg/ml in Top-Agar and blue-white screening for recombinant phages. The percentage of phages containing *M. mycetomatis* cDNA was 20%.

Immunological screening of the cDNA library

Escherichia coli Y1090 cells (Stratagene Europe) were grown overnight in Luria-Bertani broth supplemented with 0.2% (w/v) maltose, 10 mM $MgSO_4$, and 50 μg/ml ampicillin. The bacteria were pelleted for 10 min at 500 × *g* and resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.5. Two hundred thousand recombinant phages were absorbed to the cells for 20 min at room temperature. Top-Agar medium (26 ml) was added to 2.4 ml of cells and poured on a prewarmed 500-cm^2 Luria-Bertani agar plate. The plate was incubated at 37°C until the plaques were clearly visible. Subsequently they were overlaid with a Protran nitrocellulose membrane (Schleicher & Schuell) for 10 min at room temperature. The membrane was washed three times with TBS/Tween 20 (TBST) (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20 (pH 8.0)) and blocked for 30 min with 1% BSA (Sigma-Aldrich) in TBST. Mouse serum obtained from *M. mycetomatis* infected mice was diluted 1/130 in TBST and preincubated with *E. coli* Y1090 extract to absorb nonspecific Abs. This extract was prepared by pelleting an overnight *E. coli* Y1090 culture, resuspending the pellet in a third of the original volume in EDTA buffer (50 mM Tris-HCl (pH 8) and 10 mM EDTA), freezing it 1 h at −80°C, and sonicating it six times for 15 s at 10 μm. Cell remnants were pelleted at 500 × *g*, and the supernatant was used as the *E. coli* Y1090 extract. The blot was incubated for 45 min with the treated mouse serum. After washing, the membrane was incubated for 30 min with anti-IgG alkaline phosphatase conjugate. Color development was started by adding NBT/5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics). Positive phages were selected and purified by repeated screening.

Sequence analysis

To determine the nature of the insert present in antigenic phages, purified phages were boiled for 10 min. Five microliters of this crude extract was amplified in 50 μl of PCR mixture consisting of 1× Supertaq PCR buffer 1 (HT Biotechnology), 0,2 mM PCR nucleotide mix (Amersham Life Sciences), 25 pmol primer gt11fw (5′-GGTGGCGACGACTCCTGGAGC CCG-3′), 25 pmol primer gt11rv (5′-TTGACACCAGACCAACTGG TAATG-3′), and 1.2 U of Supertaq (HT Biotechnology). The PCR was composed of a predenaturation step of 4 min at 94°C and 35 cycles consisting of a 1-min denaturation at 94°C, 1-min annealing at 50°C, and 2-min elongation at 72°C. This was followed by a postelongation step of 7 min at 72°C. The PCR products were sequenced (BaseClear), and their insert sequences were compared with other sequences in the National Center for Biotechnology Information database (⟨www.ncbi.nlm.nih.gov/BLAST⟩).

Population analysis

We used the primers TCTPfw (5′-GCCGAGGAGGCTCTCGAGGA-3′) and TCTPrv (5′-ATCGGGATCCTTAGACCTTCTCCTCCTTCAG-3′) to determine presence of the TCTP sequence in the other 37 *M. mycetomatis* strains in our collection. The PCR program was identical to the one described in the next section for constructing the expression vector. The resulting PCR products were digested with the endonuclease *Alu*I to determine the variability within TCTP found in various strains.

Construction of a TCTP expression vector

The purified phages containing the complete TCTP coding region were boiled for 10 min, and 5 μl of these extracts were amplified in 50 μl of PCR mixture consisting of 1× Supertaq PCR buffer 1 (HT Biothechnology), 0.2 mM PCR nucleotidemix (Amersham Life Sciences), 25 pmol forward primer (5′-ACGTGCTAGCCATCACCATCACCATCACATTA TCTACAAGGATATTAT-3′), 25 pmol reverse primer (5′-ATCGGGAT CCTTAGACCTTCTCCTCCTTCAG-3′), and 1.2 U of Supertaq (HT Biotechnology). The PCR was composed of a predenaturation step of 5 min at 92°C and 10 cycles consisting each of 10 s denaturation at 92°C, 30-s annealing at 45°C, and 1-min elongation at 68°C followed by 15 cycles consisting each of 10 s at 92°C, 30 s at 45°C, and 1 min at 68°C, which extended with 10 s per cycle. The PCR ended with a postelongation step of 4 min at 68°C. In a 2:1 molar ratio the PCR product and vector pET11c (New England Biolabs) were restricted for 2 h at 37°C with 10 U of *Nhe*I and 10 U of *Bam*HI in buffer M (all Roche Diagnostics). After removal of excess restriction enzyme (Zymo Research) the PCR product was ligated into the pET11c vector with 3U T4 ligase (Promega Benelux BV). The

resulting plasmid was electroporated into electrocompetent *E. coli* BL21. To determine the absence of point mutations generated during the cloning process, the sequence of the insert was verified (BaseClear) by alignment with the sequence obtained from the original recombinant phages.

