Parasitol Res (2003) 91: 298–303
DOI 10.1007/s00436-003-0950-2

ORIGINAL PAPER

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Investigations on the biology, epidemiology, pathology and control of *Tunga penetrans* in Brazil: III. Cytokine levels in peripheral blood of infected humans

Received: 28 April 2003 / Accepted: 24 June 2003 / Published online: 22 August 2003

Abstract Tungiasis is caused by penetration of the female jigger flea, *Tunga penetrans*, into the skin of its host. This parasitic skin disease is almost invariably associated with intense inflammation around embedded fleas, the underlying mechanisms being unknown. A study was undertaken to determine whether the inflammatory process can be attributed to immune activation induced by a biologically active foreign body. We determined the concentrations of Th1-mediated (IFN-γ, TNF-α) and Th2-mediated (IL-4) cytokines in the sera of patients with tungiasis. The results were compared with those of controls infected with different helminths or exposed to soil-transmitted helminths. The results show that tungiasis causes a mixed Th1 and Th2 immune response, characterized by significantly increased concentrations of the pro-inflammatory cytokines IFN-γ and TNF-α, with a slightly increased concentration of IL-4. The preponderance of the Th1 immune response was indicated by a significantly increased TNF-α/IL-4 ratio in patients with tungiasis, as compared with the control groups.

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Introduction

Tungiasis is a common, but neglected health problem in deprived communities in South America, the Caribbean and sub-Saharan Africa (Heukelbach et al. 2001). This ectoparasitosis is caused by the sand flea *Tunga penetrans* (order Siphonaptera), also called the jigger flea. In contrast to almost all other flea species, the female jigger flea penetrates into the skin of its host. There, it undergoes a peculiar hypertrophy, expels several hundred eggs for a period of up to 3 weeks and then dies. Thereafter, the shriveled carcass is sloughed from the epidermis by host repair mechanisms (Eisele et al. 2003). Within 10 days, the flea increases its volume by a factor of roughly 2,000, finally reaching the size of a pea.

Via its posterior end—which serves for breathing, defecating and expelling eggs—the flea remains in contact with the air, leaving a sore of 240–500 μm in the skin, the latter being an entry point for pathogenic microorganisms (Feldmeier et al. 2002).

In a previous study, we described the natural history of tungiasis in man (Eisele et al. 2003). A constant finding was the intense inflammation around embedded fleas, as indicated by erythema, edema, warmth and pain. Frequently, bacterial superinfection of the lesion occurred, leading to pustule formation, suppuration and ulceration (Feldmeier et al. 2003). Histopathologically, the clinical findings were reflected by an intense cellular infiltrate around the lesion. These infiltrates were mainly composed of lymphocytes, macrophages, eosinophils, mast cells and histiocytes (Eisele et al. 2003).

As the mechanisms underlying the intense inflammation are unknown, a study was undertaken to determine whether the inflammatory process could be attributed to immune activation induced by a biologically active foreign body present in the skin. Since a

mixed cellular infiltrate was found in the lesions of patients (Eisele et al. 2003), we determined the concentration of cytokines considered as markers for the activation of the Th1 type (IFN-γ, TNF-α) or the Th2 type (IL-4) of help. Cytokine concentrations obtained in the sera of patients with tungiasis were compared with those in sera of endemic controls infected with different helminth species or individuals chronically exposed to soil-transmitted helminths.

Materials and methods

Groups examined

Seventy-eight individuals infected with *Tunga penetrans* were recruited from the Primary Health Center serving the inhabitants of the favela Vicente Pinzon II, at the outskirts of Fortaleza, Ceará, Northeast Brazil. The socio-economic characteristics of this poor neighborhood have been described by Eisele et al. (2003). The parasite burden varied considerably (median 12 lesions, range 1–145 lesions). The age of patients ranged from 2 years to 67 years (median 11 years) and males were more common than females (Table 1). According to a recently performed helminth survey in the area, 74% of children are infected with soil-transmitted helminths, the predominant species being *Ascaris lumbricoides*. This group is referred to as TUNG1.

