

Possible mechanisms of immune modulation in chronic dermatophytoses: an *in vitro* study

J.M.McGREGOR, A.J.HAMILTON AND R.J.HAY

Dunhill Research Laboratories, UMDS (Guy's Campus), St Thomas' Street, London SE1 9RT, U.K.

Accepted for publication 15 April 1992

Summary

It has been suggested that patients with chronic superficial *Trichophyton rubrum* infection have defective cellular immunity to dermatophyte antigens. This may be due to a selective anergy to dermatophyte antigens or reflect the activity of dermatophyte-derived lymphocyte inhibitory factors. To explore these possibilities, we assessed lymphocyte transformation to a variety of recall antigens, including a cytoplasmic and exoantigen preparation of *Trichophyton rubrum* in 15 patients with chronic dermatophyte infection and 15 age- and sex-matched positive controls. In a duplicate set of experiments, autologous serum was replaced by heat-inactivated fetal calf serum. In addition, the direct effect of *Trichophyton rubrum* extracts on lymphoproliferation was assessed *in vitro*.

Comparable lymphocyte transformation to each recall antigen was observed in patients and controls. Moreover, we found no evidence for a circulating dermatophyte-derived lymphocyte inhibitory factor in sera from patients with chronic superficial infection. A direct inhibitory effect of *Trichophyton rubrum* on lymphocyte proliferation to recall antigens was observed, however, at protein concentrations of $>10 \mu\text{g/ml}$ (exoantigen preparation) and $>25 \mu\text{g/ml}$ (cytoplasmic preparation). This inhibitory effect was rapidly reversible, not associated with loss of cell viability and maximal when added within 24 h of antigen to cultured lymphocytes. Class II MHC antigen HLA-DR, a surface marker of T-cell activation, was observed on inhibited lymphocytes co-cultured with antigen, suggesting the primary target for the inhibitory effect *in vitro* is the lymphocyte rather than the antigen-presenting cell. These data are consistent with the hypothesis that lack of cutaneous inflammation at the site of *Trichophyton rubrum* infection in the human host may reflect a local effect of dermatophyte-derived lymphocyte inhibitory factors. This contrasts with previous suggestions that patients predisposed to chronic dermatophytoses might exhibit a selective anergy to *Trichophyton rubrum*.

Dermatophytes comprise a group of superficial fungal pathogens exhibiting pronounced immunological cross-reactivity,^{1–4} yet they characteristically cause disparate patterns of infection in the human host. For example, *Trichophyton rubrum* (*T. rubrum*) elicits a weak inflammatory response, typically causing chronic or recurrent infection of skin and nails, whilst *T. verrucosum* elicits a brisk inflammatory response associated with rapid resolution and resistance to re-infection. Decreased cell-mediated immunity has been observed in patients with chronic dermatophytoses⁵ but does not appear to reflect low levels of circulating antigen, which is detectable in sera from patients with both acute and chronic infection.⁶

Intact cellular immunity is critical for eradication of dermatophytes from the skin.^{7,8} Persistence of *T. rubrum* infection in otherwise healthy individuals might therefore result from selective anergy to dermatophyte antigens as previously suggested⁹ or reflect the activity of putative dermatophyte-derived lymphocyte-inhibitory factors. In support of these proposed mechanisms, dermatophyte antigen has been shown to suppress cellular immunity in murine models of experimental dermatophyte infection.¹⁰ Moreover, autologous serum from patients with deep or unusually extensive dermatophyte infection has similarly been shown to suppress lymphocyte transformation to recall antigens *in vitro*.^{11,12} Because this phenomenon is not observed following successful treatment of infection it suggests the inhibitory effect might be due to circulating dermatophyte-derived blocking factors.

Correspondence: Professor R.J.Hay, Department of Dermatology, Guy's Hospital, St Thomas' Street, London SE1 9RT, U.K.

This study examines cellular immune responses to recall antigens and possible circulating inhibitory factors in patients with chronic *T. rubrum* infection. Lymphocyte transformation to a variety of mitogenic and antigenic stimuli, including a cytoplasmic and exoantigen preparation of *T. rubrum*, was measured in patients with chronic *T. rubrum* infection and compared with controls in the presence of either fetal calf serum or autologous serum. In addition, the ability of *T. rubrum* to suppress lymphoproliferation directly was determined *in vitro*.