Expression and purification of the recombinant fusion protein

To express the His-tagged TCTP fusion protein, a culture containing the *E. coli* BL21 strain with the recombinant plasmid was grown to an OD_{600} of 0.6. To induce expression, isopropyl β-D-thiogalactoside (Promega Benelux BV) was added to a final concentration of 1 mM, and the culture was grown for another 2 h. The culture was pelleted and resuspended in 20 mM phosphate, 0.5 M NaCl, and 10 mM imidazole. The cell suspension was sonicated at 4°C, and cell remnants were pelleted. The histidine-tagged recombinant proteins were purified using a HiTrap column (Amersham Life Sciences) and eluted (300 mM imidazole, 10 mM $Na_2HPO_4 \cdot 2H_2O$, 10 mM $NaH_2PO_4 \cdot H_2O$, 0.5 M NaCl). The eluate was dialyzed against distilled water for 6 h. The size and purity of the recombinant protein were determined on a 12% SDS-PAGE gel stained with Coomassie G250 (Bio-Rad).

Immunohistochemistry

Grains obtained from experimentally infected mice were embedded in paraffin and histological slides were prepared. The slides were rehydrated in PBS and incubated for 30 min in a blocking solution (1% BSA, 5% sucrose in PBS). The primary Ab was diluted 1/100 in blocking solution and incubated for 30 min. As primary Ab, rabbit serum raised against recombinant *M. mycetomatis* TCTP was used. This serum was obtained 6 wk after immunization. As negative control, preimmune serum from the same animal was used under the same conditions. After washing, the slides were incubated with diluted goat anti-rabbit IgG HRP-conjugated Ab (1/50; Sigma-Aldrich). The substrate NovaRed (Vector Laboratories) was as the primary stain, and hematoxylin was used as counterstain.

Recombinant protein ELISA

The purified recombinant protein (12.5 μg/well) was coated onto Maxisorp plates (Nunc) overnight at 4°C. After blocking for 2 h with 1% BSA (Sigma-Aldrich) and 5% sucrose (Mallinckrodt Baker), plates were washed and incubated for 1 h with 1/50 diluted patient serum. After washing, 1/5,000 diluted goat anti-human IgM (Sigma-Aldrich) or 1/10,000 diluted goat anti-human IgG (Sigma-Aldrich) was applied and incubated for 1 h. The reaction was developed for 15 min in tetramethylbenzidine (Meddens Diagnostics), which was stopped with 2 M H_2SO_4. Absorption was measured at 450 nm in a model 550 microplate reader (Bio-Rad).

Peptide ELISA

To bind the peptides shown in Table I to Covalink ELISA-plates (Nunc), these plates were preincubated for 30 min at 37°C with 10 mM SPDP (3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester) (Sigma-Aldrich). Each peptide (10 μM in 0.1 M Tris-HCl (pH 8.0)) was coupled to the bivalent linker via the N-terminal cysteine residue for 60 min at 37°C. Empty binding sites were blocked for 2 h at 37°C with 6% low-fat milk (Similac 2; Abbott) before 1/50 diluted patient serum was allowed to bind (45 min at 37°C). After 30 min of incubation with the secondary Ab (1/10,000, goat anti-human IgG (Sigma-Aldrich)) the reaction was developed as described above.

Statistical analysis

IgG and IgM levels raised against the TCTP Ag or the various TCTP peptides were compared between study populations by the Mann-Whitney *U* test (GraphPad Instat 3.00). A value of $p < 0.05$ was considered significant. The Mantel-Haenszel χ^2 test for trends was used to test if there was a significant trend of higher levels in bigger lesions, by including size (small, moderate, massive) as independent variables, and the log transformed Ab level as dependent variable.

Nucleotide sequence accession numbers

The sequences of the *M. mycetomatis* homologs of TCTP were deposited in the GenBank. GenBank accession numbers of the cDNA sequences for variants I of this gene are DQ218143 (strain mm55), DQ218146, (strain mm46), and DQ218147 (strain mm83). GenBank accession numbers for variants II of this gene are DQ218144 (strain mm30) and DQ218145 (strain mm45). The DNA sequences for the two variants in these strains are deposited under numbers DQ218148–DQ218152.

Results

Antigenic phages contain a M. mycetomatis *TCTP homolog*

In our search for Ags of *M. mycetomatis*, an expression library in phage λgt11 was constructed. This library consisted of 6.0×10^4 independent clones with an average insert length of 1500 bp. A total of $\sim 6 \times 10^8$ recombinant phages was screened with sera obtained from mice experimentally infected with *M. mycetomatis*. Twelve phage clones were found to significantly bind Abs and these were rescreened to purity. PCR amplification, restriction analysis, and subsequent sequencing demonstrated that all 12 immunoreactive plaques harbored the same 692-bp insert, encoding a single open reading frame of 516 nt. The open reading frame defined a 171-aa polypeptide sequence, which was followed by an untranslated sequence of ~156 bp, including a poly(A) tail of 15 nt (Fig. 2). As seen in Fig. 3 the protein showed significant homology with the TCTP gene of various organisms, including fungi and yeasts. The highest degree of homology was observed for the TCTP gene of the fungus *Neurospora crassa* (GenBank accession no. XP_326319), with 61% aa identity and 71% aa similarity. At the protein level, *M. mycetomatis* TCTP was less closely related to mammalian TCTP, with only 39% identity and 56% similarity.

To establish whether this gene was ubiquitously found in all *M. mycetomatis* strains we screened 38 isolates for its presence. After PCR screening of genomic DNA of these isolates it appeared that all strains possessed the TCTP gene homolog (data not shown). PCR RFLP exploring the genes from these isolates revealed that there was not one isoform of this gene but two. Subsequently sequencing of these genes confirmed this finding. The isoforms as shown in Fig. 4 were named variant I and II. Variant I was found in 53% of the isolates while variant II was found in 47% of the isolates. Further PCR analyses showed that both TCTP genes contained at least three introns of 64 bp (intron 1), 161 bp (intron 2), and 56 bp (intron 3) and four exons (Fig. 2).