Thirty-two individuals living in the favela Vicente Pizon II were actively recruited for a pilot study assessing the efficacy of different drugs against *T. penetrans*. All were children aged 2–14 years (median 8.5 years) and presented at least ten lesions. Of these children, 58% had pediculosis, 11.5% scabies and 8% suffered from both ectoparasitoses. As with the children in group TUNG1, it can be expected that approximately 75% of these children carry intestinal helminths. This group is referred to as TUNG2.

During a field trial to compare the efficacy of praziquantel and oxamniquine in *Schistosoma mansoni* infection, 32 individuals were identified in Aracoiaba, a small town located 60 km west of Fortaleza (Zwingenberger et al. 1987). Of these, 24 were found to be co-infected with intestinal helminths. Sera from these individuals are included in this study. The individuals ranged in age from 7 years to 33 years (median 13.5 years). The intensity of infection with *S. mansoni* was rather low: median 43 eggs (range 2.5–648 eggs) per gram of stool. Nineteen individuals were infected with *Trichuris trichiura*, 17 with *Ankylostoma duodenale*, six with *Ascaris lumbricoides*, three with *Taenia solium* and one with *Hymenolepis nana*. Polyparasitism was very common: 42% of the individuals carried two, 17% carried three and 4% carried five different intestinal helminths. *Tunga penetrans* was not observed in the area. This group is referred to as MANS + IH.

Adjacent to the town of Aracoiaba, a village was identified where—according to data of the National Health Foundation—transmission of *S. mansoni* had been interrupted for many years. From all patients presenting at the local health post during 1 day (23 individuals), a stool sample was obtained and examined. None of the stool samples contained *S. mansoni* eggs. Nineteen individuals consented to provide a blood sample; and, of these, 16 individuals were infected with *Ankylostoma duodenale*, 15 with *Trichuris trichiura*, ten with *Ascaris lumbricoides*, three with *Enterobius vermicularis* and one with *Taenia solium*. Polyparasitism was very common: 37% of the individuals were infected with two, 26% with three and 11% with four different helminth species. According to the staff of the local health post, tungiasis did not occur in the area. This group is referred to as IH.

During a study on childhood anemia in Coina, Cajamarca Province, Peru, stool samples and serum were collected from all children aged 7–14 years presenting with their parents at the outpatient clinic of the Hospital Los Andes situated at an altitude of 2,000 m. Schistosomiasis does not occur in Peru and tungiasis has never been observed in this Andean region. Only children without helminth eggs in their stool were included in this study. As it can be assumed that these children were at a permanent risk for infection with soil-transmitted helminths, this group is referred to as the intestinal helminth exposure group, IH-EXP.

The main characteristics of the five groups are summarized in Table 1.

Two expatriates, of whom the precise time of infection with *Tunga penetrans* was known and who carried no other ectoparasites or intestinal helminths, provided serial serum samples at 13 days and 19 days after the sand flea penetration.

Blood samples

Samples (10 ml) of peripheral blood were drawn and, after clotting, the blood was centrifuged and sera aliquots of 2 ml were deep-frozen. Aliquots remained for 2 months in a deep-freezer at −20 °C and thereafter at −80 °C, until thawed for the determination of cytokines.

Determination of cytokines

Cytokines were determined in serum samples using ELISA kits specific for human IL-4, IFN-γ and TNF-α (Fa. Biocarta, Hamburg, Germany). Briefly, plates were coated overnight with 100 µl of capture antibody. Following washing, plates were blocked for 1 h and 100 µl of serum sample were added. After incubation for 2 h, 100 µl of detection antibody were added, incubated for 1 h and developed with 100 µl of avidin-horseradish peroxidase and substrate. Plates were read at 450/570 nm. Cytokine concentrations were determined using standard curves for human recombinant cytokines.

