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IN VIVO AND *IN VITRO* IMMUNE RESPONSES TO TRICHOPHYTIN IN DERMATOPHYTOSIS

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Abstract. Cell-mediated immunity in patients with verified dermatophytosis was investigated by means of skin test and lymphocyte stimulation test (LST) using purified trichophytin, prepared by the ethylene glycol method, and commercially available trichophytin. The purified trichophytin seemed to be more specific and sensitive both in skin test and in LST. In the majority of cases skin-positive patients were also positive in LST, especially when *T. mentagrophytes* or *E. floccosum* was the causative organism. Patients with non-chronic infections also showed a close correlation between skin reactivity and LST. Patients with chronic mycosis or infected with *T. rubrum*, on the other hand, were often skin-negative. Their lymphocyte reactivity *in vitro*, however, was usually positive, thus showing a discrepancy in the ability of these two methods to measure cell-mediated immunity.

Key words: Dermatomycosis; Delayed hypersensitivity; Lymphocyte transformation; Skin test; Trichophytin

Cellular immunity is believed to be reflected by *in vitro* reactivity of lymphocytes, as measured for example by lymphocyte stimulation. Its clinical correlate is considered to be delayed hypersensitivity, usually demonstrable by intracutaneous test. The demonstration of specific cellular immunity *in vitro* or *in vivo* is regarded as indicative of resistance to the infectious agent in question. This should also apply to dermatophyte infection. However, while the skin test is known to correlate closely with immunity, there are conflicting reports as to the lymphocyte responsiveness *in vitro* and its relation to skin test and the clinical condition (5, 18). For this reason we have investigated a well defined group of dermatophyte infected patients and compared their *in vitro* and *in vivo* reactivity to different trichophytin antigens in various clinical conditions.

MATERIAL AND METHODS

Patients. Outpatients from the Department of Dermatology, Södersjukhuset, having clinical signs of dermatophytosis verified by positive culture, were included in the study. All patients were questioned about previous dermatophytosis and other diseases. Patients with immunological disorders or receiving immunosuppressive medication were not included. Patients with dermatophytosis of more than one year's duration were considered chronically infected.

As a control group, 11 healthy laboratory technicians were examined. They had no previous history of dermatophytoses and no skin lesions.

Culture. Skin scrapings were taken from active lesions and inoculated on both Sabouraud's glucose agar and Dermatophyte test medium (DMT) (1). Dermatophytes were identified according to their colony morphology and microscopic appearance, using standard criteria (12).

Antigens. The following antigens were used: 1) Purified trichophytin (Ag I) processed according to the ethylene glycol method as described elsewhere (10). 2) Commercial trichophytin antigen (Ag II) obtained from Sächsisches Serumverk KG, Dresden (Trichophyton Vaccine) (9). 3) Pure tuberculin (PPD 2 TU) obtained from Statens Seruminstitut, Copenhagen.

Skin tests. All subjects were tested intracutaneously on the volar side of the forearm with 0.1 ml of antigen solution, containing 0.1 mg/ml of Ag I. The stock solution of Ag II was diluted 1:200, this being the strongest concentration recommended by the manufacturer (9). Reactions were read after 48 hours and were considered to be positive when an induration and erythema of at least 4 mm diameter was present. PPD was given intracutaneously as a standard tuberculin test (2 TU).

Lymphocyte cultures. Separation of lymphocytes was obtained by gradient centrifugation of heparinized blood over Ficoll-Isopaque (2). Duplicates of 10^6 cells were cultured in glass tubes containing 1 ml of Eagle's minimal essential medium supplemented with Earle's salts, 10% heat-inactivated human serum from AB-positive donors, 1% glutamin, and antibiotics.

The strongest final concentration of Ag I used *in vitro* was the same as that employed in the skin test, i.e. 100 μg/ml. Two additional tenfold dilutions were tested. In the same way, Ag II was added so as to obtain final dilutions of 1/200 or 1/2 000. Cultures without antigen served as controls. After 4 days of incubation at 37°C in humidified 5% CO_2 atmosphere, 0.2 μCi of ^{14}C-thymidine (specific activity 54.5 mCi per mM; The Radiochemical Centre, Amersham, England) was added to each tube.