According to the PROSITE pattern database (⟨www.expasy.ch/prosite/entry, PDOC00768⟩) two signature patterns can be identified for TCTP amino acid sequences, namely TCTP1 and TCTP2.

Table I. *Peptide sequences constructed on the M. mycetomatis specific TCTP domains*[a]

Name	Variant I	Variant II
Peptide 1	VDCAMVVEDAVNA	Not determined
Peptide 2	EAEEALEDAAVKV	EAEEALED**Q**A**I**KV
Peptide 3	DAAVKVNNVVNSF	D**Q**A**I**KVN**D**V**I**H**Y**F
Peptide 4	SVKKALQDAGKSE	SVKKALQDAGKSE
Peptide 5	KSEDEVKEFETKA	KSEDEVKEFETKA
Peptide 6	TKAQAYVKDTVLP	TKAQAYVKDT**I**LP

[a] Peptide sequences constructed on the *M. mycetomatis*-specific TCTP domains. These sequences were based on nonconserved sequences of variant I of the TCTP sequence when compared with other TCTP. In this table, the homologs for variant II are also shown. Differences between this variant and variant I are highlighted in the bold font in the sequence for variant II. The peptide 1 homolog in variant II could not be determined because no sequence data were available for this area. Peptides developed for variant I are the ones constructed and used in the peptide ELISA.

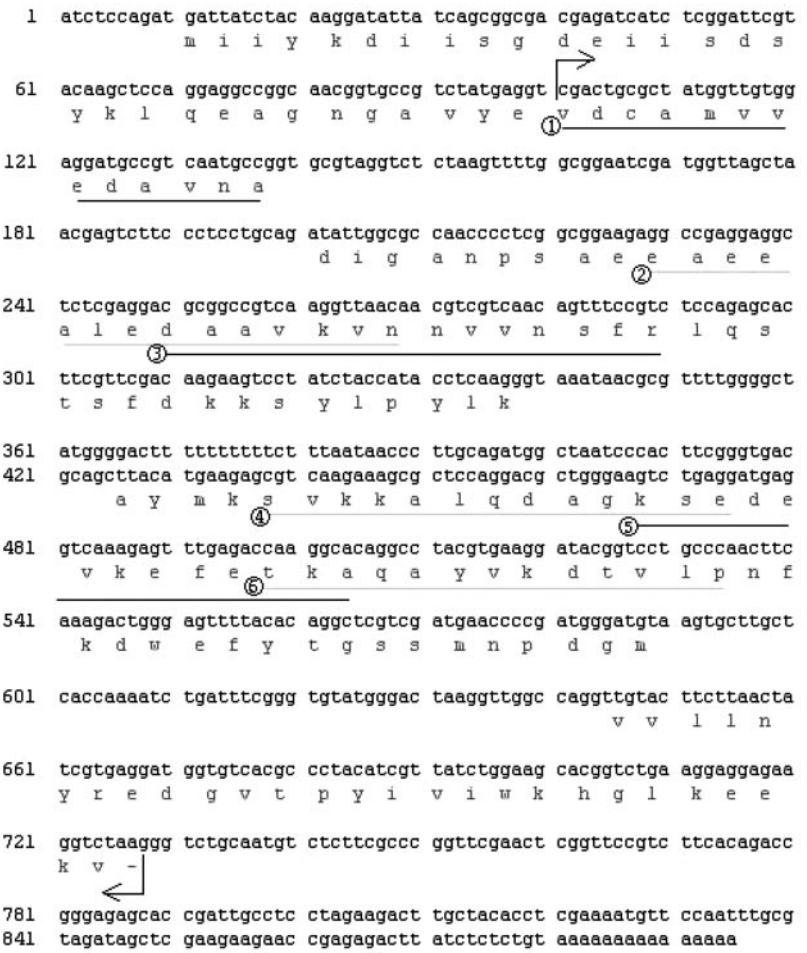

FIGURE 2. Nucleotide sequence and deduced amino acid sequence of the TCTP gene of *M. mycetomatis.* In this sequence at least four exons can be found: exon 1 (aa 99–137), exon 2 (aa 200–337), exon 3 (aa 423–585), and exon 4 (aa 644–727). These exons are shown translated and are followed by three introns: intron 1 (aa 138–199), intron 2 (aa 338–422), and intron 3 (aa 586–643). The sequence preceding the first arrow (aa 1–8) and after the second arrow (aa 728–895) are mRNA. For only the sequence between those arrows (aa 9–727) the DNA sequence is known. Peptides are underlined and numbered.

After comparing the MmTCTP amino acid sequences with the database entries it was noticed that amino acid positions 49–54 in the MmTCTP variant I were identical to the typical TCTP1 signature sequence (bold amino acids correspond to the amino acid sequence found in this particular fungus) [**I**FAE]-[**G**A]-[G**A**S]-**N**-[**P**AK]-**S**-[GT**A**]-**E**-[GD**E**V]-[P**A**GEQV]-[D**E**QGAV]. In contrast, the TCTP2 sequence of this MmTCTP variant deviated from the known signature sequence, [**F**LIV]-**x4**-[**F**LVH]-[F**Y**]-[MIVC**T**]-**G**-*E*-x(**4**,7)-[**D**ENP]-[**G**AST]-**x**-[LI**V**M]-[GAVI]-**x3**-[F**Y**QW]. For the second variant this finding was reversed. This variant had a deviating TCTP1 signature sequence ([**I**FAE]-[**G**A]-[*GAS*]-**N**-[P**A**K]-**S**-[GT**A**]-**E**-[GD**E**V]-[P**A**GEQV]-[D**E**QGAV] and a canonical TCTP2 signature sequence ([**F**LIV]-**x4**-[**F**LVH]-[F**Y**]-[MIVC**T**]-**G**-**E**-x(4,7)-[**D**ENP]-[**G**AST]-**x**-[LI**V**M]-[GA**V**I]-**x3**-[F**Y**QW]). Both deviating sequences each displayed only one amino acid difference. In the TCTP2 sequence of variant I, glutamate was replaced by serine and in the TCTP1 sequence of variant II, glutamine was found instead of either glycine, alanine, or serine.

