Circulating Antigens and Antibodies in Human and Mouse Dermatophytosis: Use of Monoclonal Antibody Reactive to Phosphorylcholine-like Epitopes

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The presence of circulating antibodies in the sera of patients infected with either *Trichophyton concentricum* or *Trichophyton rubrum*, and in the sera of BALB/c mice chronically infected with *Trichophyton quinckeanum*, was determined by ELISA. High levels of antibody to dermatophyte cytoplasmic antigens were detected both in infected humans and in mice. Partial inhibition of this reaction was observed by pretreatment of the sera with the hapten phosphorylcholine (PC). Moreover, antibodies were shown to have some reactivity with PC when tested by ELISA against PC conjugated to bovine serum albumin. Significant levels of circulating antigen were detected in patients with *T. concentricum* and *T. rubrum* infections, but not in uninfected subjects, by an immunoradiometric assay using a monoclonal antibody, Tq-1, which reacts with the PC-like epitopes of dermatophytes. It is possible that this dermatophyte antigen may play a role in modulating the cell-mediated immune responses, which would appear to be defective in most patients with these chronic forms of dermatophytosis.

INTRODUCTION

Dermatophyte fungi cause superficial infections by invading keratinized tissue of the skin, hair and nails of humans and animals. A distinctive characteristic of dermatophytes which makes them unique is that fungal invasion limited to the keratinized layers of the epidermis can elicit strong humoral and cell-mediated immune responses (Grappel *et al.*, 1974; Lepper, 1969; Jones *et al.*, 1974). However, in spite of this potential for immune expression some dermatophyte species cause persistent and recurrent infections.

In humans, two contrasting types of dermatophyte infections may occur. Zoophilic species of dermatophytes such as *Trichophyton mentagrophytes* usually cause an inflammatory infection which may heal spontaneously and results in resistance to reinfection. Anthropophilic species such as *Trichophyton rubrum* and *Trichophyton concentricum*, however, cause persistent infections, do not elicit significant inflammatory responses and are often resistant to therapy. The reasons for the disparity in the disease process caused by zoophilic and anthropophilic species are as yet unknown. However, although antibodies are produced in response to both types of infections, cell-mediated immunity, as judged by delayed-type hypersensitivity, is more readily detected in the inflammatory infections (Jones *et al.*, 1974); chronic infections have been correlated with specific defective T-lymphocyte activity (Hanifin *et al.*, 1974).

In experimentally infected mice it has been shown that adoptive immunity can be abrogated by serum from chronically infected mice (Calderon & Hay, 1984*b*) and that administration of alum-adsorbed specific antigen may prolong and exacerbate subsequent experimental infection.

Abbreviations: PC, phosphorylcholine; CA, cytoplasmic antigen; IRMA, immunoradiometric assay.

Circulating dermatophyte antigens have been detected by ELISA in infected animals and it is possible that soluble fungal antigens produced *in vivo* may play an important role in modulating the host immune response.

These observations suggest that a more detailed knowledge of dermatophyte antigens and their interaction with the cells of the host immune system is essential to understanding the immunopathogenesis of the disease. This in turn requires the ability to detect monospecific antigens. In the present study we have analysed the sera of patients infected with either *T. rubrum* or *T. concentricum*, for circulating antigen using a monoclonal antibody. This antibody, Tq-1, directed against phosphorylcholine (PC)-like epitopes common to dermatophytes is described in the accompanying paper (Calderon & Shennan, 1987). We have also investigated the presence of circulating antibodies in dermatophyte infected humans and mice, and their reactivity to PC.

METHODS

Dermatophyte species. *T. concentricum* (NCPF 567), *T. quinckeanum* (NCPF 309) and *T. rubrum* (NCPF 633) were obtained from the National Collection of Pathogenic Fungi (NCPF), Mycology Reference Laboratory, PHLS Colindale, London.

Dermatophyte antigen preparation. Fungal cytoplasmic antigens (CAs) were prepared as described in the accompanying paper (Calderon & Shennan, 1987).

Human sera. The patients with dermatophytosis comprised two groups. The first were patients with *T. rubrum* infections affecting the body (tinea corporis), palms or soles. These patients were attending the outpatient department of St John's Hospital for Diseases of the Skin, London. The second group were patients with tinea imbricata, an extensive and chronic infection caused by *T. concentricum*. Sera were obtained from patients residing in an area (Milne Bay and West Sepik Provinces, Papua New Guinea) in which tinea imbricata is endemic. Clinical features of these infections were described by Hay *et al.* (1984). Control sera were obtained from individuals residing in London or Papua New Guinea with no history of dermatophyte infection.