Methods

Patients

Fifteen healthy adults (10 males, five females) with culture-proven *T. rubrum* skin infection were recruited. They comprised 13 patients with dry-type tinea pedis and two patients with extensive tinea corporis. All had either failed to respond to, or had relapsed within 3 months of stopping griseofulvin treatment. No patient had been on oral medication, including antifungal treatment, for at least 6 weeks prior to the study. As positive controls 15 age- and sex-matched healthy adults were recruited; all had a previously documented history of inflammatory-type tinea pedis caused by *T. interdigitale*. Despite a high prevalence of atopy in patients predisposed to chronic dermatophyte infection, no patient in our study was atopic.

Fetal cord blood samples ($n = 2$) were used as negative controls.

Isolation of peripheral blood mononuclear leucocytes (PBML)

Blood was collected by venepuncture from each volunteer into sterile universal containers with preservative-free heparin. PBML were isolated from each sample by density-gradient centrifugation using Lymphoprep

(Nycomed, U.K.). Lymphocytes stained with eosin were counted using a haemocytometer, and cells resuspended in a concentration of 10^6 cells/ml in RPMI (Gibco, U.K.) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μ g/ml streptomycin and penicillin, buffered with 1% Hepes. In addition either 5% heat-inactivated fetal calf serum (FCS) or 5% autologous serum was added prior to use in proliferation assays.

Cultures of *Trichophyton rubrum*

An isolate of *T. rubrum* was inoculated into autoclaved medium containing sodium β -glycerophosphate, D-glucose and peptone. Cultures were aerated using mechanical stirrers and allowed to grow in 250 ml tissue-culture flasks for 21 days at room temperature. Surface cultures were then harvested by filtration through Whatman's No. 2 filter paper and the supernatants retained. Mycelial suspensions were washed in phosphate-buffered saline (PBS 0.01 M, pH 7.4), homogenized using sterile glass beads (Biospec Products) and the cell extract, cytoplasmic antigen (CA), centrifuged at 1500 *g* for 7 min. Culture supernatant (exoantigen) and CA preparations of *T. rubrum* were dialysed against 100% polyethylene glycol (PEG) and then isometrically dialysed against PBS at 4°C. In addition, dialysates of *Epidermophyton floccosum* and *T. mentagrophytes* were similarly prepared. Protein determinations were estimated by the Coomassie blue method¹³ and antigens were stored at -70°C until required. Dialysed uninoculated medium was used as a negative control in each experiment.

Lymphoproliferation assays

Cells (10^5 cells/100 μ l) cultured in either 5% FCS or 5% autologous serum, were added to round-bottomed 0.2 ml 96-well microtitre plates (Flow Laboratories, High Wycombe, U.K.) containing either antigen or mitogen (Table 1). Parallel experiments were also performed using EBV-transformed B lymphocytes (M958/ATCC

Table 1. Antigens and mitogens employed in lymphoproliferation assays

Protein	Antigen/mitogen	Concentration (μ g/ml)	Source
Purified protein derivative (PPD 289)	Antigen	50–200	Department of Agriculture, Fisheries and Food, Surrey, U.K. Mycological Reference Laboratory, Public Health Service Laboratories, Colindale, London, U.K.
<i>Candida albicans</i> (NCPF 3153)	Antigen	5–500	
TR cytoplasmic antigen	Antigen	0.046–460	As described
TR exoantigen	Antigen	0.025–250	As described
Phytohaemagglutinin (PHA, L8754)	Mitogen	20–200	Sigma, U.K.

Laboratories, U.S.A.) in place of PBML. Cultures were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide for 4 days. On day 4, 0.2 μCi ^3H -thymidine (Amersham, specific activity 185 GBq/mmol) was added to each well and cells were incubated for a further 16 h before harvesting. Cells were harvested on to glass-fibre filter paper (G-10, Inotech, New Hampshire) using a semi-automatic 96-well cell harvester (Inotech), dried at 40°C and placed in vials containing scintillation fluid (Wallac, Sweden). Radioactive uptake by cells was measured using a liquid scintillation beta counter (Wallac). All experiments were performed in triplicate and a mean value expressed as disintegrations per minute—background (d.p.m. — Δ). Statistical analysis was performed on all results using an unpaired Student's *t*-test.

