The Journal of Immunology

Polymorphisms in Genes Involved in Innate Immunity Predispose Toward Mycetoma Susceptibility

Wendy W. J. van de Sande, Ahmed Fahal, Henri Verbrugh and Alex van Belkum

J Immunol 2007; 179:3065-3074; ;
doi: 10.4049/jimmunol.179.5.3065
http://www.jimmunol.org/content/179/5/3065

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Polymorphisms in Genes Involved in Innate Immunity Predispose Toward Mycetoma Susceptibility

Wendy W. J. van de Sande,[1]* Ahmed Fahal,† Henri Verbrugh,* and Alex van Belkum*

***Madurella mycetomatis* is the main causative agent of mycetoma, a tumorous fungal infection characterized by the infiltration of large numbers of neutrophils at the site of infection. In endemic areas the majority of inhabitants have Abs to *M. mycetomatis,* although only a small proportion of individuals actually develop mycetomal disease. It therefore appears that neutrophils are unable to clear the infection in some individuals. To test this hypothesis, 11 single nucleotide polymorphisms involved in neutrophil function were studied in a population of Sudanese mycetoma patients vs geographically and ethnically matched controls. Significant differences in allele distribution for IL-8 (CXCL8), its receptor CXCR2, thrombospondin-4 (TSP-4), NO synthase 2 (NOS2), and complement receptor 1 (CR1) were found. Further, the NOS2$^{\text{Lambaréné}}$ polymorphism was clearly associated with lesion size. The genotypes obtained for CXCL8, its receptor CXCR2, and TSP-4 all predisposed to a higher CXCL8 expression in patients, which was supported by the detection of significantly elevated levels of CXCL8 in patient serum. The NOS2 genotype observed in healthy controls was correlated with an increase in NOS2 expression and higher concentrations of nitrate and nitrite in control serum. We present the first evidence of human genetic predisposition toward susceptibility to mycetoma, a neglected infection of the poor. *The Journal of Immunology,* 2007, 179: 3065–3074.**

Madurella mycetomatis is the most common fungal causative agent of eumycetoma in Sudan (1). This agent is abundantly present in the soil and on the vegetation in the endemic region (2). Chances for coming into contact with this fungus are, therefore, high for inhabitants of these areas. When using an ELISA system based on crude fungal extracts, all individuals in the Sudanese endemic regions seemed to posses IgG Abs against this fungus (1). With an ELISA based on a specific Ag of *M. mycetomatis*, the translationally controlled tumor protein TCTP, Ab levels were found to be elevated in endemic control populations as well, although these levels were lower than those for the patient population. No Abs were found in Caucasian controls from Europe (3). This implies that most of the individuals living in endemic regions are regularly exposed to this pathogen but that only a small percentage of them actually develop the disease. A predisposing factor could be that the immune status of mycetoma patients is impaired (4). Using the tuberculin test, 2,4-dinitrochlorobenzene sensitization, and lymphocyte proliferation induced by phytohemagglutinin, deficiencies in cell-mediated immunity were previously documented among patients (4). Also, differences in blood group Ags between mycetoma patients and a matching healthy control population were investigated, but no correlation with blood group type and development of mycetoma was reported (5). Investigators did not note defects in HLA-mediated presentation of pathogen-derived peptides to T cells (5). It is currently not known whether mycetoma patients suffer from substantial immune defects.

In previous reports (6–8) it was shown that large numbers of neutrophils are present in the mycetoma lesion. Apparently, neutrophils are important in the early defense against mycetoma. Because the neutrophils are unable to clear the infection, it was hypothesized that there might be genetic impairment in neutrophil function in mycetoma patients.

Neutrophils and monocytes are attracted to the site of infection by either Ags secreted by the invading microorganism or by locally produced host chemokines such as IL-8 (CXCL8), MCP-1, and TNF-α. In mycetoma it has previously been shown that neutrophils are actively attracted by Ags secreted by *M. mycetomatis* in a complement-dependent manner (9). Normally, when neutrophils arrive at the site of infection they will eliminate the pathogen through phagocytosis. To ingest pathogens, neutrophils are equipped to directly recognize either molecules on the surface of invading microbes or their opsonization with serum host proteins including complement factors, mannose binding lectin (MBL),[2] or Abs. An example of such a receptor is complement receptor 1 (CR1), which is also the determinant for the Swain-Langley (Sl) blood group Ag and the McCoy (McC) blood group Ag (10, 11). After pathogen recognition, reactive oxygen species including hydrogen peroxide, superoxide, and NO are formed, which effectively kill ingested microorganisms (12). This process is summarized in Fig. 1.

Many genetic polymorphisms that influence phagocytosis and killing by neutrophils have been described. For instance, a point mutation in the NO synthase (NOS) type 2 promotor, namely NOS2$^{\text{Lambaréné}}$, has been shown to be associated with a 7-fold higher NOS activity (13). This genotype and, subsequently, its higher NO levels were shown to offer protection against severe malaria to a level similar to that of the sickle cell trait (13). Because neutrophils attracted to the mycetoma lesion apparently are

*Erasmus MC, University Medical Centre Rotterdam, Department of Medical Microbiology and Infectious Diseases, Rotterdam, The Netherlands; and †Mycetoma Research Centre, University of Khartoum, Khartoum, Sudan

Received for publication April 19, 2007. Accepted for publication June 19, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Wendy van de Sande, Erasmus MC, PO Box 2040, Rotterdam, The Netherlands. E-mail address: w.vandesande@erasmusmc.nl

[2] Abbreviations used in this paper: MBL, mannose-binding lectin; CR1, complement receptor 1; HWE, Hardy-Weinberg equilibrium; McC, McCoy (blood group); NOS, NO synthase; Sl, Swain-Langley (blood group); SNP, single nucleotide polymorphism; TSP, thrombospondin.

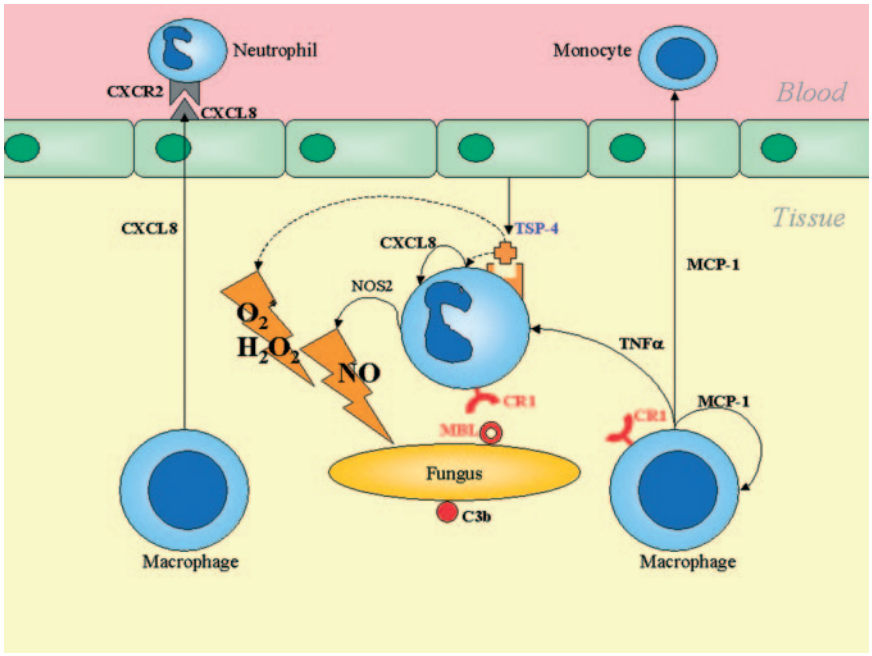

FIGURE 1. A simplified scheme of the innate immune response against fungi. When a fungus enters the body the innate immune system will be activated. Complement is activated via the alternative and lectin binding pathways by the binding of C3 and MBL, respectively, to the fungal surface. Both pathways lead to the formation of the opsonin C3b on the fungal surface, which is recognized by the complement receptor CR1 expressed by macrophages and neutrophils. MBL is also recognized by this receptor. Macrophages are already present in healthy tissue and will release a number of cytokines to kill the invading pathogen and attract more monocytes and neutrophils from the bloodstream to the site of infection. Monocytes are attracted by the macrophage chemoattractant protein MCP-1 and will mature into macrophages in the tissue. Neutrophils are attracted by IL-8 (CXCL8). CXCL8 is recognized by neutrophils via the receptors CXCR1 and CXCR2. Both chemokines also activate their target cells, in particular CXCL8 and TNF-α, which activate neutrophils to generate among others NO via NOS2. TSP-4 is secreted by endothelial cells to stimulate the production of oxygen radicals and the excretion of CXCL8.

unable to clear the infection, we investigated whether selected single nucleotide polymorphisms (SNPs) in genes involved in neutrophil function were more commonly found in the patient population than in an endemic reference population or vice versa.