Since we obtained a complete cDNA sequence for variant I we used this to express and purify the recombinant TCTP variant I gene product as a histidine-tagged fusion protein. The yield of this recombinant protein was 50 mg/L *E. coli* culture. SDS-PAGE gel analysis of the purified recombinant protein with the histidine tag revealed one clear band with a molecular mass of ~26 kDa (Fig. 5). This was slightly larger than the expected molecular mass but still in full accordance with the molecular mass of other cloned TCTP (22). As seen in Fig. 5, the purified protein was essentially free of contaminating proteins.

TCTP is expressed in the developing grain

To assess whether TCTP was expressed in vivo we isolated grains in their surrounding tissues from mice infected with *M. mycetomatis.* These grains were embedded in paraffin, and Abs raised against TCTP were used to demonstrate the presence of TCTP in these grains. The results obtained are shown in Fig. 6. The grains in the i.p. mouse tissues are typically filamentous and are surrounded by three zones of inflammatory cells, a type I tissue reaction as described by Fahal and El Hassan (3, 23, 24). The zone directly around the grain was composed mainly of neutrophils. This zone was followed by a region of histiocytes and an outer vascular zone. The fungal cells in the grain itself are mostly embedded in brown-pigmented cement material. It was clearly seen that TCTP is not evenly expressed in the grain. In the grain itself, three stages of grain development are seen. The first stage (Fig. 6*A*, *number 1*) is the stage in which the grain is still forming. The brown-pigmented cement material in this part of the grain is not present yet (Fig. 6, *B* and *C*), and the neutrophil zone surrounding the grain is not completely formed and only a few cell layers thick. This zone is invaded by the grain and fungal cells are in direct physical contact with the immune cells. The fungi are mostly seen as complete hyphae. In this part of the grain, TCTP was expressed abundantly (Fig. 6*B*). Most cells express the protein but some exceptions are noticed, even within hyphae where other cells do express the protein. In the second stage of the grain formation (Fig. 6*A*, *number 2*), the cement material is more developed but individual fungal cells are still seen (Fig. 6, *D* and *E*). Some of the neutrophils are also still found between the fungal cells. The number of cells