HLA-DR expression by PBML using a double-labelling immunofluorescence technique

PBML were incubated for 3 days with either RPMI, PHA, PHA and TR exoantigen, or TR exoantigen alone, and then fixed in 0.01% azide in PBS. Non-specific binding was blocked using heat-inactivated normal human serum, and cells were labelled first with unconjugated monoclonal antibody (MAb) to HLA-DR (L243)(ATCC, U.S.A.) and then with fluorescein isothiocyanate-conjugated (FITC) polyclonal goat anti-mouse immunoglobulin antibody (Becton Dickinson, Mount View, CA, U.S.A.). Following incubation with normal mouse serum, cells were subsequently labelled with a phycoerythrin-conjugated MAb to CD4 or CD8 (Becton Dickinson). Samples were washed twice between each step. Controls included irrelevant antibodies for both red and green fluorescence, and single-stain analysis for each antibody was used. Cytofluorometric analysis, counting 5×10^3 cells per sample, was performed on a fluorescence-activated cell sorter (FACStar, Becton Dickinson). Appropriate forward and 90 light scatter gates were set to exclude dead cells and non-lymphoid cells. Positive cells were determined by setting a threshold with reference to the relevant negative controls.

Results

Using this protocol, optimal (highest mean value) lymphoproliferative responses were obtained at the following concentrations: phytohaemagglutinin 40 $\mu\text{g}/\text{ml}$, purified protein derivative 100 $\mu\text{g}/\text{ml}$, *T. rubrum* (TR) exoantigen 2.5 $\mu\text{g}/\text{ml}$, TR CA 10 $\mu\text{g}/\text{ml}$ and *Candida albicans* 56 $\mu\text{g}/\text{ml}$. Individual responses to antigens and

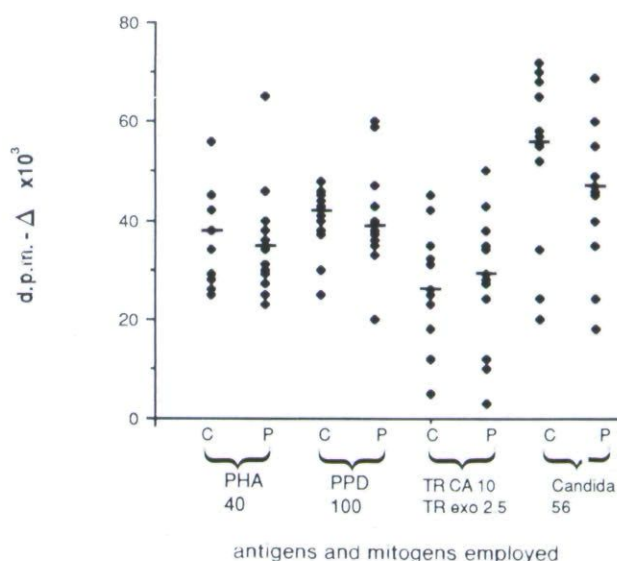


Figure 1. Scattergraph demonstrating lymphocyte proliferation to recall antigens and PHA: a comparison of patients (P) with chronic *T. rubrum* infection and controls (C). Statistical analysis performed using the Student's unpaired *t*-test demonstrates no significant difference between the groups.

mitogens varied, but no one individual was a consistently low responder to all stimuli. No significant difference in mean lymphoproliferative response to any mitogen or antigen was found between patients and controls when compared using a Student's unpaired *t*-test (Fig. 1). Furthermore, equivalent lymphoproliferative responses to all antigens were seen using either FCS or autologous serum from patients with chronic *T. rubrum* infection.

These results indicate that PBML from patients with chronic *T. rubrum* infection respond normally to PHA and to recall antigens, as well as to both an exoantigen and a cytoplasmic antigen preparation of TR. The possibility that TR preparations had a mitogenic effect was excluded by the absence of lymphoproliferation using PBML from fetal cord blood (Fig. 2), although these data do not exclude a possible superantigen effect of TR preparations. There was no evidence to support the presence of a circulating inhibitory factor in sera from patients with chronic superficial *T. rubrum* infection.