Materials and Methods

Study cohort

Blood samples were taken from patients and a matched control population in the Sudan endemic area between 2001 and 2004. Furthermore, from some of the patients biopsies were taken as well. No coinfections were recorded. Serum was stored at −20°C until further use. For patients the duration of disease, the size of the lesion, and the site of infection were recorded. The mean age of the patients was 27.4 years (7–80 years) and 72.8% of the patients were male, figures comparable to those of the matched endemic control population (mean age 28.6 and 73.8% male). For the patients, the mean duration of the disease was 6.98 years (<1–27 year). Eighty nine point six percent of the patients had eumycetoma, 8% had actinomycetoma, and for 2.4% the type of mycetoma was not known. Seventy eight point four percent of the patients had a lesion on the foot, 9.6% on the hand, 11.2% on the lower leg, and 0.8% (1 patient) had a lesion on both a foot and a hand. Fifty one point two percent of the patients had a small lesion, and 48.8% had a moderate to large lesion. Lesion size was measured in a comparable and standardized manner among mycetoma patients. A more clear definition of size was not possible because mycetoma lesions are diffuse and have a mass and ill-defined margins.

DNA isolation

Genomic DNA was isolated from 265 blood samples (125 patients and 140 controls) with the large volume kit for the MagNA Pure system (Roche) according to the manufacturer's descriptions. DNA was stored at −20°C until further use.

Genotyping

All PCR primers and amplification conditions are stated in Table I. Genotyping of CXCR2, CXCL8, and TNF-α was performed using a PCR tetra-primer amplification refractory mutation system (11). Genotyping of the other genes was performed with classical PCR-RFLP methods. Restriction enzymes used are also shown in Table I and were obtained either from New England Biolabs or Fermentas. All restriction endonucleases were used as described by the manufacturer.

CXCL8 expression

CXCL8 expression was measured in serum from 43 patients and 37 healthy controls with a CXCL8 ELISA (Diaclone) according to the manufacturer's instructions.

NOS production

The concentrations of nitrite and nitrate in serum from 43 patients and 37 healthy controls were determined as a reflection of NOS activity. Serum was diluted 4-fold in a solution containing 50 μM NAPDH (Sigma-Aldrich), 5 μM flavin adenine dinucleotide (Sigma-Aldrich), and 200 U/L nitrate reductase (Sigma-Aldrich). To convert nitrate into nitrite, the sample was incubated for 20 min at 37°C. Excess NADPH was oxidized by adding 7760 U/L lactate dehydrogenase (Fluka Biochemicals), and 10 mM sodium pyruvate (Sigma-Aldrich) during a further incubation for 5 min at 37°C. Finally, the sample was deproteinized by adding 10 μl of 300 g/L zinc sulfate. The sample was centrifuged for 10 min at 10,000 rpm and 100 μl of the supernatant was used to determine the nitrite concentration colorimetrically using the Griess reagent system (Promega). Concentrations of nitrite were estimated by comparing absorbance readings at 540 nm against those of standard solutions of sodium nitrite.

In situ analysis

Five mycetoma biopsies and one biopsy from the uninfected part of the foot of a mycetoma patient were embedded in paraffin. The mean age of the patients was 25.4 years (22–30 years) and two of the patients were male. The mean duration of the disease was 5.3 years (2.5–9 years). All patients were infected with *M. mycetomatis* and had a lesion on a foot. One of the patients had a small lesion and the others had large lesions. Biopsies taken from the infected parts of the foot were chosen on the basis of the visible presence of grains to ascertain that grains were present in stained slides.

Table I. *PCR conditions for the different polymorphisms*[a]

Polymorphism	Primer	Sequence (5′→3′)	PCR Program	Restriction Endonuclease	Allele	Length (bp)	Ref.
CXCR2							
+785C→T	CXCR2-in fw (C)	TCTTTGCTGTCGTCCTCATCTTCCTGATC	5 × (1′ 94°C + 1′ 67°C + 1′ 72°C) → 30 ×	None	C	451 + 226	11
	CXCR2-in rv (T)	AGGACCAGGTTGTAGGGCAGCCAGAAA	(1′ 94°C + 1′ 62°C + 1′ 72°C)		T	451 + 281	
	CXCR2-out fw	CTGCTTGTCTTACTTTTCCGAAGGACCG					
	CXCR2-out rv	TCTTGAGGAGTCCATGGCGAAACTTCTG					
CXCL8							
−251T→A	CXCL8-in fw (T)	GTTATCTAGAAATAAAAAAGCATACAA	5 × (1′ 94°C + 1′ 52°C + 1′ 72°C) → 30 ×	None	T	349 + 169	11
	CXCL8-in rv (A)	CTCATCTTTTCATTATGTCAGAG	(1′ 94°C + 1′ 47°C + 1′ 72°C)		A[b]	349 + 228	
	CXCL8-out fw	CATGATAGCATCTGTAATTAACTG					
	CXCL8-out rv	CACAATTTGGTGAATTATCAAA					
MCP-1							
−2518	MCP-1-fw	GCTCCGGGCCCAGTATCT	5 × (1′ 94°C + 1′ 52°C + 1′ 72°C) → 30 ×	*Pvu*II	A	236	38
	MCP-1-rv	ACAGGGAAGGTGAAGGGTATGA	(1′ 94°C + 1′ 47°C + 1′ 72°C)		G[b]	182 + 54	
TNF							
−308G→A	TNF-308-fw in	TGGAGGCAATAGGTTTTGAGGGGCAGGA	5 × (1′ 94°C + 1′ 67°C + 1′ 72°C) → 30 ×	None	G[b]	323 + 224	11
	TNF-308-rv in	TAGGACCCTGGAGGCTGAACCCCGTACC	(1′ 94°C + 1′ 62°C + 1′ 72°C)		A	323 + 154	
	TNF-308-fw out	ACCCAAACACACGCCTCAGGACTCAAC					
	TNF-308-rv out	AGTTGGGGACACGCAAGCATGAAGGATA					
MBL							
54	MBL-fw	GTAGGACAGAGGGCATGCTC	35 × (30″ 94°C + 1′ 58°C + 1′ 72°C)	*Ban*I	w[b]	245 + 84	39
	MBL-rv	CAGGCAGTTTCCTCTGGAAGG			m	329	
57	MBL-fw	GTAGGACAGAGGGCATGCTC	35 × (30″ 94°C + 1′ 58°C + 1′ 72°C)	*Mbo*II	w[b]	329	
	MBL-rv	CAGGCAGTTTCCTCTGGAAGG			m	248 + 81	
XY	MBL prom-fw	GTTTCCACTCATTCTCATTCCCTAAG	35 × (30″ 94°C + 30″ 60°C + 45″ 72°C)	*Bsa*JI	X	242 + 108	40
	MBL prom-rv	GAAAACTCAGGGAAGGTTAATCTCAG			Y[b]	166 + 108 + 76	
CR1							
S1	24KnNde	ACCAGTGCCACACTGGACCAGATGGAGAACAGCTGTTTGAGCAT	44 × (1′ 94°C + 1′ 58°C + 1′ 72°C)	*Mfe*I	w	305	10
	25Rb	GGAGGAGTGTGGCAGCTTG			m	261 + 44	
McC	24KnNde	ACCAGTGCCACACTGGACCAGATGGAGAACAGCTGTTTGAGCAT	44 × (1′ 94°C + 1′ 58°C + 1′ 72°C)	*Bsm*I	w	305	
	25Rb	GGAGGAGTGTGGCAGCTTG			m	166 + 139	
NOS2							
Lambaréné	NOS-F	CATATGTATGGGAATACTGTATTTCAG	40 × (30″ 94°C + 1′ 60°C + 1′ 72°C)	*Bsa*I	G	680	41
	NOS-4	TCTGAACTAGTCACTTGAGG			C[b]	490	
TSP-4							
29926G→C	TSP4-fw	AATTCCGCATCTTCACTTCAC	32 × (40″ 94°C + 30″ 59°C + 40″ 72°C)	*Ava*II	C[b]	143 + 78	(42)
	TSP4-rv	AACCGGTTCTGCTTTGATAAC			G	221	