Table I. *Results of intracutaneous tests (48 h)*

EF=*Epidermophyton floccosum*, TR=*Trichophyton rubrum*, TM=*Trichophyton mentagrophytes*

Pat.	Dermatophyte	Ag I (Mean diam. mm)	Ag II (Mean diam. mm)	PPD (Mean diam. mm)
Chronic				
1	TR	0	0	25
2	TR	7	7	75
3	TR	12	10	35
4	TR	0	0	15
5	TR	0	0	15
6	TR	0	0	35
7	TR	0	0	10
8	EF	0	0	20
Non-chronic				
1	TR	11	8	ND
2	TR	9	0	0
3	TR	0	0	30
4	TR	0	0	15
5	TR	0	0	ND
6	EF	15	10	ND
7	EF	11	12	0
8	EF	6	0	15
9	EF	12	0	30
10	EF	0	0	30
11	TM	20	7	12
12	TM	7	0	15
13	TM	22	12	50
14	TM	13	11	10
15	TM	9	0	0
16	TM	11	0	10

Twenty-four hours later, cultures were terminated and DNA synthesis was measured as described elsewhere (10). Values obtained from control cultures were subtracted before the means were calculated. Ratios of counts per minute (cpm) with and without antigen (lymphocyte stimulation index, LSI) were also calculated.

Statistical method. Student's *t*-test was applied to evaluate differences between mean values.

RESULTS

Skin tests

The results of the intradermal tests read after 48 hours are summarized in Table I. In 7 of the 8 patients with chronic mycoses, *T. rubrum* was isolated. In this group only 2 patients were skin-positive reactive to both trichophytin antigens. All patients in the chronic group reacted positively to tuberculin.

The non-chronic patients showed a more varied pattern—the three most common dermatophytes were isolated in nearly the same frequency (Table I). The delayed skin reactions here were predominantly positive to purified trichophytin (12 of 16 patients) while the commercial trichophytin caused fewer reactions (6 of 16 patients). Positive reactions to tuberculin occurred in 10 out of 13 cases.

Lymphocyte stimulation (LST)

The results expressed in cpm are shown in Table II. The reactivity was significantly greater in skin-positive patients than in controls. This was true for all concentrations of Ag I and Ag II. Also, in skin-negative patients reactivity was greater than in controls. For these two groups the difference was significant for Ag I in concentration of 100 μg/ml.

The reactivity of skin-positive patients was greater than that of skin-negative patients. The difference was significant when Ag I was used at a concentration of 10 μg/ml.

Essentially the same results as mentioned above were obtained when lymphocyte stimulation was expressed as an index between cpm values obtained in stimulated and unstimulated cultures (LSI). Figs. 1*a* and 1*b* show the LSI values obtained with Ag I and Ag II at a concentration of 100 μg/ml

Table II. *Lymphocyte stimulation with trichophytin*

Antigen	^{14}C-thymidine uptake, cpm (corrected) Patient's (+) skin test (1) $n=14$	Patient's (−) skin test (2) $n=10$	Controls (3) $n=11$	Statistical differences (1)–(2)	(1)–(3)	(2)–(3)
Ag I 100 μg/ml	5 260±2 441	1 928±1 720	168±130	NS	$P<0.005$	$P<0.05$
Ag I 10 μg/ml	2 226±1 157	614± 666	220±287	$P<0.05$	$P<0.01$	NS
Ag I 1 μg/ml	968± 642	195± 135	76± 63	NS	$P<0.05$	NS
Ag II 1/200	1 774±1 207	775±1 022	49± 43	NS	$P<0.02$	NS
Ag II 1/2 000	1 103± 689	575± 938	47± 98	NS	$P<0.02$	NS

Means and 95% confidence limits of counts per minute (cpm). NS = not significant.