Concentration-dependent inhibition of lymphoproliferative responses by TR (Fig. 3)

PBML from five patients previously found to have strong proliferative responses to TR preparations were used, and each experiment was performed in triplicate. Maximal stimulation of PBML by TR CA was observed at

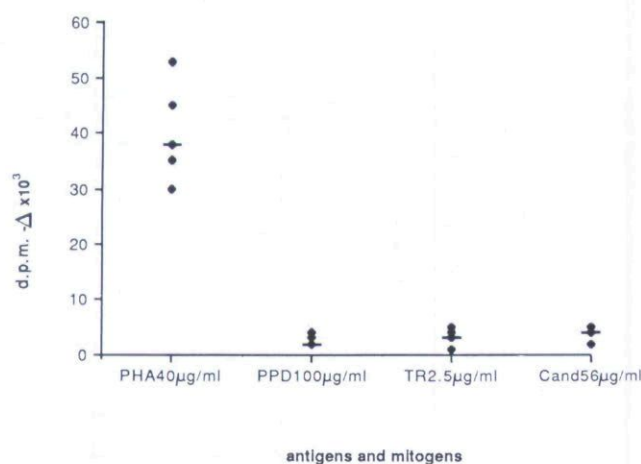


Figure 2. Scatter graph demonstrating proliferation of PBML from fetal cord blood to PHA. Proliferation was not observed with any antigen, including TR exoantigen and TR cytoplasmic antigen.

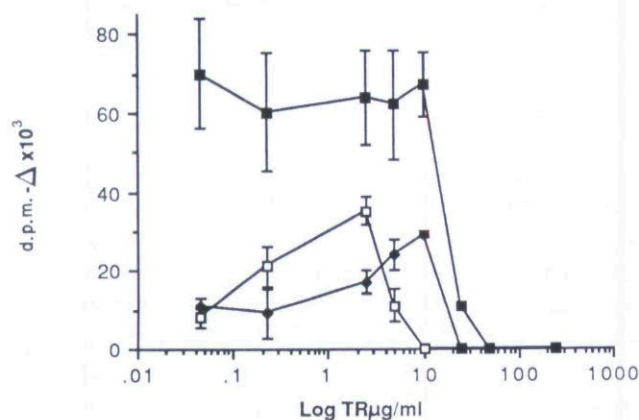


Figure 3. Line graph demonstrating lymphoproliferation to TR exoantigen (□) and TR cytoplasmic antigen (◆). Maximal proliferation is observed at 2.5 μg/ml and 10 μg/ml, respectively. Conversely, complete inhibition of lymphoproliferation is seen at >10 μg/ml TR exoantigen and >25 μg/ml TR cytoplasmic antigen. Inhibition of EBV-transformed B lymphocytes by equivalent concentrations of TR inhibitor is similarly observed (■).

protein concentrations of 10 μg/ml and by TR exoantigen at 2.5 μg/ml. In contrast, at concentrations >25 μg/ml and >10 μg/ml, respectively, complete inhibition of proliferation was observed using both PBML and EBV-transformed B lymphocytes. No inhibition was observed using dialysed uninoculated culture medium alone. This suggests a dermatophyte inhibitory factor (DIF) is present in both preparations. TR exoantigen preparation has both increased stimulatory and inhibitory effects compared with equivalent protein concentrations of TR CA, suggesting that both major stimulatory antigens and immunoinhibitory factors derived from *T. rubrum* are secreted by the organism.