[a] All polymorphisms described here resulted not only in a nucleotide change but also in functional amino acid change, except for CXCR2. For most genes, the difference in amino acids resulted in differences in the expression of the protein encoded. Primer abbreviations: fw, Forward; rv, reverse; prom, promoter.

[b] The allele with the higher expression of the corresponding protein.

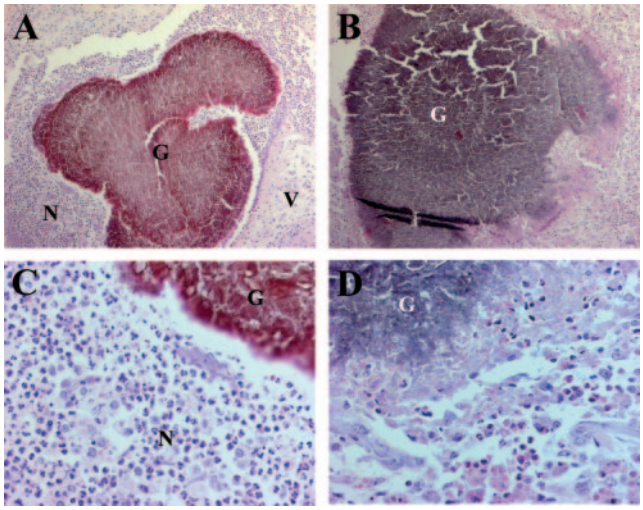

FIGURE 2. Two different inflammation reaction types in mycetoma (HE stained). *A*, Type I inflammation reaction, characterized by an inner zone of neutrophils (N) surrounding the grain embedded in cement material (G) and an outer vascular zone (V) (original magnification: ×100). *B*, Type II inflammation reaction in which the neutrophil zone is absent and is replaced by histiocytes and multinucleated giant cells (original magnification: ×100). *C*, Type I inflammation reaction (original magnification: ×400). *D*, Type II inflammation reaction (original magnification: ×400).

Slides were deparaffinized in xylene, dehydrated through a graded ethanol series, and washed in distilled water. To retrieve the Ag epitopes, slides were heated for 10 min at 650 W in a microwave oven in 10 mM citrate buffer (pH 6.4). Endogenous peroxidase was blocked by immersing the slides in 0.3% H_2O_2 in methanol for 30 min at room temperature. Nonspecific binding sites were blocked with 1/50 diluted normal goat serum (Vector Laboratories) for 1 h at room temperature. Then the sections were incubated with the primary Ab at 4°C overnight. Anti-CXCL8 (H-60, catalog no. sc-7922; Santa Cruz Biotechnology) was used at a concentration of 4 μg/ml, anti-NOS2 (N-20, catalog no. sc-651; Santa Cruz Biotechnology) in a concentration of 2 μg/ml. Both primary Abs were rabbit polyclonal Abs. Sections were further incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) for 1 h at room temperature and another 30 min in the ABC reagent (Vector Laboratories). Peroxidase was developed with 3-amino-9-ethylcarbazole (Sigma-Aldrich) for 12 min. Development was stopped by washing for 15 min in PBS with 0.05% Tween 20. Sections were counterstained with hematoxylin (Sigma-Aldrich). For reference purposes, some of the slides were also stained with H&E or Grocott stain. As a control, two *M. mycetomatis* isolates in vitro cultured on Sabouraud agarose and one *Candida albicans* isolate were stained for endogenous CXCL8-like molecules following the same procedure.

Statistical analysis

Verification of Hardy-Weinberg equilibrium (HWE) was performed with Pearson's χ^2 test. The effect of human polymorphisms in susceptibility to mycetoma was assessed with the logistic regression model (SPSS 11.0). Differences in allele frequency were analyzed with the Fisher's exact test (GraphPad Instat software). The significance of differences in CXCL8 and nitrite/nitrate concentrations in serum was calculated with the Mann-Whitney Test (GraphPad Instat software). $p < 0.05$ were considered significant.

Results

Neutrophils are attracted to the site of infection

As is seen in Fig. 2, neutrophils are attracted to the site of infection. Around the fungal grain two main types of inflammatory reaction can be observed (6). The first was the type I reaction, where *M. mycetomatis* grains were surrounded by a large zone of neutrophils (Fig. 2*A*) (6). The second reaction was characterized by the presence of histiocytes and multinucleated giant cells and a small number of neutrophils (Fig. 2*B*).

CXCR2, CXCL8, thrombospondin-4 (TSP-4), NOS2, and CR1 polymorphisms are differentially distributed among mycetoma patients vs healthy endemic matched controls

To elucidate the possible deficiencies in neutrophil function among mycetoma patients, genotype and allele frequencies for the genes encoding for CXCR2, CXCL8, MCP-1, TNF-α, MBL, MBL promoter, CR1, NOS2, and TSP-4 were determined. As shown in Table II, genotype distributions for all SNPs reached HWE except for CXCL8. Genotype distribution for CXCL8 in the control population was in disequilibrium (HWE; $p = 0.003$) but was in equilibrium in the patient population (HWE; $p = 0.81$). Differences in genotype distributions were found for CR1, CXCR2, and NOS2 (Table II). Significant differences in allele frequencies were found for CR1, CXCR2, CXCL8, TSP-4, and NOS2 (Table III). Obviously, the statistical significances of the CR1, CXCL8, and NOS2 polymorphisms are the most important ones, given the low p values. No significant differences were found for MCP-1, TNF-α, MBL, and MBL promoter SNPs.

Mycetoma patients more often posses the Sl2 and McC^a genotypes of CR1

In the gene encoding for CR1 two different polymorphisms were determined, namely the Sl polymorphism and the McC polymorphism. These polymorphisms were previously shown to be associated with resistance to severe malaria (10). The allele *Sl2* was more often found in the patient population than in the control population (Tables II and III). The *McC^b* allele was more dominant in the control population. To assess whether one of these polymorphisms in the gene encoding CR1 was also associated with disease progression, the allele frequencies obtained for the patients were divided into three groups according to lesion size. Allele frequencies for the *Sl* allele and the *McC* allele were compared between the group with the largest lesions and the group with the smallest lesions. It appeared that in both groups no differences in allele frequencies for the *Sl* allele ($p = 0.47$; Table IV) and the *McC* allele ($p = 0.45$; Table IV) were found.