Mouse sera. The methods of induction of chronic dermatophyte infection in BALB/c mice (obtained from the National Institute for Medical Research, London) with *T. quinckeanum*, and mouse immunization with CAs are described in the accompanying paper (Calderon & Shennan, 1987).

Treatment of human and mouse sera with PC. In some experiments human and mouse sera and monoclonal antibody Tq-1 were pretreated with cytidine-5'-diphosphocholine (Sigma) to give a final concentration of 10 nM, and incubated for 1 h at 37 °C. Samples were then centrifuged at 20000 g for 10 min and the supernatant used for subsequent antibody assays.

Radioiodination of Tq-1 monoclonal antibody. Protein-A affinity purified Tq-1 monoclonal antibody (Calderon & Shennan, 1987) was labelled with [¹²⁵I]iodide (Amersham) by the Iodo-gen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril) technique described by Markwell & Fox (1978) and summarized in the accompanying paper (Calderon & Shennan, 1987). The radiolabelled antibody showed a precipitability index with TCA of 98%.

ELISA. This was used to detect circulating antibodies to fungal CAs and to PC in humans and mice. Microtitre plates were coated with 100 μ l (10 μ g protein ml⁻¹) of *T. concentricum*, *T. rubrum* or *T. quinckeanum* CA or with 100 μ l (1 μ g ml⁻¹) cytidine-5'-diphosphocholine conjugated to bovine serum albumin (BSA-PC) (donated by Dr R. M. Maizels, Imperial College, London). The procedure and reagents are described in the accompanying paper (Calderon & Shennan, 1987). Peroxidase-conjugated goat anti-mouse polyvalent Ig (Sigma), goat anti-human IgG, and goat anti-human IgM (Sigma), and the substrate *o*-phenylenediamine were used in this study.

Immunoradiometric assay (IRMA). This was done with the modification of Forsyth *et al.* (1985) and was used to detect circulating dermatophyte antigens in humans. Briefly, polyvinylchloride (PVC) plates (Dynatech) were incubated overnight with 50 μ l per well of a 10 μ g ml⁻¹ solution of protein-A-affinity-purified Tq-1 monoclonal antibody in PBS. Plates were washed three times with 0.05% (v/v) Tween 20/PBS, and incubated with 50 μ l 0.5% (w/v) BSA in PBS for 30 min at room temperature. In the wells of an uncoated duplicate PVC plate 60 μ l of ¹²⁵I-labelled monoclonal antibody containing 6×10^4 c.p.m. were mixed with 60 μ l of human serum diluted 1:5 in 5% normal rabbit serum in 0.05% Tween/PBS and incubated for 6 h at 37 °C; samples of equal volume from each well were transferred to two wells (50 μ l per well) of a washed Tq-1 coated plate. The plate was incubated overnight at room temperature and washed three times with 0.05% Tween/PBS; individual wells were excised, placed in vials and counted in a gamma counter (LKB 1280 Ultrogamma).

Statistics. Standard error was used as an estimate of variance, and means were compared by Student's two-tailed *t*-test for independent means.

Fig. 1. Effect of PC treatment of mouse anti-dermatophyte antibody on antigen binding by ELISA. Microtitre plates were sensitized with *T. quinckeanum* CA ($10 \mu\text{g ml}^{-1}$). Reaction was measured with peroxidase-conjugated goat anti-mouse polyvalent Ig. (a) Sera from immune mice; (b) sera from chronically infected mice; (c) monoclonal antibody Tq-1. ○, Mouse anti-dermatophyte antibody after treatment with 10 nM-PC for 1 h at 37°C; ●, control done in the absence of PC.

RESULTS

*Detection of antibodies to PC in serum of *T. quinckeanum* immune and infected mice*

The presence of circulating antibodies with specificity to PC in the serum of mice chronically infected (8 weeks) with *T. quinckeanum*, or immunized with fungal CA, was determined by ELISA using BSA-PC coated microtitre plates and peroxidase-conjugated goat anti-mouse IgG. Although sera from both immune and chronically infected mice were reactive with PC, the chronic sera showed stronger reactivity ($P < 0.001$). The sera from uninfected mice, however, did not react with PC, indicating that induction of antibodies to PC was the result of specific dermatophyte antigen stimulation.

Effect of PC treatment of immune and infected mouse sera on reactivity to dermatophyte antigens

To estimate the binding activity to PC-like dermatophyte antigens, sera from immune or chronically infected mice were pretreated with PC hapten and tested in an ELISA system using fungal CA-coated microtitre plates. High titres of circulating antibody were detected in chronic sera. A partial but significant inhibition of binding to fungal antigens was observed after treatment of serum with PC ($P < 0.001$ at 1:20 and 1:40, and $P < 0.05$ at 1:80–320 serum dilutions) (Fig. 1b). In immune sera antibody was also detected, but here inhibition by PC was less pronounced ($P < 0.05$ at 1:20 and 1:40 serum dilution) (Fig. 1a). On the other hand, the binding activity of Tq-1 monoclonal antibody to fungal CA was completely inhibited by PC (Fig. 1c). Sera from uninfected mice did not react with fungal CA in an ELISA, and background readings of 0.08 to 0.12 at A_{490} were regularly obtained.