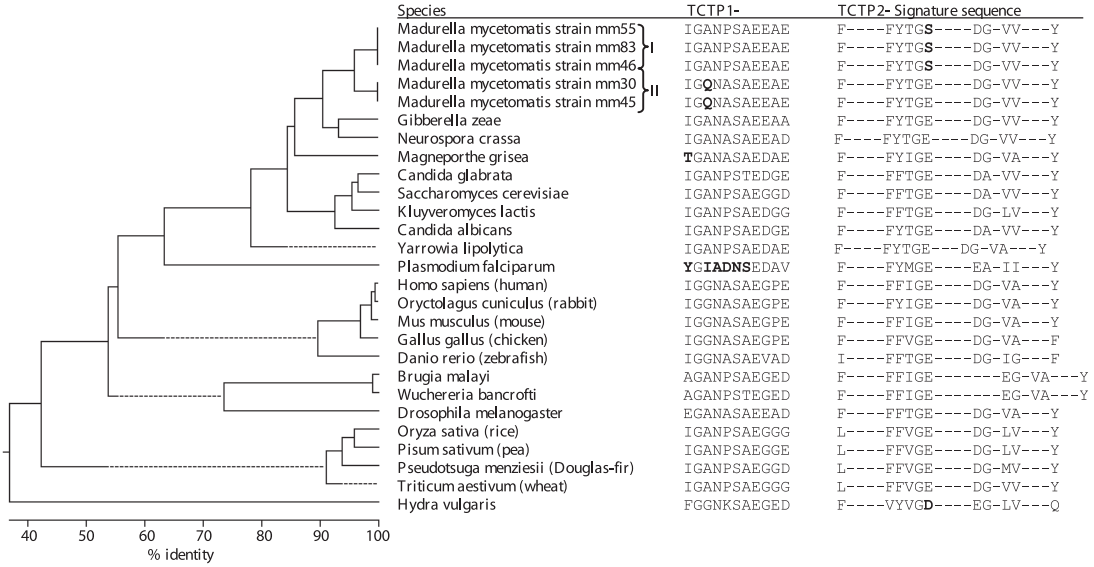

FIGURE 3. Phylogenetic tree analysis of TCTP protein sequences from different species. The tree distances were calculated using the Clustal V algorithm in the programm MegAlign (DNAstar). Both *M. mycetomatis* TCTP variants (I and II) (GenBank accession no. DQ218143–DQ218147) were compared with TCTP sequences obtained from *Brugia malayi* (GenBank accession no. AAC47622), *Candida albicans* (EAK99010), *Candida glabrata* (XP_449328), *Danio rerio* (NP_937783), *Drosophila melanogaster* (DmTCTP, NP_650048), *Gallus gallus* (NP_990729), *Gibberella zeae* (XP_382699), *Homo sapiens* (NP_003286), *Hydra vulgaris* (AAB18413), *Kluyveromyces lactis* (CAH01617), *Magnaporthe grisea* (EAA56278), *Mus musculus* (p63028), *Neurospora crassa* (XP_326319), *Oryctolagus cuniculus* (CAA12650), *Oryza sativa* (P35681), *Pisum sativum* (P50906), *Plasmodium falciparum* (NP_703454), *Pseudotsuga menziesii* (CAA10048), *Saccharomyces cerevisiae* (P35691), *Triticum aestivum* (AAM34280), *Wuchereria bancrofti* (AAK71499), and *Yarrowia lipolytica* (XP_504451). For each of the organisms the TCTP1 and TCTP2 signature sequences are given. In this sequence amino acids that do not correspond to the signature sequence (given in bold) and amino acids that do not belong in the signature sequence (−) are shown as well.

expressing TCTP was inversely related to the amount of cement material present. In the last stage (Fig. 6*A*, *number 3*) the grain is fully formed. The fungus is embedded into cement material and no neutrophils are seen within the grain (Fig. 6, *F* and *G*). In this stage no visible TCTP expression was documented (Fig. 6*F*).

Association between lesion size, duration of the disease, and TCTP Ab levels

The phage containing the TCTP insert was selected because of its reactivity with sera from infected mice. Mice infected with *M. mycetomatis* had Abs against the protein while healthy control mice did not (results not shown). To test the antigenicity of MmTCTP in humans, an ELISA was developed. The IgA, IgM, and IgG levels against the protein were measured. None of the patients or controls displayed an IgA level against this protein, but clear IgM and IgG responses were detected. Since only comparative optical densities were measured, a prozone phenomenon could not be ruled out. In Fig. 7 IgM and IgG levels against TCTP are summarized. Both in the patient group as well as in the Sudanese control group, significant IgM and IgG levels were measured against the protein, however, in a lower percentage of controls than patients, and not all patients developed elevated levels. All individuals in the Dutch healthy control group had low anti-TCTP levels overall. For some of the patients it was known whether the fungus causing the infection produced TCTP variant I or II. Patients infected with a type II variant had detectable Abs in an ELISA coated with the variant I protein. No significant difference in Ab binding was noticed when serum of variant II patients was used (data not shown). It was seen that the IgM level in the *M. mycetomatis*-infected people was significantly higher compared with the Sudanese controls (Mann-Whitney *U* test, $p = 0.03$) or to the Dutch control group ($p = 0.003$). The IgG levels of the *M. mycetomatis*-infected people were only significantly higher compared with the Dutch control group ($p = 0.002$). It was also noted that the patients with massive eumycetoma lesions had the highest absorbances and that patients with the smaller or cured lesions had lower IgG levels (Mantel-Haenszel test for trend, $p = 0.030$). No significant trend was documented for the IgM immune response. Furthermore, it was seen that after a disease duration of 6–10 years anti-TCTP levels were highest.

Since some individuals in our healthy control group from Sudan had raised levels of Abs against the recombinant TCTP protein we analyzed whether these elevated levels were due to the *M. mycetomatis* homolog of this Ag or due to cross-reactivity. Cross-reactivity could occur with TCTP from other infectious agents, such as *Plasmodium falciparum* and *Leishmania spp.,* which are locally endemic pathogens in the Sudan region as well. To test this, known TCTP sequences for various species were compared and we designed six *M. mycetomatis* specific peptides based upon nonconserved sequences of variant I of the TCTP sequence (see Fig. 2 for the position, code, and sequence of the peptides). As a precaution we also compared the peptides designed to variant II of the *M. mycetomatis* TCTP protein. Five of the six peptides were conserved in *M. mycetomatis*, with a maximum of 2 aa difference. Only one peptide, peptide 3, was not conserved. For this peptide, 6 of the 13 aa were different between the two variants. With these peptides, ELISA were performed and both the patient and the control sera were retested. In Fig. 8 the Ab levels against the six individual peptides are shown. Peptides 1, 2, 3, 4, and 5 can clearly differentiate the patient population from the healthy Sudanese control population, but with peptide 6 the mean IgG levels of the patient population and the healthy Sudanese control population were not significantly different. When the patients were stratified according to massive lesions, moderate lesions, small lesions and cured patients the same picture was obtained for the peptides as

FIGURE 4. Comparison of the two TCTP variants. *A*, Alignment of the two TCTP variants of *M. mycetomatis* based on the BLOSUM 62 algorithm. Identical amino acid residues are shown as a dot. *B*, Banding patterns obtained for each variant after treatment with the endonuclease *Alu*I.

with the whole TCTP protein (data not shown). Overall, the patients with the largest lesions had the highest Ab levels against the respective peptides, the level lowered with the decreasing size of the lesion. After 6–15 years of disease duration levels were the highest.

Discussion

It has been demonstrated before that specific Abs are raised against the agents of mycetoma. It was, however, never clear what the nature of the immunogenic epitopes was (9, 11, 12). In the present study we completely identify and characterize the first immunoreactive Ags from a *M. mycetomatis* isolate namely TCTP. Many functions have been recorded for TCTP. This protein appears to be involved in cell cycle control, stress responses, histamine release, and IL production (25, 26). It is also known to bind the antimalarial and anticancer drug artemisinin (27, 28). In our search for Ags, we constructed an expression library in phage λgt11, which was screened with sera obtained from *M. mycetomatis* infected mice. Screening the library resulted in 12 antigenic phages, which all contained the same insert, namely the *M. mycetomatis* homologue of TCTP. This finding differs from results obtained for other pathogens such as *Mycobacterium tuberculosis* for which large numbers of independent and clearly different antigenic phages were found (29). The antigenicity of *M. mycetomatis* TCTP was confirmed by rabbit immunization. Other parasites are also known to produce TCTP. Some examples are *P. falciparum*, *Brugia malayi*, *Wuchereria bancrofti*, and *Schistosoma mansoni* (22, 30–32). These TCTP have been documented to be antigenic as well (22, 30–32).