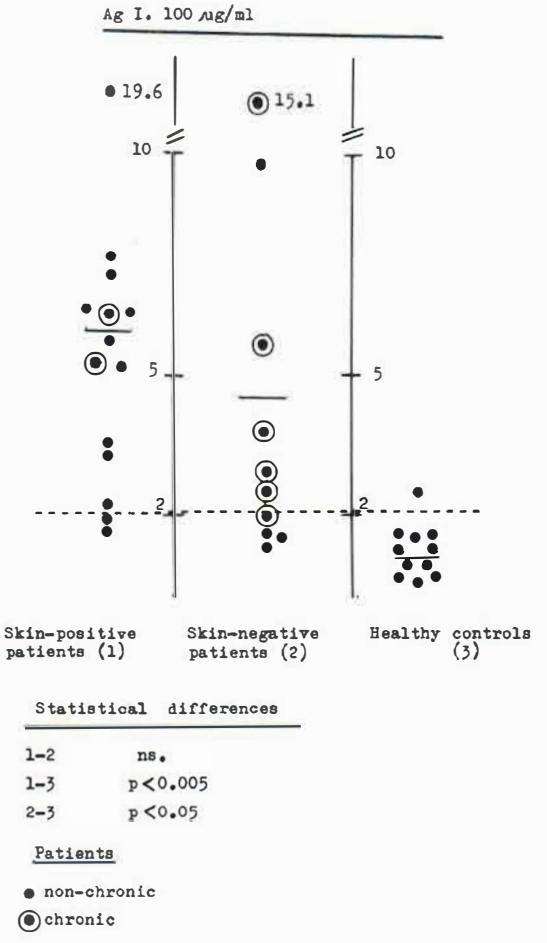

Fig. 1 a. Lymphocyte stimulation index (LSI) with purified trichophytin.

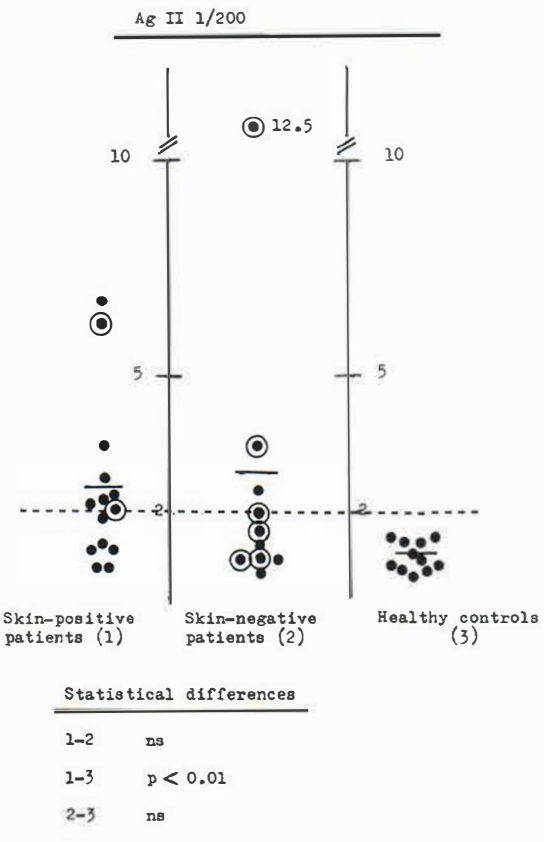

Fig. 1 b. Lymphocyte stimulation index (LSI) with commercial trichophytin.

and dilution 1/200, respectively. It can be seen that there were more positive LSI, and that the mean index values were higher when Ag I was used, than with Ag II. This applies both to skin-positive and skin-negative patients, indicating lower sensitivity of the commercial antigen in LST.

With Ag I, lymphocytes from 12 out of 14 skin-positive patients and 6 of the 10 skin-negative patients reacted positively. Thus, when using Ag I, both skin test and LST showed a better correlation in skin-positive than in skin-negative patients.