Detection of antibodies to PC in serum of infected patients

In a similar manner to mouse sera, antibodies to PC were detected in sera of human subjects infected with *T. concentricum*. In these patients, IgM class antibodies to PC were present at higher levels than IgG antibodies (Fig. 2a, b). Human sera from uninfected subjects also showed reactivity to PC; however, the antibody titre was significantly lower than in patients, with $P < 0.001$ for IgM at 1:10–1:160 serum dilution and $P < 0.03$ for IgG at 1:10. Similar results were obtained with sera of patients infected with *T. rubrum*. For instance, the A_{490} values for anti-IgG response were: 1:10 (0.28 ± 0.09), 1:40 (0.23 ± 0.10), and 1:160 (0.15 ± 0.03); and for anti-IgM were: 1:10 (0.85 ± 0.14), 1:40 (0.72 ± 0.19) and 1:160 (0.49 ± 0.10).

Fig. 2. Detection of anti-PC antibody in human sera by ELISA. Plates were sensitized with BSA-PC ($1 \mu\text{g ml}^{-1}$) and reaction was measured with peroxidase-conjugated goat anti-human IgG (a) or IgM (b). ●, Sera from patients infected with *T. concentricum*; ○, normal human sera. Values represent the arithmetic mean $\pm$ SEM of 40 patients and 20 uninfected subjects.

Fig. 3. Effect of PC treatment of human anti-dermatophyte antibody on antigen binding by ELISA. Microtitre plates were sensitized with *T. concentricum* CA ($10 \mu\text{g ml}^{-1}$). Reaction was measured with goat anti-human IgG (a) or IgM (b). □, Sera from *T. concentricum* patients after treatment with PC; ■, normal human sera after treatment with PC; △, Normal human sera after treatment with PC; ▲, control done in the absence of PC. Values represent the arithmetic mean $\pm$ SEM of 40 patients and 20 uninfected subjects.

Effect of PC treatment of sera of infected patients on reactivity to CAs

Circulating antibody to CAs was detected in the sera of patients infected either with *T. concentricum* or with *T. rubrum*. The results in Fig. 3 show a strong IgG and IgM antibody reaction in sera of *T. concentricum* patients. This binding activity was partially but significantly reduced by pretreatment of sera with PC hapten, with $P < 0.001$ for IgM, and $P < 0.02$ for IgG.

Fig. 4. Detection of Tq-1 target antigen in the sera of *T. concentricum* and *T. rubrum* patients by IRMA. IRMA was carried out by a sequential sandwich procedure as detailed in Methods. Values represent the arithmetic mean $\pm$ SEM of 40 patients with *T. concentricum* (▩), 20 patients with *T. rubrum* (▨) and 20 uninfected subjects (□).

Similar results were obtained with sera of *T. rubrum* infected patients. For instance, the A_{490} values for anti-IgG response were: 1:40 (0.95 ± 0.20 ; +PC 0.70 ± 0.12), 1:160 (0.70 ± 0.17 ; +PC 0.42 ± 0.09), 1:640 (0.45 ± 0.07 ; +PC 0.26 ± 0.04); and for anti-IgM were: 1:40 (0.91 ± 0.21 ; +PC 0.76 ± 0.10), 1:160 (0.63 ± 0.15 ; +PC 0.29 ± 0.05), 1:640 (0.54 ± 0.12 ; +PC 0.27 ± 0.17). A less pronounced, but nonetheless significant reaction to CA was found in the sera of uninfected subjects. This result was not unexpected since these sera showed some reactivity to PC (Fig. 2), known to be present in fungal antigens.

Detection of circulating Tq-1 target antigen in the sera of dermatophyte infected patients

Antigen cross-reactivity between species is a characteristic of dermatophytes. Previous work using crossed-immunoelectrophoresis has shown that whole-cell antigens derived from dermatophyte species contain numerous antigenic peaks and that there is strong cross reactivity between different organisms (Svejgaard, 1985). Thus, in this study the Tq-1 antibody, which has been demonstrated to react with both *T. concentricum* and *T. rubrum* CAs, was used as a probe for detection of circulating antigens in patients infected with these organisms. Sera were tested in a one-antibody two-site IRMA for Tq-1 target antigens in circulation. The results of this study showed significant levels of circulating antigen in patients with *T. rubrum* and in those with *T. concentricum* infections (Fig. 4). Low antigen titres were found in the sera of uninfected subjects with $P < 0.0001$ compared to infected patients. It was also found that by the IRMA technique Tq-1 can detect dermatophyte antigen in the presence of CA ($12 \text{ ng protein ml}^{-1}$) (Fig. 4).