In the present study we found that the TCTP gene isolated from the expression library was one of two variants present in the species *M. mycetomatis*, which again further corroborates the genetic heterogeneity within this species (33). Although TCTP have been found in a great variety of eukaryotic organisms no one has yet reported the presence of multiple variants of this gene within one species. In the mouse, rabbit and human genomes there are indeed pseudogenes of this protein known (34–36). But in the more ancient *Hydra vulgaris* and *Labeo rohita* no doublet TCTP mRNA is

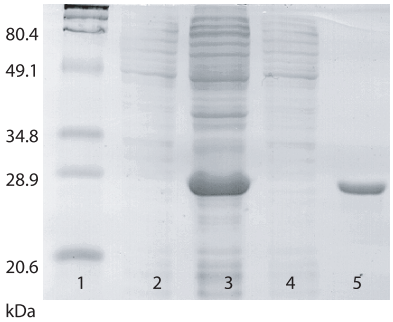

FIGURE 5. Twelve percent Coomassie-stained SDS-PAGE of the recombinant his-tagged *M. mycetomatis* protein. *Lane 1*: SDS-Page broad range marker; *lane 2*: total *E. coli* extract before inducement of the TCTP expression; *lane 3:* total *E. coli* extract after inducement of the TCTP expression; *lane 4*: protein extract after binding the his-tagged recombinant TCTP protein to the HiTrap column; and *lane 5*: eluted his-tagged recombinant TCTP protein.

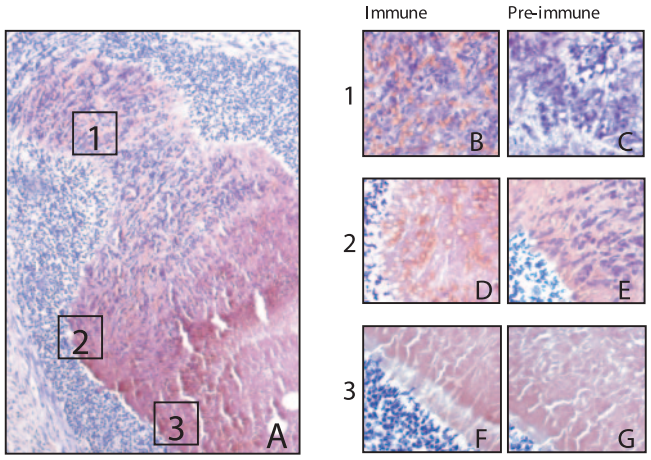

FIGURE 6. *A*, Stained histological section of a grain isolated from a mouse i.p. inoculated with 120 mg of fungal mycelia and soil adjuvant (×100). Immunohistochemistry with anti-TCTP Abs and counterstained with hematoxylin. The grain is surrounded by a type 1 host reaction. The grain is surrounded by three zones of host inflammation cells. The first zone, closest to the grain, is composed of neutrophils, and the second zone is composed of histiocytes. The outer layer is the vascular layer. The grain itself is divided into three developmental stages. The first stage is the early developing stage (*1*), the second stage is the intermediate stage (*2*), and the third stage is the developed stage (*3*). *B*, TCTP is highly expressed in first stage of development. Immunohistochemistry performed with immune serum. *C*, First stage of development with preimmune serum. *D*, TCTP expression in second stage of development, immune serum. *E*, Second stage of development, preimmune serum. *F*, No TCTP expression in third stage of development, immune serum. *G*, Third stage of expression, preimmune serum. *B–G* are photographed at ×400. TCTP expression is stained with the substrate NovaRed (orange).