Stool examination

Stool examination was performed using the Kato–Katz thick smear technique (Katz et al. 1972). A single thick smear examined 40 mg of stool. Stool samples were also examined by the merthiolate-iodine-formaldehyde concentration technique (Blagg et al. 1955). This technique was more sensitive than the Kato–Katz technique, although eggs could not be quantified.

Clinical examination

As tungiasis may occur at any topographic site, the whole body surface of the patient was examined for the presence of immature, egg-producing or dead fleas. Lesions were classified according to

Table 1 Characteristics of control groups and patients with tungiasis

Group designation	*n*	Age	Female/male	Infection status (with presumed additional infection in parentheses)
TUNG1	78	11 (2–67)	28/50	Tungiasis (intestinal helminths)
TUNG2	32	8.5 (2–14)	14/18	Tungiasis, pediculosis, scabies (intestinal helminths)
SMANS + IH	24	13.5 (7–33)	13/11	Schistosomiasis, intestinal helminths
IH	19	14 (6–38)	10/9	Intestinal helminths
IH-EXP	29	12 (7–14)	14/15	Exposure to soil-transmitted helminths

the "Fortaleza classification", a recently elaborated staging system (Eisele et al. 2003). The following findings were considered diagnostic for tungiasis:

1. Flea seen in the act of penetration (stage I)
2. Occurrence of a dark and itching spot in the epidermis with a diameter of 1–2 mm, with or without local pain (early lesion, stage II)
3. Lesions presenting as a white halo with a diameter of 3–10 mm with a central black dot (mature egg-producing flea, stage III)
4. Brownish-black circular crust with or without surrounding necrosis of the epidermis (dead parasite, stage IV)
5. Circular "punched-out" residue in the keratin layer of the sole or irregular thickening of the nail rim (stage V).

Lesions altered through manipulation by the patient (such as partly or totally removed fleas, leaving a characteristic crater-like sore in the skin) and suppurating lesions (frequently caused by the use of non-sterile perforating instruments, such as needles or thorns) were also documented. The localization and the number of lesions were noted.

Statistical analysis

As data were not normally distributed and variances varied considerably, the median and percentiles were used to indicate the average and the dispersion of data. Cytokine concentrations in the different groups were compared with the Wilcoxon signed-rank test. The correlation between two variables was calculated using the Spearman rank correlation coefficient.

Ethical considerations

Informed consent was obtained from each study participant before taking a blood sample. In the case of minors, the parents or guardians were asked for their consent.

Results

Concentrations of peripheral IFN-γ are shown in Fig. 1a. In both tungiasis groups, the median serum IFN-γ concentration was 0.1 ng/ml. However, whereas in the group TUNG1 the dispersion of the concentrations was rather narrow around the average, there was a wide variation in group TUNG2. The concentration of IFN-γ in both tungiasis groups was significantly higher, as compared with the IH and IH-EXP groups ($P < 0.001$). There was no difference in IFN-γ levels

Fig. 1a–d Concentration of IFNγ, TNFα and IL-4 in the serum of control groups and patients with tungiasis. *TUNG1*, *TUNG2* Patients with tungiasis, *MANS+IH* patients infected with *Schistosoma mansoni* and intestinal helminths, *IH* patients infected with intestinal helminths, *IH-EXP* individuals exposed to soil-transmitted helminths. The median, the interquartile range and the 10th/90th percentiles *are indicated*. The TNFα/IL-4 ratio in patients with tungiasis and in the control groups is shown in **d**