Mycetoma patients express high levels of CXCL8 during infection

Different allele frequencies were also found in the genes encoding for the neutrophil attractant CXCL8, its receptor CXCR2, and TSP-4. The genotypes for these genes, which were more often encountered in the patient population, were all correlated with phenotypes expressing high CXCL8 levels. When comparing the allele frequencies of the patients with large lesions with the allele frequencies of the patients with the small lesions, no correlation was found between these allele frequencies and the size of the lesion (Table IV).

To analyze whether the neutrophils present at the site of infection did indeed express CXCL8, lesion tissue was stained for CXCL8. CXCL8-producing cells were found in all samples. Grains surrounded by a so-called type I tissue-reaction (Fig. 1) had only a few CXCL8-positive cells; neutrophils generally produced no CXCL8. More CXCL8-positive cells were noticed during a type II tissue reaction. Cells expressing CXCL8 were mainly macrophages, especially macrophages with hemosiderin deposits or cells surrounding them (Fig. 3). Interestingly, CXCL8 was found on ~50% of the hyphae within the grain (Fig. 3*A*). This was not found when cultured *M. mycetomatis* was stained with an Ab to CXCL8. In contrast, cultured *C. albicans* did stain with an Ab to CXCL8, which agrees with previously published data (14). CXCL8 only appeared to be present at the site of infection ($n = 5$),

Table II. *Genotype distributions of mycetoma patients in comparison with a matched healthy control population*[a]

Gene	Allele	Mycetoma Genotype: Patients $n = 125$ (%)	Controls $n = 140$ (%)	HWE	Binary Logistic Regression OR (95% CI)
CR1					
Sl	11	46 (36.8)	63 (45.0)	0.83	1
	12	53 (42.4)	61 (37.9)		**2.23 (1.1–4.6)**
	22	26 (20.8)	16 (11.4)		1.19 (0.7–2.0)
McC	aa	105 (84.0)	93 (66.4)	0.40	**aa vs ab and bb**
	ab	19 (15.2)	44 (31.4)		**0.38 (0.21–0.68)**
	bb	1 (0.8)	3 (2.1)		
CXCL8					
−251	AA	82 (65.6)	78 (55.7)	0.003	AA versus AT and TT
	AT	39 (31.2)	43 (30.7)		0.66 (0.4–1.0)
	TT	4 (3.2)	19 (13.6)		
CXCR2					
+785	TT	8 (6.4)	13 (9.3)	0.78	TT versus TC and CC
	TC	38 (30.4)	57 (40.7)		**0.38 (0.21–0.68)**
	CC	79 (63.2)	70 (50.0)		
MBL					
54	ww	113 (90.4)	132 (94.3)	0.73	ww versus wm
	wm	12 (9.6)	8 (5.7)		1.76 (0.7–4.4)
	mm	0 (0)	0 (0)		
57	ww	95 (76.0)	104 (74.3)	0.06	ww versus wm
	wm	28 (22.4)	30 (21.4)		0.91 (0.5–1.6)
	mm	2 (1.6)	6 (4.3)		
XY	XX	19 (15.2)	21 (15.0)	0.05	1
	XX	48 (38.4)	52 (37.1)		1.05 (0.5–2.1)
	YY	58 (46.4)	67 (47.9)		1.07 (0.6–1.8)
MCP1					
2518	AA	79 (63.2)	91 (65.0)	0.46	AA versus AG and GG
	AG	36 (28.8)	42 (30.0)		1.08 (0.7–1.8)
	GG	10 (8.0)	7 (5.0)		
NOS2					
Lambaréné	GG	107 (85.6)	94 (67.1)	0.21	**GG versus GC and CC**
	GC	18 (14.4)	44 (31.4)		**0.34 (0.2–0.6)**
	CC	0 (0)	2 (1.4)		
TNF-α					
−308	AA	106 (84.8)	117 (83.6)	0.29	AA versus AT and TT
	AT	17 (13.6)	23 (16.4)		0.91 (0.5–1.8)
	TT	2 (1.6)	0 (0)		
TSP-4					
29926	GG	83 (66.4)	108 (77.1)	0.96	GG versus GC and CC
	GC	36 (14.4)	30 (21.4)		1.71 (1.0–2.9)
	CC	6 (2.4)	2 (1.4)		

[a] HWE and binary logistic regression analyses are shown. Values in the boldfaced type are considered significant. OR, Odds ratio; CI, confidence interval.

because in control tissue from a noninfected part of the foot no CXCL8 expression was noted ($n = 1$).

Because of the presence of CXCL8 at the site of infection, it was presumed that it was also secreted in serum. Therefore, a CXCL8-specific ELISA was performed to measure the amount of CXCL8 present in serum. As is seen in Fig. 4, serum CXCL8 levels were significantly elevated in mycetoma patients (mean = 431.2 pg/ml). This increase was statistically highly significant when compared with the matched endemic population (Mann-Whitney; $p < 0.0001$). To assess whether the CXCL8 concentration was also an indication of the severity of the disease, it was analyzed whether the CXCL8 serum concentrations found in patients with large lesions were higher than the concentrations found in patients with small lesions. No significant correlation with the size of the lesion and the amount of CXCL8 present in serum was found (Mann-Whitney; $p = 0.0973$).

NOS2 is expressed at the site of infection

The last polymorphism that was not equally distributed between patients and the endemic control populations was in the gene encoding NOS2. The $NOS2^{Lambaréné}$ polymorphism appeared to be more common in the control population as compared with the patient population. With immunohistochemistry it was shown that NOS2 was present at the site of infection (Fig. 3). NOS2 was expressed throughout the entire dermis and epidermis. NOS2 production was found in the stratum corneum and stratum spinosum in both infected and uninfected parts of the foot and was therefore probably not specific for mycetoma (Fig. 3*B*). NOS2 expression

Table III. *Allele frequencies of mycetoma patients in comparison to a matching healthy control population as assessed with Fisher's exact test*

Gene	Allele	Mycetoma Allele Frequency: Patients $n = 125$ (%)	Controls $n = 140$ (%)	p Value[a]	OR (95% CI)[a]
CR1					
Sl	1	145 (58.0)	187 (66.8)	**0.0390**	**0.68 (0.48–0.98)**
	2	105 (42.0)	93 (33.2)		
McC	A	229 (91.6)	230 (82.1)	**0.0014**	**2.37 (1.38–4.08)**
	B	21 (8.4)	50 (17.9)		
CXCL8					
−251	A	203 (81.2)	199 (71.1)	**0.0081**	**1.76 (1.17–2.65)**
	T	47 (18.8)	81 (28.9)		
CXCR2					
+785	T	54 (21.6)	83 (29.6)	**0.0372**	**0.65 (0.44–0.97)**
	C	196 (78.4)	197 (70.4)		
MBL					
54	W	238 (95.2)	272 (97.1)	0.2618	0.58 (0.23–1.45)
	M	12 (4.8)	8 (2.9)		
57	W	218 (87.2)	238 (85.0)	0.5306	1.20 (0.73–1.97)
	M	32 (12.8)	42 (15.0)		
XY	X	86 (34.4)	94 (33.6)	0.3988	1.04 (0.72–1.49)
	Y	164 (65.6)	186 (66.4)		
MCP-1					
2518	A	194 (77.6)	224 (80.0)	0.5236	0.87 (0.57–1.32)
	G	56 (22.4)	56 (20.0)		
NOS2					
Lambaréné	G	232 (92.8)	232 (83.6)	**0.0006**	**2.67 (1.51–4.72)**
	C	18 (7.2)	48 (16.4)		
TNF-α					
−308	A	229 (91.6)	257 (91.8)	1.0000	
	T	21 (8.4)	23 (8.2)		
TSP-4					
29926	G	202 (80.8)	246 (87.9)	**0.0301**	**0.58 (0.36–0.94)**
	C	48 (19.2)	34 (12.1)		

[a] The p values and odds ratios (OR) are given. Significant p values are highlighted in the boldfaced letters. CI, Confidence interval.

was also found in phagocytic cells. The number of NOS2-positive cells differed per patient and per grain but were clearly present in all five patients. The closer the grain was to the dermis, the more NOS2-positive cells were detected.