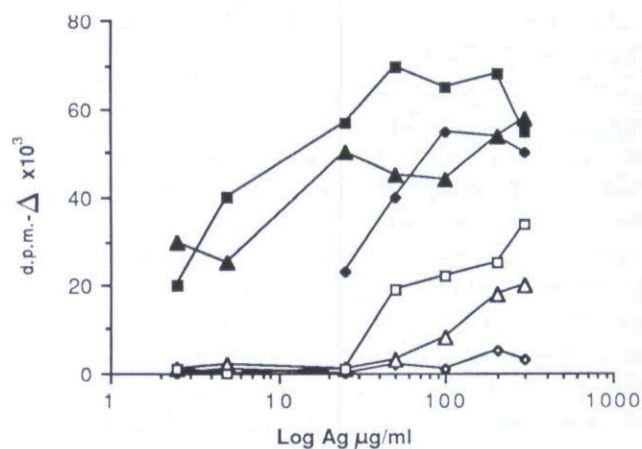


Figure 4. Demonstration of dose-related lymphoproliferation to PHA(□), PPD(Δ) and *C. albicans*(◇) with (open figures) and without (solid figures) the presence of TR inhibitor. Inhibition of lymphoproliferation to each antigen and PHA is observed on addition of TR inhibitor. Escape from inhibition occurs when sufficiently high concentrations of PHA and PPD are employed (PHA >100 μg/ml, PPD >200 μg/ml, *C. albicans* >168 μg/ml).

Mechanism of inhibition

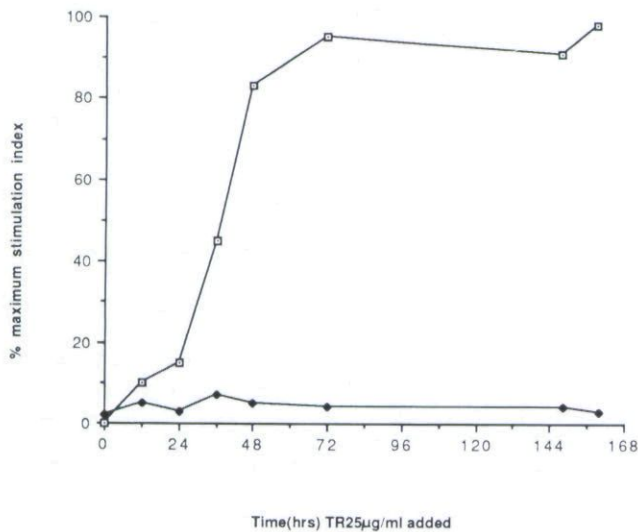
Lymphoproliferation to recall antigens and PHA inhibited by TR (Fig. 4). Experiments were performed in triplicate on PBML from five patients. Very similar patterns of response were observed in each case, but statistical analysis of pooled data was not performed due to the large inter-patient variability in lymphoproliferative responses to antigen preparations. Figure 4 represents the mean of three experiments performed on PBML from one patient.

Lymphoproliferation to recall antigens and to PHA was abrogated by co-culturing with DIF (TR CA >25 μg/ml and TR exoantigen >10 μg/ml). This inhibitory effect was not observed using dialysed uninoculated medium. Increasing concentrations of mitogen or antigen titrated against a fixed concentration of DIF overrode the inhibitory effect, indicating that it was not due to loss of cell viability. This is supported by the demonstration that the inhibitory effect of DIF on lymphocytes could be rapidly reversed by washing inhibited lymphocytes in PBS and resuspending them in RPMI containing either antigen or mitogen (data not shown).

HLA-DR expression by lymphocytes cultured with DIF and PHA (Table 2). Three experiments were performed on PBML from one patient. Results represent the mean of these data. HLA-DR expression (measured as mean intensity of fluorescence using FITC-DR) was markedly upregulated on lymphocytes stimulated with PHA and

Table 2. Flow cytometric analysis of HLA-DR antigen expression on CD4⁺ PBML

Culture medium	RPMI	PHA	PHA and TR inhibitor	TR inhibitor
FITC-DR mean intensity of fluorescence	53.5	547	701	83.3
d.p.m. (mean)	500	56,000	8000	243

**Figure 5.** Addition of TR inhibitor to cultured lymphocytes(□) at $t = 0-160$ h. Maximal inhibition of antigen-driven lymphoproliferation is seen when TR inhibitor is added at $t < 24$ h. EBV-transformed B lymphocytes(◆) are consistently inhibited by addition of TR ($t = 0-160$ h).

on lymphocytes co-cultured with both PHA and DIF, despite inhibition of cellular proliferation. In contrast, lymphocytes cultured in RPMI or DIF alone did not express HLA-DR, suggesting that some events required for T-lymphocyte activation, such as DR synthesis, proceed normally in the presence of DIF and PHA.

Time-dependent effect of TR inhibition on lymphoproliferation (Fig. 5). Lymphocytes cultured with PPD were maximally inhibited when DIF was added to the culture at $t = 0-24$ h. Thereafter, addition of DIF had no significant effect on lymphoproliferation. In contrast, addition of DIF to EBV-transformed B lymphocytes consistently inhibited lymphoproliferation at all times ($t = 0-160$ h).