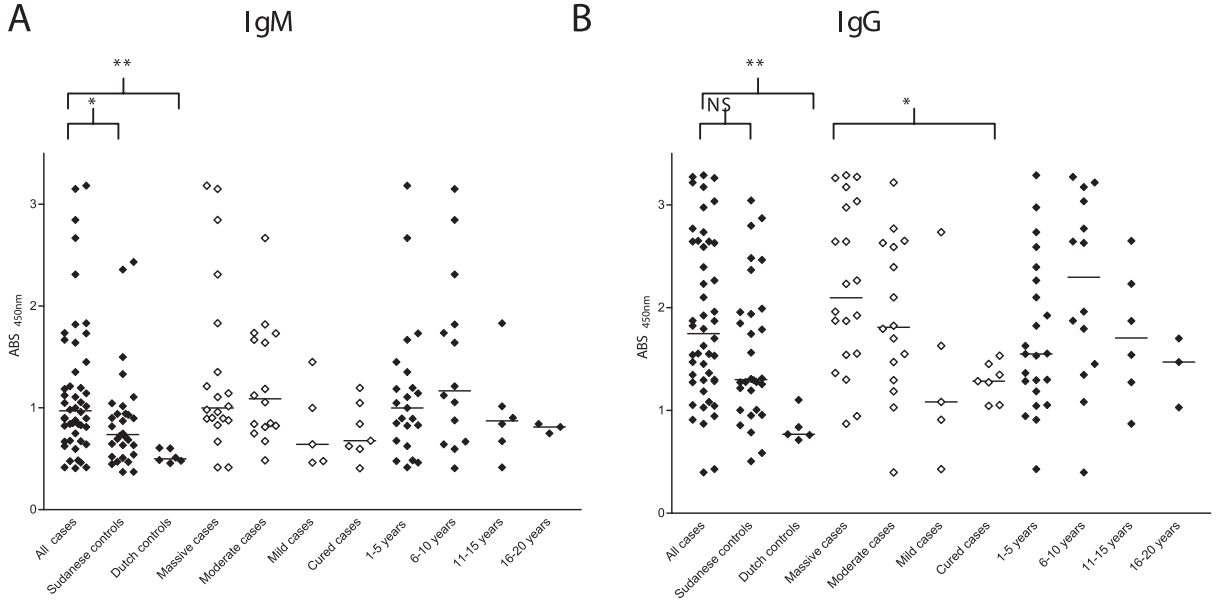

FIGURE 7. IgG levels against the purified recombinant his-tagged TCTP protein measured at a wavelength of 450 nm in an ELISA system. Each point represents the mean of three independent measurements per patient or a healthy control. *A*, IgM levels in correlation of the size of the lesion and the duration of the disease. *B*, IgG levels in correlation of the size of the lesion and the duration of the disease. The differences between the geometric means were tested for significance with the Mann-Whitney *U* test. Furthermore it was tested if there was a significant trend or higher level in bigger lesions by including size (small, moderate, massive) as an independent variable, and log-transformed level as a dependent variable in a linear regression model. The *p* values are stated as follows: $p \leq 0.0100$ (**), $p \leq 0.05$ (*), p = NS.

found (26, 37). This raises questions on the possibly different physiological findings of the TCTP variants.

To test its antigenicity in humans an ELISA was developed with variant I of MmTCTP. A significant number of the patients seen in the Mycetoma Research Centre had raised Abs against mmTCTP variant I, but, unfortunately, not all the patients. Reasons for this restriction are currently unknown but subject to further study. It also appeared that some individuals of the Sudanese healthy control population had an elevated Ab level against TCTP as well. The most simple and also likely explanation would be that these individuals raised Abs after environmental exposure to the Ag. It could also be possible, since mycetoma has a long incubation time, that some of these individuals had subclinical or early *M. mycetomatis* infections. Another plausible explanation is cross-reactivity with TCTP from other endemic infectious organisms. Although Rao demonstrated that Abs raised against filarial TCTP did not react against recombinant *S. mansoni* TCTP, Gnanasekar showed that antisera raised against *B. malayi* could detect recombinant *W. bancrofti* TCTP and visa versa (22, 32). These latter filarial TCTP were closely related to each other, with 98% sequence homology at the amino acid level (22). From the phylogenetic tree shown (Fig. 3), it can be easily seen that the closest homology was found with TCTP genes of other fungi. To determine whether cross reactivity with TCTP from other species were causing the high levels in the healthy Sudanese control population, specific TCTP peptides which showed as little homology as possible with other TCTP sequences were developed. In the peptide ELISA there was a clear difference between the mean IgG levels raised against the different peptides in the patient population and the healthy Sudanese control population. Only with peptide 6, no statistic significant difference in the mean IgG levels was detected. Apparently, peptides provide more specific ELISA targets than the full protein. This is biologically plausible and suggests that cross-reactivity between TCTP from various organisms is occurring in humans. Pathological effects of this cross-reactivity deserve additional research.

Our experiments showed that the TCTP gene is present in all *M. mycetomatis* strains tested. Also, the immunogenic epitopes of variants I and II of the protein seems to be overlapping. This overlap was proven by comparing sera obtained from patients of whom it was known with which TCTP variant of *M. mycetomatis* they were infected. No statistically significant difference in Ab level was detected between the two variants. By developing peptides specific for conserved *M. mycetomatis*-specific regions between the two variants, Ab responses could be compared. Since no difference in response between patients infected with variant I or variant II were found in the ELISA, we could conclude that differences found in Ab level were not caused by the variability in the TCTP gene. This difference must be due to other factors involved in TCTP expression. For instance, it is possible that not all strains transcribe this protein in vivo (although they do so in vitro) and even if TCTP is transcribed it is likely that not all patients form Abs against this protein to the same extent.

To investigate TCTP expression in vivo, grains in surrounding tissues from infected mice were isolated. In these tissues TCTP expression could be demonstrated. TCTP expression was very high in the first stage of *M. mycetomatis* grain development, almost all fungal cells in this stage of development expressed TCTP. For other TCTP it is known that the expression is also dependent on the developmental stage of the organism (22, 32, 35). TCTP is highly expressed in mitotically active stages while in postmitotic tissue it is hardly expressed at all (22, 32, 35). In the developing stage of the *M. mycetomatis* grain the fungal cells are probably mitotically active. Expression was less in the second stage of grain development and almost nonexistent when the grain was completely formed. In this latter stage the fungus is probably not mitotically active anymore and the fungal cells are completely embedded in cement material. This latter could also result in shielding of the Ag. In the first 15 years of infection, the Ab levels were elevated. This finding was seen both for the whole protein as for the individual peptides. Levels drop after prolonged infection