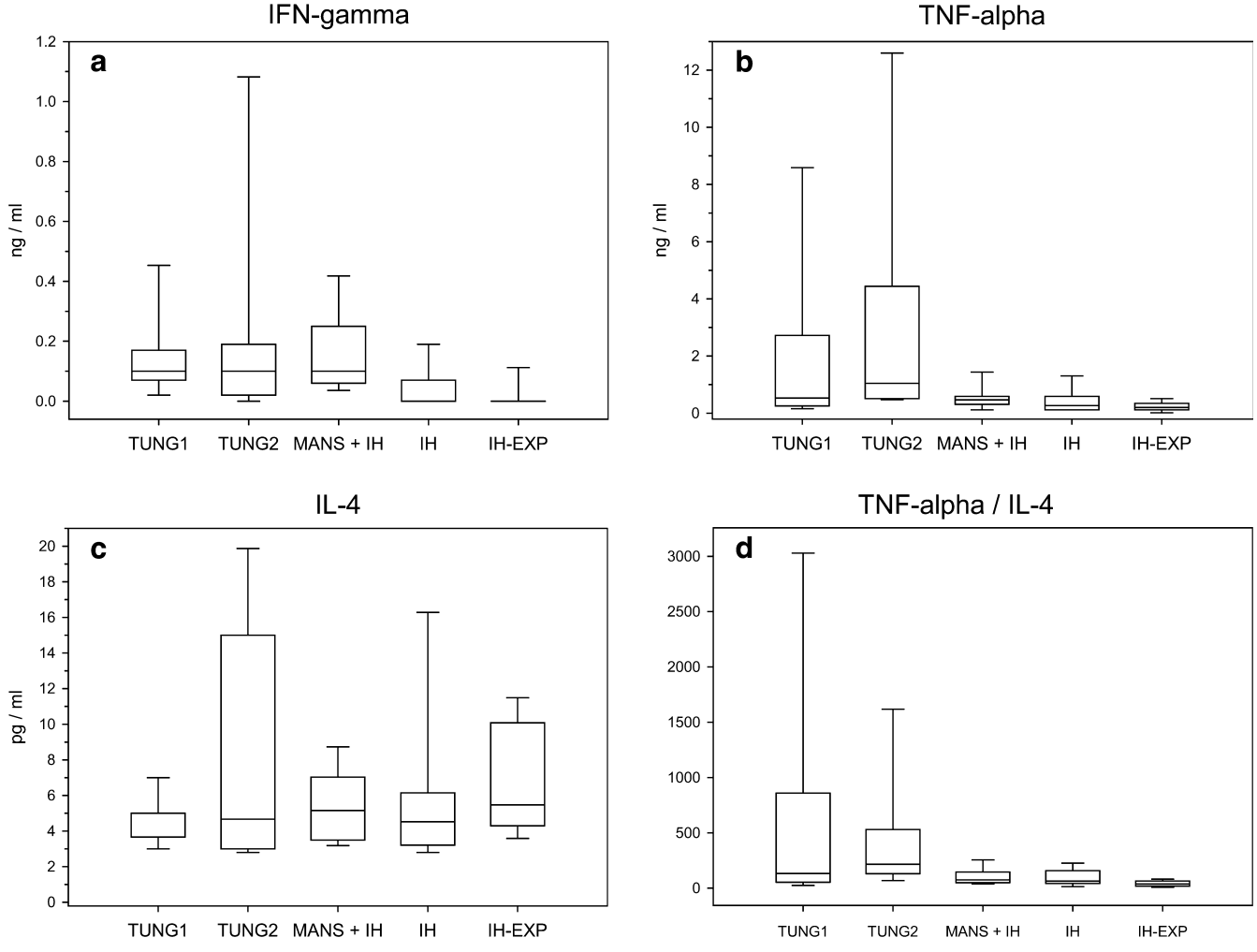

Table 2 Concentration of IFN-γ, TNF-α and IL-4 in patients with tungiasis according to age groups. Since there were no adults in group TUNG2 and over-proportionately few children in group TUNG1, the data for both tungiasis groups were combined. Data-ranges for the 25th/75th percentiles are given in parentheses

Age group (years)	IFN-γ (ng/ml)	TFNα (ng/ml)	IL-4 (pg/ml)	TNFα/IL-4
<9	0.12 (0.07–0.19)	0.65 (0.30–3.42)	4.33 (3.67–7.50)	130
10–19	0.08 (0.05–0.12)	0.63 (0.35–2.16)	3.67(3.00–5.00)	160
>19	0.12 (0.07–0.17)	0.84 (0.44–3.60)	3.67 (3.00–4.33)	260

between the tungiasis groups and the patients infected with *Schistosoma mansoni* and intestinal helminths (MANS + IH).