The frequency of the $NOS2^{Lambaréné}$ polymorphism differed among subgroups. Not only was the polymorphism more often found in the control population, but it was also more frequent among patients with the largest lesions. Only three of 64 patients with small lesions displayed this polymorphism, whereas the frequency of this polymorphism was much higher in the patient group with the largest lesions (in 15 of 61 patients ($p = 0.0027$; Table IV)). Because the $NOS2^{Lambaréné}$ polymorphism was more frequent in the control population, we expected to find higher NOS activity in the control sera. Therefore, the nitrite and nitrate concentrations in serum were determined. As seen in Fig. 3 the serum nitrite and nitrate concentrations were significantly lower among patients (mean = 2.83 μM) than in the matched endemic control population (mean = 9.28 μM). Because the $NOS2^{Lambaréné}$ polymorphism was also found more often in patients with larger lesions, it was determined whether the nitrite/nitrate concentration in the serum of these patients were higher than in the serum of patients with smaller lesions. Although the patients with the larger lesions did indeed have a higher nitrite/nitrate concentration in their serum (mean = 4.36 μM vs mean = 2.55 μM), the difference was not statistically significant (Mann-Whitney; $p = 0.6028$).

Discussion

In this study it was shown that neutrophils are attracted to the mycetoma grains in situ. Two main types of inflammatory reaction were observed. Both reactions could be seen in the same lesion and are not unique to *M. mycetomatis.* They are also observed in mycetoma caused by *Petriellidium boydii*, *Neotestudina rosatii*, *Fusarium* spp., and *Acremonium* spp. (7). It has been suggested that the type I reaction is an early response to grain formation that is succeeded by the type II reaction (8). Apparently, neutrophils are important in the early defense against mycetoma. Some differences in genotype distributions between patients and a matched endemic population for some genes involved in neutrophil function were observed. The control individuals who were sampled were living in the same region as the patients and had similar tribal and ethnic backgrounds. Unfortunately, at the time of collection of the samples we were not in a position to collect extensive amounts

Table IV. *Gomparison of frequencies for CR1, CXCL8, CXCR2, NOS2, and TSP-4 alleles between the group with the largest lesions and the group with the smallest lesions*[a]

Genotype	Lesion Size: Massive $n = 41$ (%)	Lesion Size: Moderate $n = 20$ (%)	Lesion Size: Small $n = 64$ (%)	Allele	Lesion Size: Massive $n = 41$ (%)	Lesion Size: Moderate $n = 20$ (%)	Lesion Size: Small $n = 64$ (%)	p Value (Massive Compared to Small)	OR (95% CI)
CR1 SI									
11	14 (34.1)	8 (40.0)	24 (37.5)	1	44 (53.7)	25 (62.3)	76 (59.4)	0.4753	0.79 (0.45–1.39)
12	16 (39.0)	9 (45.0)	28 (43.8)	2	38 (46.3)	15 (37.5)	52 (40.6)		
22	11 (26.8)	3 (15.0)	12 (18.8)						
CR1 McC									
aa	36 (87.8)	17 (85.0)	52 (81.3)	a	77 (93.9)	36 (90.0)	116 (90.6)	0.4487	1.59 (0.54–4.70)
ab	5 (12.2)	2 (10.0)	12 (18.8)	b	5 (6.1)	4 (10.0)	12 (9.4)		
bb	0 (0.0)	1 (5.0)	0 (0.0)						
CXCL8									
AA	31 (75.6)	11 (55.0)	40 (62.5)	A	72 (87.8)	30 (75.0)	101 (78.9)	0.1368	1.93 (0.88–4.23)
AT	10 (24.3)	8 (40.0)	21 (32.8)	T	10 (12.2)	10 (25.0)	27 (21.1)		
TT	0 (0.0)	1 (5.0)	3 (4.7)						
CXCR2									
CC	2 (4.9)	1 (5.0)	5 (7.8)	C	64 (78.0)	30 (75.0)	102 (79.7)	0.8623	0.91 (0.46–1.79)
TC	14 (34.1)	8 (40.0)	16 (25.0)	T	18 (22.0)	10 (25.0)	26 (20.3)		
TT	25 (60.9)	11 (55.0)	43 (67.2)						
NOS2									
GG	31(75.6)	15 (75.0)	61 (95.3)	G	72 (87.8)	35 (87.5)	125 (97.7)	**0.0063**	**0.17 (0.05–0.65)**
GC	10 (24.3)	5 (25.0)	3 (4.7)	C	10 (12.2)	5 (12.5)	3 (2.3)		
CC	0 (0.0)	0 (0.0)	0 (0.0)						
TSP-4									
GG	28 (68.3)	12 (60.0)	43 (67.2)	G	67 (81.7)	30 (75.0)	105 (82.0)	1.0000	0.98 (0.48–2.01)
GC	11 (26.8)	6 (30.0)	19 (29.7)	C	15 (18.3)	10 (25.0)	23 (18.0)		
CC	2 (4.9)	2 (10.0)	2 (3.1)						

[a] The p values and odds ratios (OR) are given. Significant p values are highlighted in the boldfaced letters. CI, Confidence interval.

of demographic and health-related data and we therefore cannot be totally sure that the control group was a complete match to the patient population. When the mycetoma patients were compared with the control population, differences were found for CR1, CXCL8, CXCR2, TSP4, and NOS2. No differences in the distribution of SNPs in MCP-1, TNF-α, MBL, and MBL promoter were

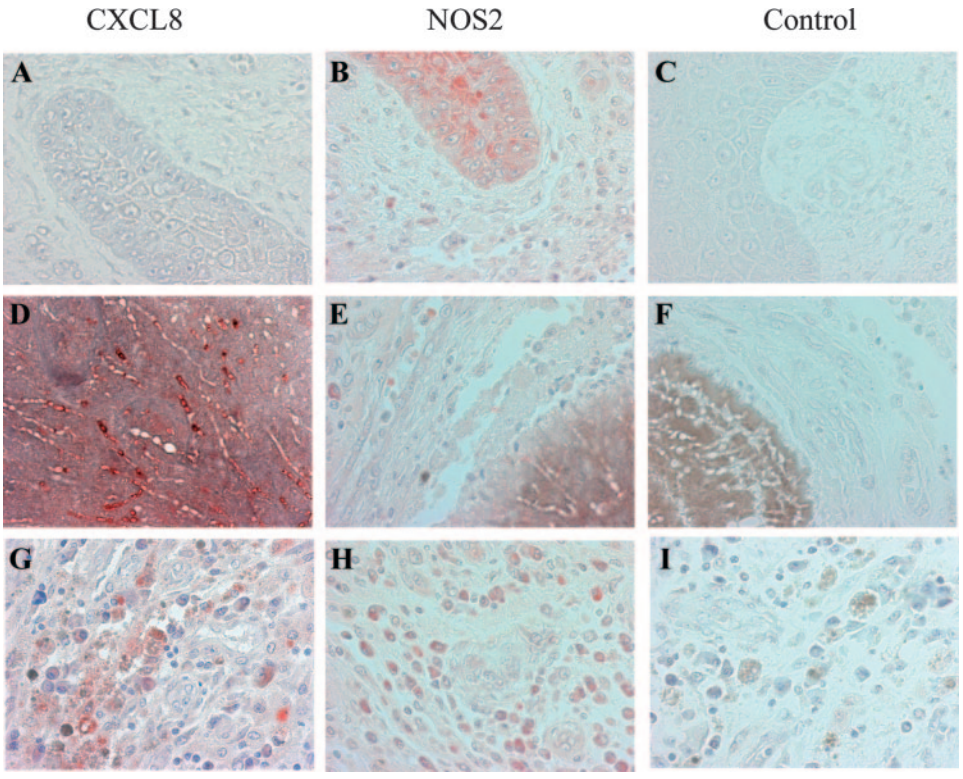

FIGURE 3. CXCL8 and NOS2 production in *M. mycetomatis* mycetoma-infected skin (original magnification ×400). *A*, No CXCL8 is found in the epidermis. *B*, NOS2 expression in the epidermis. *C*, No expression is found when the primary Ab is replaced with PBS or normal rabbit serum. *D*, Binding of CXCL8 Abs to individual hyphae in the grain. *E*, No expression of NOS2 on hyphae, but some expression found in cells surrounding the grain. *F*, No expression is found when the primary Ab is replaced with PBS or normal rabbit serum. *G*, High CXCL8 expression in macrophages with hemosiderin deposits and the surrounding cells in the vascular zone. *H*, NOS2 expression in the vascular zone. *I*, Macrophages with deposits in the vascular zone of the PBS-treated control.