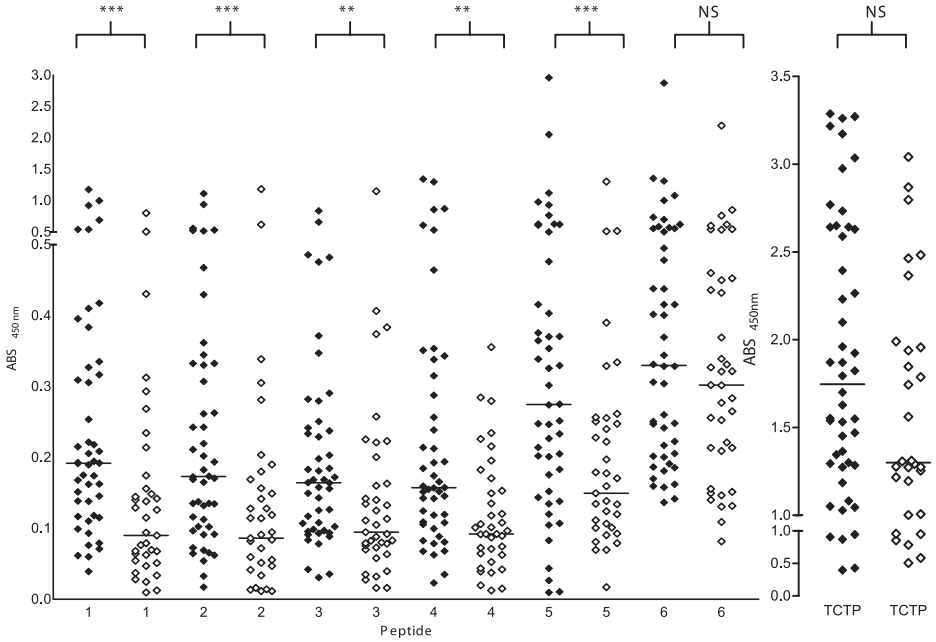

FIGURE 8. IgG level generated against the different TCTP peptides. In this graph the Abs raised against the different peptides (numbers 1–6) in the *M. mycetomatis*-infected patients (♦) are plotted next to Abs found in the Sudanese healthy control group (◇). For reference the results obtained with the complete TCTP protein as shown in Fig. 7 are repeated in this figure as well (TCTP). The differences between the geometric means were tested for significance with the Mann-Whitney *U* test. The *p* values are stated as follows: $p \leq 0.0010$ (***), $p \leq 0.0100$ (**), $p \leq 0.05$ (*), p = NS.

(>15 years). Since eumycetoma is a chronic infection with a long incubation time and a slow progression, *M. mycetomatis* may well reside in the host for a prolonged period as a mitotically less active organism. Combining the in vivo TCTP expression and the serological data this could indicate that after establishing the infection the organism becomes less reproductively active and less TCTP is expressed. The same can be said for the size of the lesion. It was demonstrated that the Ab response was associated with the size of the lesion since the patients with the largest lesions had the highest Ab levels. In patients with large massive mycetoma, many s.c. lesions exist in different stages of development, involving elevated high expression of TCTP. In the largest lesions probably most mitotically active cells are found. In these lesions the highest anti-IgG levels were found. Patients with only small lesions had similar Ab levels as the Sudanese controls. A correlation between the size of the lesion and the immune response was also reported for the crude Ags used in the double immunodiffusion method developed by Murray and Mahgoub (10). Murray and Mahgoub (10) found a correlation with the number of precipitin bands and volume of the tissue involved in the disease. The TCTP Ab levels were also correlated with the duration of the disease. After 6–10 years anti-TCTP responses were the highest, whereas no correlation with duration of the disease was observed by Murray and Mahgoub (10) when using crude cell extracts of *M. mycetomatis*.

Eukaryotic TCTP are reported to respond to a wide range of extracellular signals and cellular conditions (25, 38–40). Growth factors, cytokines, starvation, heat shock, heavy metal stress, calcium stress, but also viral infections all have been reported to either induce or repress the formation of TCTP in various organisms (25, 38–40). Since TCTP is known to be expressed as part of a protective mechanism in many other eukaryotes, it could be involved in the pathogenesis of mycetoma caused by *M. mycetomatis. M. mycetomatis* DNA has been found in Sudanese soil and vegetation (41). When the fungus enters the body it will have to readapt, and TCTP expression might be part of the adaptation process. A similar phenomenon has been noted for the parasite *B. malayi* (22). In this parasite no TCTP is found in the preinfective stages but upon entering the host, TCTP becomes strongly up-regulated. TCTP was also found to be up-regulated by higher temperatures (42). It was hypothesized by Gnanasekar et al. (22) that the entry of the parasite *B. malayi* from a cold-blooded insect vector to a warm-blooded host could trigger higher expression of BmTCTP. However, for *S. mansoni* TCTP expression was significant in all developmental stages, including the preinfective ones (32). In the developing stages of the *M. mycetomatis* grain, TCTP expression is high. This high TCTP expression is typical for mitotically active tissues and could be associated with stress conditions imposed by the host immunesystem or nutrient depletion during the invasive growth (35, 37, 43).

In conclusion, we discovered that *M. mycetomatis* TCTP is antigenic in mice, rabbits and humans. The TCTP protein was found mainly at the cell surface of the fungus and primarily in the developing part of the grain. TCTP has two variants in the species *M. mycetomatis,* and in an ELISA system, disease stage-dependent immuneresponses were documented. Although TCTP may not be the best diagnostic tool, the ELISA presented here could be useful in seroprevalence studies. In addition, studies into the influence of TCTP on mycetoma development or its use as a therapeutic vaccine or a vaccine in the prevention of infection are urgently warranted.

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Disclosures

The authors have no financial conflict of interest.

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