The TNF-α concentrations are depicted in Fig. 1b. They were significantly higher in group TUNG2 than in group TUNG1 (median 1.05 ng/ml vs 0.53 ng/ml; $P = 0.01$). The median concentration of the former group was also significantly higher than in the three other groups (MANS + IH 0.47 ng/ml, IH 0.27 ng/ml, IH-EXP 0.20 ng/ml; $P < 0.001$, as compared with all control groups). In group TUNG1, the TNF-α concentration was significantly higher than in groups IH ($P = 0.02$) and IH-EXP ($P < 0.001$).

In all groups, the concentration of IL-4 was elevated (Fig. 1c). The lowest increase was observed in group TUNG1 and the highest in group IH-EXP. The difference was significant (medians 3.67 pg/ml, 5.47 pg/ml, respectively; $P < 0.001$). Concentrations of IL-4 did not differ between groups TUNG2, MANS + IH and IH.

For the TUNG1 group, the precise number of embedded fleas was available and a regression analysis between cytokine concentration and the parasite burden was therefore performed. However, no significant relationship was obtained ($\rho < 0.18$, $P > 0.10$ in all cases).

In order to analyze whether the cytokine concentrations in the sera of patients with tungiasis were related to the age of the infected individuals, the results of cytokine testing were stratified into three age groups: <9 years, 10–19 years and >19 years (Table 2). The results show that the TNF-α and IL-4 levels tended to increase with the age of infected individuals. However, a significant difference was only observed for IL-4 levels between the youngest and the oldest age group ($P = 0.04$).

To investigate whether the different infections were due to a characteristic pattern of Th1 vs Th2 immune response, the ratio between TNF-α and IL-4 was determined. As Fig. 1d shows, the ratio was highest in the TUNG2 group and lowest in the IH-EXP group (medians 250 and 60, respectively; $P < 0.001$). In patients with tungiasis, the ratio tended to increase with age, from a median of 130 in the <9 years group, to 260 in the >19 years group (Table 2).

Sera were available from two expatriates in whom the precise time of flea penetration was known and in whom the lesions were not superinfected. These individuals had neither intestinal helminths nor ectoparasites. In both patients, the serum concentration of IFN-γ and IL-4 increased during the observation period, the increase of IL-4 being particularly obvious (Fig. 2). With regard to TNF-α, there was no consistent pattern.

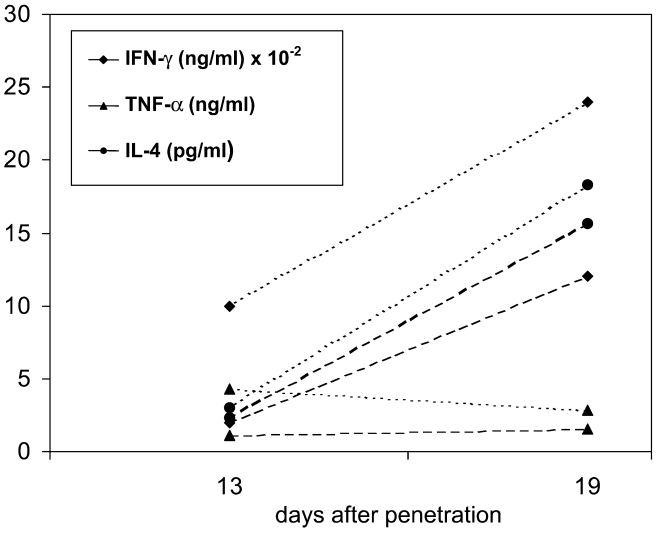

Fig. 2 Concentration of IFNγ, TNFα and IL-4 in the serum of two expatriates after first infection with *Tunga penetrans*. *Dotted line* Patient 1, *dashed line* patient 2