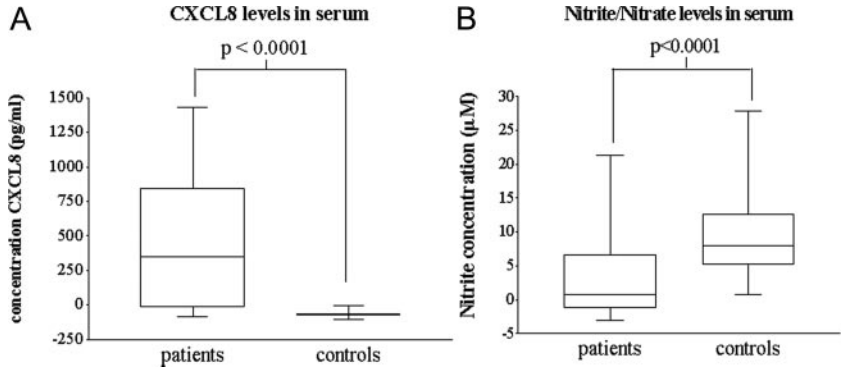

FIGURE 4. CXCL8 and nitrite/nitrate levels in serum. *A*, CXCL8 levels (pg/ml) determined in the serum of patients ($n = 43$) and the healthy endemic control population ($n = 39$). *B*, Nitrite/nitrate levels (μM) determined in the serum of patients and a healthy Sudanese control population. Significance was calculated with the Mann-Whitney *U* test.

found. In mycetoma patients the Sl2 and McC^a genotypes of CR1 were more common than in the endemic control population. The CXCL8, CXCR2, and TSP-4 genotypes correlated with a higher CXCL8 production, and the NOS2 SNP correlated with lower NOS2 secretion. The latter were confirmed by physiological measurements of higher CXCL8 levels and lower nitrate/nitrite levels in patient serum.

Sl2 and McC^a genotypes of CR1 in mycetoma patients

From the data presented in the present work it appeared that having a deviating CR1 could enhance the chance of developing a mycetoma infection. This observation was in agreement with our hypothesis that mycetoma patients have a genetic impairment in neutrophil function. Although CR1 is expressed on neutrophils, it is not unique for this cell type. In fact, CR1 is a receptor expressed by a whole range of other cells including follicular dendritic cells, macrophages, T and B lymphocytes, and erythrocytes. Two of the polymorphisms in the CR1 gene are responsible for the Sl blood group Ag and the McC blood group Ag, both members of the Knops blood group typing system (15). Here we showed that the Sl2 and the McC^a alleles were more often found in mycetoma patients than in the matched endemic control population. This was unique, because with other blood group typing systems such as the ABO blood groups and Rhesus factors no association was found with a predisposition to develop mycetoma (5). The McC^a allele associated with mycetoma has already been described as being associated with severe cerebral malaria caused by *Plasmodium falciparum* (10). In contrast, the Sl2 allele offered some protection against this type of malaria (10).

As is seen in Fig. 5, the Sl and McC polymorphisms are present in the long, homologous repetitive D region of the CR1 gene. This is the region that codes for the binding structure of the protein in which MBL and C1q binding occurs and could therefore cause conformational changes that could influence the function of the molecule, not only as executed on erythrocytes but also on other cell types (10, 16). On neutrophil surfaces, CR1 binds pathogens such as *Escherichia coli* and *Staphylococcus aureus* and presents them to phagocytic cells (17, 18). It is, therefore, conceivable that conformational changes in the receptor also influence the efficacy of *M. mycetomatis* phagocytosis (17, 18). However, this remains to be determined because the effects of these polymorphisms on the function of the receptors has not yet been defined in full detail.

Mycetoma patients express high levels of CXCL8 during infection

Additional differences in allelic distributions were found for the genes encoding CXCL8, its receptor CXCR2, and TSP-4, which implied that alterations in neutrophil attraction are associated with the development of mycetoma. In mycetoma patients the CXCL8 *−251A* allele, the CXCR2 *+785C* allele, and the TSP-4 *29929C* allele (also known as the 387P variant) were found more often than in the control population. These alleles are all associated with an increased production of CXCL8 (11). CXCL8 is produced by many cell types, including macrophages, as a chemokine to attract neutrophils to the site of infection. TSPs are also secreted at the site of injury and stimulate the chemotactic response of neutrophils (19). CXCR2 is activated by CXCL8 and this activation enhances the generation of reactive oxygen species and the phagocytosis of pathogens (20). CXCL8 is usually barely detectable in the normal skin, but strong CXCL8 production can be observed in psoriasis, atopic dermatitis, and acute generalized exanthematous pustulosis (21). In this report we show that CXCL8 is also abundantly present in the mycetoma lesions. The extent of CXCL8 expression appeared to be dependent on the inflammation type, with more

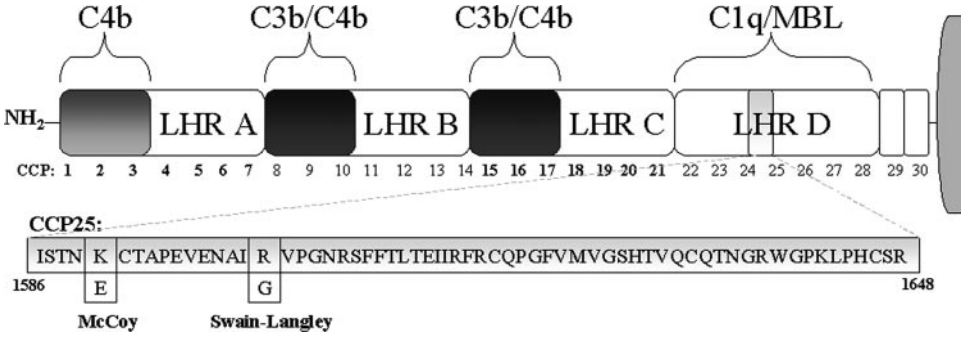

FIGURE 5. A schematic figure of the gene organization of the complement receptor CR1. CR1 is composed of 20 complement control protein repeats (CCP) that are arranged in four long homologous regions (LHR A–D). CCP1–3 represents a C4b binding site and CCP8–10 and CCP15–17 represent two identical copies of a C3b/C4b binding site. In LHR D an additional binding site exists for both C1q and MBL. Knops blood group type polymorphisms are found in CCP25 at amino acid positions 1590 and 1601.

CXCL8-positive cells present during the type II reaction, a reaction characterized by a higher amount of histiocytes and giant cells. Macrophages with hemosiderin deposits and high CXCL8 expression were found in the vascular zone (see Fig. 3*G*). CXCL8 was also found on hyphae within the grain, suggesting that CXCL8 is bound to the *M. mycetomatis* hyphae to prevent either neutrophil attraction or activation. Another explanation could be that CXCL8 simply becomes trapped when the cement material is formed. Cement material is composed of remnants of fungal and host cells. It is not expected that *M. mycetomatis* forms CXCL8 analogues such as *C. albicans* does, because cultured *M. mycetomatis* did not react with anti-CXCL8 Ab (14).