DISCUSSION

The present study examined the significance of the PC hapten, present in dermatophyte antigens, in relation to the humoral response of infected humans and experimentally infected mice. In the accompanying paper (Calderon & Shennan, 1987) we showed that a monoclonal antibody, designated Tq-1, derived from mice immunized with an alum-adsorbed fraction of dermatophyte CA had a strong affinity for PC-bearing fungal components. Here, antibodies to PC were detected both in the serum of mice infected with *T. quinckeanum* and in those immunized with fungal antigens, but none were detected in uninfected mice, indicating that PC-

reactive antibodies were elicited by dermatophyte antigens. Furthermore, the reaction of dermatophyte antigen with specific antibody from infected or immunized mice was partially inhibited with the PC hapten (Fig. 1), suggesting that a proportion of this antibody response was directed to the PC epitopes.

In humans, IgM antibodies with affinity for PC were found in both uninfected and dermatophyte infected individuals. However, this activity was significantly more pronounced in patients with dermatophytosis (Fig. 2*a*). It is not known whether there is any cross-reactivity with bacteria containing PC-like antigens which might explain the levels of antibodies occurring in controls. Anti-IgG antibodies, on the other hand, were present in low concentration in patients, but absent in uninfected individuals (Fig. 2*b*). Specific IgG and IgM antibody responses to CAs were present in patients infected with either *T. rubrum* or *T. concentricum* (Fig. 3) and this antigen-antibody reaction was partially inhibited with PC hapten; PC binding inhibition of patient sera was, however, more significant than in controls.

Antibody responses to antigens occur during dermatophyte infections in humans and animals (Grappel *et al.*, 1974). Thus, in order to establish their significance in the clinical course of the infection the nature of dominant antigenic determinants needs to be defined. In our accompanying paper (Calderon & Shennan, 1987) the results indicated that the hapten PC, a structural component of dermatophytes (Baldo *et al.*, 1977), appeared to be a dominant antigen during dermatophyte infections in mice. The hapten PC, found also in bacteria (Brundish & Baddiley, 1968) and parasites (Pery *et al.*, 1974), binds to the acute phase C-reactive protein and the resulting complex is known to elicit some immunological reactions (Kaplan & Volanakis, 1974).

Dermatophyte infections in humans and animals often result in temporal suppression of specific and non-specific cell-mediated immune functions such as lymphocyte blastogenesis to T-cell and B-cell mitogens (Hanifin *et al.*, 1974; Green & Balish, 1979; Calderon & Hay, 1984*a*). S. Mayou, R. A. Calderon, R. J. Hay & A. Goodfellow (unpublished data) showed that the *in vitro* non-specific and specific blastogenic responses to the T-cell mitogen concanavalin A and to dermatophyte CA were severely reduced in a patient with deep dermatophytosis only in the presence of autologous, but not of heterologous serum, suggesting the presence of humoral blocking factors. The presence of blocking factors in serum of dermatophyte infected patients has been reported (Walters *et al.*, 1974; Allen *et al.*, 1977), but the putative blocking factors in human dermatophytosis have not yet been defined. In mice experimentally infected with *T. quinckeanum* the adoptive cell-mediated immunity was abrogated with specific antigens (Calderon & Hay, 1984*b*). In the present study, circulating Tq-1 target antigens, PC-bearing components, were found in patients with infections caused by *T. rubrum* localized to the palms or soles as well as in those with more widespread tinea corporis caused by *T. rubrum* and *T. concentricum* (Fig. 4). This is apparently the first time that circulating antigens of dermatophyte fungi have been detected in infected patients. Whether the PC-bearing dermatophyte antigens in circulation are present alone and/or complexed to antibody, or whether they may in any way be responsible for the impairment of cell-mediated immune function is not yet clear; studies of this question are in progress. However, the patients investigated all had titres of circulating PC-bearing antigen in association with chronic dermatophytosis, and patients with *T. rubrum* or *T. concentricum* infections have defective cell-mediated immune responses (Hanifin *et al.*, 1974; Hay *et al.*, 1983). It could be interesting to compare these findings with patients infected with dermatophytes such as *T. mentagrophytes* where lesions are more inflammatory and result in effective cell-mediated immune responses.

Dermatophyte serology has concentrated previously on the detection of antibodies. However, it is possible that detection of specific fungal antigens in circulation may lead to a closer understanding of clinical events such as the dermatophyte intradermal reactions as well as immune function, including cellular immunity, in infected subjects.

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