Discussion

Almost invariably, tungiasis is associated with intense inflammation. Very shortly after penetration, erythema and pain develop which are later followed by considerable swelling (Eisele et al. 2003). Histopathologically, the inflammation is reflected by dilated blood vessels in the dermis, an inflammatory infiltrate surrounding the embedded fleas (mainly consisting of lymphocytes, macrophages, eosinophils, mast cells, histiocytes) and spongiosis of the stratum corneum (Eisele et al. 2003). Systematic bacteriological investigations have shown that, in the endemic area, most lesions become superinfected (Feldmeier et al. 2002). Clinically, this is indicated by pustule formation, suppuration and ulceration (Feldmeier et al. 2003). In this stage, a massive influx of neutrophils can be seen in histological sections, forming a dense layer around the surface of the parasite and later transforming into microabscesses (Eisele et al. 2003). Eventually, neutrophils invade the dead parasite and form an abscess through which the carcass is sloughed from the epidermis (Eisele et al. 2003). Thus, clinical and histopathological observations point to a rigorous inflammatory response against embedded sand fleas. How the inflammation is triggered and which mediators are involved has not yet been studied.

Immune responses against microorganisms can be divided into those mechanisms induced by extracellular bacteria (e.g. neutrophil-, complement-activation), by intracellular microorganisms (e.g. Th1 cells that produce IFN-γ, TNF-α) and by helminths (e.g. Th2 cells that produce IL-4, IL-5, IL-13). In a first step to analyze the inflammatory reaction directed towards embedded sand fleas, cytokine levels were determined in patients with a chronic exposure to *Tunga penetrans*, showing up to 145 lesions when admitted to this study.

The results indicate that the majority of patients with tungiasis had elevated serum concentrations of the pro-inflammatory cytokines IFN-γ and TNF-α, but only a moderate increase in the concentration of IL-4. In fact, the concentration of IFN-γ was significantly higher than in the IH or IH-EXP individuals. TNF-α levels were also higher in the group of tungiasis patients than in patients with helminth infection or exposed to helminths. In contrast, the concentration of the Th2 cytokine IL-4 was significantly lower in patients with tungiasis than in the three control groups. In both tungiasis groups, but particularly in group TUNG2, the concentration of all cytokines varied considerably. This is not surprising, as supposedly at least some of the children were also infected with intestinal helminths, *Pediculus capitis* or *Sarcoptes scabiei*. At least scabies is known to trigger both Th1 and Th2 immune responses (Morsy et al. 1995), whereas intestinal helminths preferably activate Th2 immune responses (Yazdanbakhsh et al. 2001). In our study groups, the cytokine responses to these pathogens are likely superposed on the immune response provoked by *T. penetrans*. This would explain the wide variation in the cytokine concentrations in individual patients.

Moreover, tungiasis is a dynamic process characterized by a rapid increase in size and metabolic activity of the sand flea, the subsequent death of the parasite and then involution of the lesion. Conceivably, different stages of development should be reflected by different patterns of inflammatory response. However, as almost all patients harbored parasites in different stages of development and since circulating cytokines not only reflected present but to a certain degree also previous immune activation, a wide variation of cytokine concentration in peripheral blood has to be expected in patients with tungiasis, even if they do not carry other parasites.

That both Th1 and Th2 immune responses are induced in patients with tungiasis is demonstrated by the results for two expatriates in whom the precise time of flea penetration was known and who had no other ectoparasites and were not infected with intestinal helminths. In both cases, both IFN-γ and IL-4 clearly increased over 13–19 days after flea penetration. We do not know how early after penetration cytokines are produced and released locally. There are, however, hints that very shortly after an ectoparasite has entered the epidermis, immune cells are activated and start to release cytokines. Measuring cytokine-specific messenger RNA in models for myiasis caused by *Lucilia cuprina* and scabies, two ectoparasitic diseases with similar features as tungiasis, Egan et al. (1996) and Arlian et al. (1996) observed the release of IL-1 and IL-8 within just a few hours after penetration.