Discussion

Lymphocyte transformation *in vitro* has previously been employed to examine cellular immunity in human dermatophytoses. Although some studies suggest a selective impairment of lymphoproliferation to dermatophyte antigens in patients with chronic dermatophyte infection,⁹ most studies have shown normal cellular responses to all recall antigens, including dermatophyte antigen.¹⁴ Our data, which demonstrate normal lymphocyte transformation to TR antigen in patients with chronic dermatophytosis, are in agreement with recent studies,¹⁴ and do not therefore support the hypothesis that individuals predisposed to chronic dermatophyte infection exhibit selective anergy to *T. rubrum* antigen. This does not exclude, however, the possibility that lymphocyte function may be defective in these individuals.

Our study demonstrates that a dermatophyte-derived inhibitory factor, or factors (DIF), present in both TR CA and supernatant (exoantigen) preparations inhibits lymphocyte proliferation to a variety of recall antigens *in vitro*. Studies of murine dermatophytoses similarly suggest that dermatophyte antigen may directly suppress lymphocyte function.¹⁰ Because dermatophyte antigen can be demonstrated both in the serum⁶ and in the epidermis during human cutaneous infection,¹⁵ it is possible that a local inhibitory effect on epidermal lymphocytes might explain the inability of patients chronically infected with *T. rubrum* to eradicate fungus from the skin, even in the presence of normal cell-mediated immunity. The observation that patients with normal *in vitro* lymphocyte transformation to *T. rubrum* characteristically lack delayed-type hypersensitivity responses to intradermal dermatophyte antigen is also consistent with this hypothesis. It is not currently known, however, whether dermatophyte antigen is present in the epidermis in sufficient quantities to inhibit lymphoproliferation *in vivo*. Nor is it known whether a putative local immunosuppressive effect is of any clinical relevance in dermatophyte infection.

Inhibition of lymphoproliferation was observed in this study by addition of TR preparation to EBV-transformed B lymphocytes and also to lymphocytes stimulated with PHA. These data suggest that the target cell for the inhibitory effect *in vitro* may be the lymphocyte rather than the antigen-presenting cell. The observation that lymphocytes co-cultured with DIF and PHA express HLA-DR antigen, a surface marker of T-cell activation, also suggests the inhibitory effect may occur at a step common to many dividing cells, independent of antigen

presentation. Because the inhibitory effect is readily reversible by washing, a cell-surface interaction may be more likely than an intracellular event. We cannot currently explain why EBV-transformed B lymphocytes are consistently inhibited by DIF whilst antigen-driven lymphoproliferation escapes the inhibitory effect after a critical 24 h in culture, but consider that this observation may provide an important clue to the mechanism of inhibition.

We were unable to demonstrate an inhibitory effect on lymphocyte proliferation using autologous serum from any patient with chronic superficial *T. rubrum* infection. Serum inhibitory factors previously described,^{11,12} might therefore reflect particularly high levels of circulating antigen in patients with unusually extensive disease. Reduced proliferation of spleen and lymph node lymphocytes observed in mice with *T. quinckeanum* infection in the presence of autologous serum correlated with the level of circulating dermatophyte antigen,¹⁰ suggesting that antigen load may be critical for an inhibitory effect to be observed *in vivo*.

We have observed similar lymphocyte inhibition using exoantigen preparations of *T. mentagrophytes* and *E. floccosum* (unpublished data), two organisms typically associated with more inflammatory infections. Moreover, inhibitory effects on lymphoproliferation strikingly similar to our own¹⁶ were reported using mannan extracted from both yeasts¹⁷ and *T. rubrum*,¹⁸ suggesting that many fungi may synthesize factors with lymphocyte-inhibitory properties. The outcome of an infective episode may thus be determined by the net effect of both dermatophyte-derived inhibitory and stimulatory factors.