CXCL8 was not only expressed in the skin, but high amounts of CXCL8 were also detected in the serum of the mycetoma patients. CXCL8 concentrations normally found in infectious diseases are 5–10 times lower than those reported here (22–24) except for very severe infections such as Gram-negative bacteremia (25). The concentrations of CXCL8 found in the serum of the mycetoma patients were even 10 times higher as concentrations found in skin diseases like psoriasis (26). However, such elevated concentrations of CXCL8 are not exceptional, because in skin diseases such as dermatitis herpetiformis, a skin condition characterized by the accumulation of neutrophils, comparable concentrations of CXCL8 were found (27). Apparently, neutrophil accumulations are accompanied by high CXCL8 concentrations in serum, which supports the data suggesting that CXCL8 production is indeed important during the innate immune response to mycetoma.

NOS2 is expressed in lower amounts in patients than in controls

The fifth gene with SNP frequency differences between patient and controls was *NOS2*. This gene encodes a synthase involved in generating NO, a radical toxin to most microorganisms (28). However, NO can play a dual role in infections. NO defends the host against various microbial agents, but sometimes the NO-mediated inflammation causes too much damage to host cells and thereby conversely supports microbial invasion (28). Comparing allelic distribution of the NOS2 gene variants, it appeared that the NOS2 G954C mutation was more common among Sudanese healthy controls than in mycetoma patients. This genotype was considered beneficial because the substitution from G to C results in a phenotype with a 7-fold higher baseline NOS activity (13). Indeed, a higher NOS activity in the control population was confirmed by an elevated nitrate/nitrite concentration measured in the sera as compared with that in the patients. Although for most infections nitrite and nitrate levels are increased, especially in active infections, reduced nitrite and nitrate levels were also found in patients with chronic hepatitis, tuberculosis, or malaria (29–31). Apparently, a high concentration of NO in serum offers protection against mycetoma.

High CXCL8 production and low NOS2 expression delay wound healing

Both CXCL8 and NOS2 levels influence acute inflammation and repair of damaged tissues in the skin (4). By attracting neutrophils to the site of infection, CXCL8 codetermines efficient killing of invading microbes either by phagocytosis or by secreting oxygen or nitrogen radicals (32). This is confirmed by the high NOS2 expression during wound repair (33). If for some reason NOS2 expression is suppressed, wound repair is much slower (33). Too many nitrogen and especially oxygen radicals can also cause serious tissue damage (34, 35). This will hamper wound healing as was shown by improved wound healing in neutrophil-depleted mice as compared with control mice (34). Mycetoma is thought to develop after the traumatic inoculation of a causative agent by, for instance, a thorn prick. If too much CXCL8 is produced after this thorn prick at the site of entry, too many neutrophils are attracted that could result in additional tissue damage. Patients tend to have low levels of NO, which could result in less efficient killing. NOS2 has shown to be of importance in another cutaneous infection, namely leishmaniasis. For this infection it has been shown that inhibition of NOS2 results in nonhealing cutaneous leishmanial lesions and even in reactivation of latent leishmaniasis (36, 37).

In conclusion, functional expression differences in genes involved in neutrophil function were documented for mycetoma patients. The Sl2 and McC^a blood group Ags were more often found in the patient population than in the endemic Sudanese reference population. Deviations in genes encoding for CXCL8, CXCR2, and TSP-4 were found that resulted in a higher CXCL8 production in mycetoma patients. Altered allele frequencies in the *NOS2* gene resulted in a lower NO production in mycetoma patients. Higher CXCL8 production and lower NO production are both implicated in less efficient wound healing, which could be a significant risk factor for developing mycetoma.

Acknowledgments

We thank Hafiz Bashir for this help with collecting blood samples, Marieke Emonts for statistical support, and Jon Laman and Pieter Leenen for comments and suggestions for this manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Ahmed, A. O., W. van Leeuwen, A. Fahal, W. van de Sande, H. Verbrugh, and A. van Belkum. 2004. Mycetoma caused by *Madurella mycetomatis*: a neglected infectious burden. *Lancet Infect. Dis.* 4: 566–574.
2. Ahmed, A., D. Adelmann, A. Fahal, H. Verbrugh, A. van Belkum, and S. de Hoog. 2002. Environmental occurrence of *Madurella mycetomatis*, the major agent of human eumycetoma in Sudan. *J. Clin. Microbiol.* 40: 1031–1036.
3. van de Sande, W. W., D. J. Janse, V. Hira, H. Goedhart, R. van der Zee, A. O. Ahmed, A. Ott, H. Verbrugh, and A. van Belkum. 2006. Translationally controlled tumor protein from *Madurella mycetomatis*, a marker for tumorous mycetoma progression. *J. Immunol.* 177: 1997–2005.
4. Mahgoub, E. S., S. A. Gumaa, and A. M. El Hassan. 1977. Immunological status of mycetoma patients. *Bull. Soc. Pathol. Exot. Filiales* 70: 48–54.
5. Fahal, A. H., M. E. Sadig, S. H. Suliman, and S. A. el Razig. 1996. Lack of association between ABO blood groups and Rh factor and the tendency to develop mycetoma. *East Afr. Med. J.* 73: 771.
6. Fahal, A. H., E. A. el Toum, A. M. el Hassan, E. S. Mahgoub, and S. A. Gumaa. 1995. The host tissue reaction to *Madurella mycetomatis*: new classification. *J. Med. Vet. Mycol.* 33: 15–17.
7. Hay, R. J., and M. J. Collins. 1983. An ultrastructural study of pale eumycetoma grains. *Sabouraudia* 21: 261–269.
8. El-hassan, A. M., A. Fahal, and I. El-hag. 1994. The pathology of mycetoma. *Sudan Med. J.* 32: 23–45.
9. Yousif, M. A., and R. J. Hay. 1987. Leucocyte chemotaxis to mycetoma agents–the effect of the antifungal drugs griseofulvin and ketoconazole. *Trans. R. Soc. Trop. Med. Hyg.* 81: 319–321.
10. Thathy, V., J. M. Moulds, B. Guyah, W. Otieno, and J. A. Stoute. 2005. Complement receptor 1 polymorphisms associated with resistance to severe malaria in Kenya. *Malar. J.* 4: 54.
11. Matheson, M. C., J. A. Ellis, J. Raven, E. H. Walters, and M. J. Abramson. 2006. Association of IL8, CXCR2 and TNF-α polymorphisms and airway disease. *J. Hum. Genet.* 51: 196–203.
12. Kobayashi, S. D., J. M. Voyich, and F. R. DeLeo. 2003. Regulation of the neutrophil-mediated inflammatory response to infection. *Microbes Infect.* 5: 1337–1344.
13. Kun, J. F., B. Mordmuller, D. J. Perkins, J. May, O. Mercereau-Puijalon, M. Alpers, J. B. Weinberg, and P. G. Kremsner. 2001. Nitric oxide synthase 2(Lambarene) (G-954C), increased nitric oxide production, and protection against malaria. *J. Infect. Dis.* 184: 330–336.
14. Ali, A., R. Rautemaa, J. Hietanen, A. Jarvensivu, M. Richardson, and Y. T. Konttinen. 2006. Expression of interleukin-8 and its receptor IL-8RA in chronic hyperplastic candidosis. *Oral Microbiol. Immunol.* 21: 223–230.
15. Moulds, J. M., P. A. Zimmerman, O. K. Doumbo, D. A. Diallo, J. P. Atkinson, M. Krych-Goldberg, D. E. Hourcade, and J. J. Moulds. 2002. Expansion of the Knops blood group system and subdivision of Sl(a). *Transfusion* 42: 251–256.
16. Krych-Goldberg, M., J. M. Moulds, and J. P. Atkinson. 2002. Human complement receptor type 1 (CR1) binds to a major malarial adhesin. *Trends Mol. Med.* 8: 531–537.
17. Gyimesi, E., A. J. Bankovich, T. A. Schuman, J. B. Goldberg, M. A. Lindorfer, and R. P. Taylor. 2004. *Staphylococcus aureus* bound to complement receptor 1