In the case of *S. scabiei*, the sources of these pro-inflammatory cytokines are thought to be fibroblasts from the keratin layer (Arlian et al. 1996). In our patients, histological sections showed the presence of lymphocytes, macrophages, eosinophils and mast cells in the inflammatory infiltrates within 24 h after penetration (Eisele et al. 2003). Presumably, IFN-γ was most likely produced by lymphocytes, whereas TNF-α production could result from activated lymphocytes, macrophages, or mast cells. The presence of IL-4 may be the result from the activation of lymphocytes or mast cells (Falcone et al. 1996).

The observation that the highest concentration of IL-4 was detected in the group without any parasites seems paradoxical. However, the socio-economic situation in which these children lived in Peru suggests that they are constantly exposed to the infection with intestinal helminths (H. Feldmeier, unpublished data). As Th2 cytokines, such as IL-4 and IL-5, are known to be main players in protective immune responses against these pathogens (Hoffmann et al. 2002), the high values of IL-4 observed in this group could point to a successful immune response against invading nematodes.

In chronic infection with *Schistosoma mansoni*, IFN-γ and IL-4 are co-modulated in opposite directions (Hoffmann et al. 2002; Ilma Araújo et al. 1996). Whereas IFN-γ and TNF-α are down-regulated, the production of IL-4 is up-regulated and the dichotomy increases with heavy infection and the simultaneous presence of intestinal helminths (Zwingenberger et al. 1991). In addition, the infection with intestinal helminths also results in a predominant Th2 response, with increased concentrations of IL-4 (van der Biggelaar et al. 2000). These observations are corroborated in our study, in which patients infected with *S. mansoni* and intestinal helminths showed higher values of IL-4 than those only infected with intestinal helminths.

We have to admit that the interpretation of the results is confounded by the fact that the tungiasis patients were concomitantly infected with other ectoparasites and partly also with intestinal helminths. However, as tungiasis is an ectoparasitic disease which occurs in deprived populations and is almost invariably associated with other parasitic diseases related to poverty (Muehlen et al. 2003) it is difficult to conceive how a representative sample of such a population could be studied which is only infected with *T. penetrans*. A possibility could be to selectively and successively eliminate intestinal helminths and other ectoparasites by appropriate chemotherapy, prevent re-infection by these pathogens for a period of several months and than study the immune response in those individuals with a current *T. penetrans* infection. However, in practice such an approach is hardly feasible. We therefore took refuge in

a comparison of the cytokine patterns between polyparasitized individuals with and without tungiasis and a group of children exposed to the infection with intestinal helminths. Another possibility for studying the cytokine pattern associated with tungiasis would be an experimental infection of laboratory-raised animals, such as Wistar rats. Such a study is now under way.

Taken in total, our study shows that tungiasis results in mixed Th1and Th2 immune response characterized by increased concentrations of IFN-γ and TNF-α and a slight increase in IL-4. Future studies will analyze whether the ectoparasite itself or the bacterial superinfection triggers the different immune responses observed in this study.

Acknowledgements This study was partly supported in part by the Ärztekomittee für die Dritte Welt, Frankfurt (Germany), Solvay Farma Ltda., São Paulo (Brazil) and by the World Health Organization, Geneva (Switzerland). We are indebted to the Associação dos Moradores do Sandra's for supporting the study and we are grateful to Vania Santos de Andrade and Walter Antônio da Silva for skilful assistance. The data are part of a medical thesis by M.E. The secretarial assistance of Michi Feldmeier and the technical assistance of Solvy Wolcke are gratefully acknowledged.

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