T. rubrum accounts for approximately 90% of chronic superficial dermatophyte infections in the human host. Although some patients appear to have a particular susceptibility to chronic and recurrent disease, our *in vitro* data suggests this is unlikely to result from an endogenous inability to respond to dermatophyte antigen. Instead, it is possible that persistence of *T. rubrum* in the human host could result from a local inhibitory effect of dermatophyte antigen *in vivo*, as demonstrated to occur *in vitro*, on lymphocytes recruited to the epidermis. This putative suppression of lymphocyte activity at the site of infection might thus prevent eradication of fungus from the skin, even in the presence of normal *in vitro* cellular responses to dermatophyte antigen. Further research is necessary to establish whether the inhibitory properties of dermatophyte antigen observed *in vitro* do have a role to play in the chronicity of human cutaneous fungal infections.

Acknowledgment

This work was sponsored by a research grant donated by Sandoz Pharmaceuticals, U.K.

References

- Andrieu S, Biguet J, Laloux B. Analyse immunoélectrophoretique comparée des structures antigéniques de 17 espèces de dermatophytes. *Mycopathologia* 1968; **34**: 161–85.
- Philpot CM. Serological differences among the dermatophytes. *Sabouraudia* 1978; **16**: 247–56.
- de Sanchez IT, Mackenzie DW. Exoantigens of dermatophytes. *Sabouraudia* 1983; **21**: 159–61.
- Kaufman L, Lopez RB. Immunodiffusion studies of morphologically similar dermatophyte species. In: *Proceedings of the 5th International Conference on the Mycoses*. Washington DC: Pan American Health Organisation, 1980; 159.
- Hay RJ, Brostoff J. Immune responses in patients with chronic *Trichophyton rubrum* infections. *Clin Exp Dermatol* 1977; **2**: 373–80.
- Calderon RA, Hay RJ, Shennan GI. Circulating antigens and antibodies in human and mouse dermatophytosis. Use of monoclonal antibody reactive to phosphorylchlorine-like epitopes. *J Gen Microbiol* 1987; **133**: 2699–705.
- Jones HE, Reinhardt JH, Rinaldi MG. Acquired immunity to dermatophytes. *Arch Dermatol* 1974; **109**: 840–8.
- Calderon RA, Hay RJ. Cell-mediated immunity in experimental murine dermatophytosis. II. Adoptive transfer of immunity to dermatophyte infection by lymphoid cells from donors with acute or chronic infections. *Immunology* 1984; **53**: 465–72.
- Hanifin JM, Ray LF, Lobitz WC Jr. Immunological reactivity in dermatophytosis. *Br J Dermatol* 1974; **90**: 1–8.
- Calderon RA, Hay RJ. Cell-mediated immunity in experimental murine dermatophytosis. I. Temporal aspects of T-suppressor activity caused by *Trichophyton quinckeanum*. *Immunology* 1984; **53**: 457–64.
- Mayou SC, Calderon RA, Hay RJ, Goodfellow A. Deep (subcutaneous) dermatophyte infection presenting with unilateral lymphoedema. *Clin Exp Dermatol* 1987; **12**: 385–8.
- Sherwin WK, Ross TH, Rosenthal CM, Petrozzi JW. An immunosuppressive serum factor in widespread cutaneous dermatophytosis. *Arch Dermatol* 1979; **115**: 600–4.
- Read SM, Northcote DH. Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for proteins. *Anal Biochem* 1981; **116**: 53–64.
- Sveigaard E. Immunological investigations of dermatophytes and dermatophytosis. *Seminars in Dermatology* 1985; **4**: 201–21.
- Holden CJ, Hay RJ, MacDonald DM. The antigenicity of *T. rubrum*: *in situ* studies by an immunoperoxidase technique in light and electron microscopy. *Acta Derm Venerol (Stockh)* 1981; **61**: 207–11.
- McGregor JM, Hamilton AJ, Hay RJ. Inhibition of human lymphocyte blastogenesis by a factor derived from *Trichophyton rubrum*. *Br J Dermatol* 1990; **123**: 826.
- Nelson RD, Herron MJ, McCormack RT, Gehrzi RC. Two mechanisms of inhibition of human lymphocyte proliferation by soluble yeast mannan polysaccharide. *Infect Immun* 1984; **43**: 1041–6.
- Blake JS, Dahl MV, Herron MJ, Nelson RD. An immunoinhibitory cell wall glycoprotein (mannan) from *Trichophyton rubrum*. *J Invest Dermatol* 1991; **96**: 657–61.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.