on human erythrocytes by bispecific monoclonal antibodies is phagocytosed by acceptor macrophages. *Immunol. Lett.* 95: 185–192.
18. Kuhn, S. E., A. Nardin, P. E. Klebba, and R. P. Taylor. 1998. *Escherichia coli* bound to the primate erythrocyte complement receptor via bispecific monoclonal antibodies are transferred to and phagocytosed by human monocytes in an in vitro model. *J. Immunol.* 160: 5088–5097.
19. Mansfield, P. J., L. A. Boxer, and S. J. Suchard. 1990. Thrombospondin stimulates motility of human neutrophils. *J. Cell Biol.* 111: 3077–3086.
20. Rambeaud, M., R. Clift, and G. M. Pighetti. 2006. Association of a bovine CXCR2 gene polymorphism with neutrophil survival and killing ability. *Vet. Immunol. Immunopathol.* 111: 231–238.
21. Keller, M., Z. Spanou, P. Schaerli, M. Britschgi, N. Yawalkar, M. Seitz, P. M. Villiger, and W. J. Pichler. 2005. T cell-regulated neutrophilic inflammation in autoinflammatory diseases. *J. Immunol.* 175: 7678–7686.
22. Vaisman, N., E. Leibovitz, R. Dagan, and V. Barak. 2003. The involvement of IL-6 and IL-8 in acute invasive gastroenteritis of children. *Cytokine* 22: 194–197.
23. Lyke, K. E., R. Burges, Y. Cissoko, L. Sangare, M. Dao, I. Diarra, A. Kone, R. Harley, C. V. Plowe, O. K. Doumbo, and M. B. Sztein. 2004. Serum levels of the proinflammatory cytokines interleukin-1 β (IL-1β), IL-6, IL-8, IL-10, tumor necrosis factor α, and IL-12(p70) in Malian children with severe *Plasmodium falciparum* malaria and matched uncomplicated malaria or healthy controls. *Infect. Immun.* 72: 5630–5637.
24. Jensen, P. O., C. Moser, A. Kharazmi, T. Presler, C. Koch, and N. Hoiby. 2006. Increased serum concentration of G-CSF in cystic fibrosis patients with chronic *Pseudomonas aeruginosa* pneumonia. *J. Cyst. Fibros.* 5: 145–151.
25. Kern, W. V., M. Heiss, and G. Steinbach. 2001. Prediction of Gram-negative bacteremia in patients with cancer and febrile neutropenia by means of interleukin-8 levels in serum: targeting empirical monotherapy versus combination therapy. *Clin. Infect. Dis.* 32: 832–835.
26. Arican, O., M. Aral, S. Sasmaz, and P. Ciragil. 2005. Serum levels of TNF-α, IFN-γ, IL-6, IL-8, IL-12, IL-17, and IL-18 in patients with active psoriasis and correlation with disease severity. *Mediators Inflamm.* 2005: 273–279.
27. Hall, R. P., III, F. Takeuchi, K. M. Benbenisty, and R. D. Streilein. 2006. Cutaneous endothelial cell activation in normal skin of patients with dermatitis herpetiformis associated with increased serum levels of IL-8, sE-Selectin, and TNF-α. *J. Invest. Dermatol.* 126: 1331–1337.
28. Zaki, M. H., T. Akuta, and T. Akaike. 2005. Nitric oxide-induced nitrative stress involved in microbial pathogenesis. *J. Pharmacol. Sci.* 98: 117–129.
29. Fiorenza, G., L. Rateni, M. A. Farroni, C. Bogue, and D. G. Dlugovitzky. 2005. TNF-α, TGF-β and NO relationship in sera from tuberculosis (TB) patients of different severity. *Immunol. Lett.* 98: 45–48.
30. Anstey, N. M., J. B. Weinberg, M. Y. Hassanali, E. D. Mwaikambo, D. Manyenga, M. A. Misukonis, D. R. Arnelle, D. Hollis, M. I. McDonald, and D. L. Granger. 1996. Nitric oxide in Tanzanian children with malaria: inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression. *J. Exp. Med.* 184: 557–567.
31. Amaro, M. J., J. Bartolome, M. Pardo, T. Cotonat, A. Lopez-Farre, and V. Carreno. 1997. Decreased nitric oxide production in chronic viral hepatitis B and C. *J. Med. Virol.* 51: 326–331.
32. Martin, P., and S. J. Leibovich. 2005. Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends Cell. Biol.* 15: 599–607.
33. Mahoney, E., J. Reichner, L. R. Bostom, B. Mastrofrancesco, W. Henry, and J. Albina. 2002. Bacterial colonization and the expression of inducible nitric oxide synthase in murine wounds. *Am. J. Pathol.* 161: 2143–2152.
34. Dovi, J. V., L. K. He, and L. A. DiPietro. 2003. Accelerated wound closure in neutrophil-depleted mice. *J. Leukocyte Biol.* 73: 448–455.
35. Dovi, J. V., A. M. Szpaderska, and L. A. DiPietro. 2004. Neutrophil function in the healing wound: adding insult to injury? *Thromb. Haemost.* 92: 275–280.
36. Stenger, S., N. Donhauser, H. Thuring, M. Rollinghoff, and C. Bogdan. 1996. Reactivation of latent leishmaniasis by inhibition of inducible nitric oxide synthase. *J. Exp. Med.* 183: 1501–1514.
37. Diefenbach, A., H. Schindler, N. Donhauser, E. Lorenz, T. Laskay, J. MacMicking, M. Rollinghoff, I. Gresser, and C. Bogdan. 1998. Type 1 interferon (IFNα/β) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. *Immunity* 8: 77–87.
38. Flores-Villanueva, P. O., J. A. Ruiz-Morales, C. H. Song, L. M. Flores, E. K. Jo, M. Montano, P. F. Barnes, M. Selman, and J. Granados. 2005. A functional promoter polymorphism in monocyte chemoattractant protein-1 is associated with increased susceptibility to pulmonary tuberculosis. *J. Exp. Med.* 202: 1649–1658.
39. Wang, Z. Y., A. Morinobu, S. Kanagawa, and S. Kumagai. 2001. Polymorphisms of the mannose binding lectin gene in patients with Sjogren's syndrome. *Ann. Rheum. Dis.* 60: 483–486.
40. Roelofs, R. W., T. Sprong, J. B. de Kok, and D. W. Swinkels. 2003. PCR-restriction fragment length polymorphism method to detect the X/Y polymorphism in the promoter site of the mannose-binding lectin gene. *Clin. Chem.* 49: 1557–1558.
41. Levesque, M. C., M. R. Hobbs, N. M. Anstey, T. N. Vaughn, J. A. Chancellor, A. Pole, D. J. Perkins, M. A. Misukonis, S. J. Chanock, D. L. Granger, and J. B. Weinberg. 1999. Nitric oxide synthase type 2 promoter polymorphisms, nitric oxide production, and disease severity in Tanzanian children with malaria. *J. Infect. Dis.* 180: 1994–2002.
42. Zhou, X., J. Huang, J. Chen, J. Zhao, W. Yang, X. Wang, and D. Gu. 2004. Thrombospondin-4 A387P polymorphism is not associated with coronary artery disease and myocardial infarction in the Chinese Han population. *Clin. Sci.* 106: 495–500.