Fig. 1 *a* also shows the results of LST in chronic and non-chronic patients investigated with Ag I. The majority of the chronic patients (6 out of 8) were skin-negative, but 5 of them had positive LST. Two patients were positive in both skin test and LST. On the other hand, most of the non-chronic patients (10 out of 12) were skin-positive and displayed a positive LST. Only 4 were skin-negative and 3 of them had also negative LST. Thus, the non-chronic patients showed a close correlation between skin test and LST, while the chronic patients did not.

In Fig. 2 the patients are grouped according to the dermatophyte isolated. Patients with *E. floccosum* and *T. mentagrophytes* showed similar results—both groups showed a close correlation between skin test and LST. All 6 patients with *T. mentagrophytes* were skin-positive and had a positive LST. Two *E. floccosum* infected patients were negative in skin test and LST and the remaining 4 patients were positive in both skin test and LST.

Of 12 patients with *T. rubrum*, 4 were skin-positive and 3 of them had positive LST. Of the skin-negative patients, on the other hand, 6 developed positive LST. Thus, the correlation between skin test and LST was good in patients with *T. menta-*

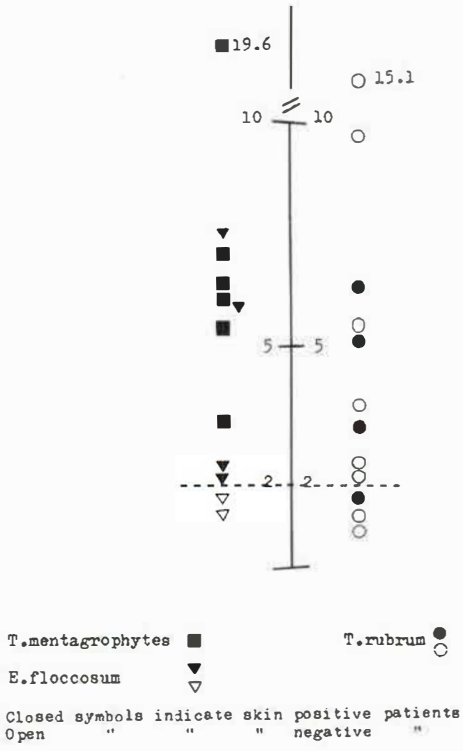

Fig. 2. Lymphocyte stimulation index (LSI) in patients to purified trichophytin according to the dermatophyte isolated.

grophytes and *E. floccosum* infections, while there was a discrepancy between skin and lymphocyte reactivity in *T. rubrum* patients.

DISCUSSION

There is considerable agreement that delayed hypersensitivity to trichophytin reflects cell-mediated immunity in dermatophytosis (4). It is obvious that the quality of the trichophytin antigen in studies of this kind is of great importance. We used purified trichophytin according to the ethylene glycol method as this antigen is considered to be both specific and sensitive (5, 8, 16). In present experiments—unlike in many other studies—the same antigens were used in both cutaneous test and *in vitro* studies. The commercial antigen (Ag II) was employed at the strongest concentration recommended for skin testing. The potency of the purified trichophytin (Ag I) was first determined in guinea pigs, as described elsewhere (10) and standardized on a weight basis to give the optimal concentration for cutaneous use. In lymphocyte studies the starting concentration of both preparations was that giving optimal skin sensitivity. In this way it was possible to compare the relative *in vivo* and *in vitro* reactivity of each antigen. The purified antigen turned out to be more reliable in skin test, giving positive reactions in 12 of 16 non-chronic patients as compared with 6 positive reactions with the commercial antigen (Table I). These data confirm results obtained in an earlier clinical study (11). Measurements of lymphocyte reactivity revealed the same tendency as in skin tests, i.e. the purified antigen caused more positive results and higher lymphocyte stimulation (Table II, Fig. 1) than the commercial antigen, thus indicating greater sensitivity. Lymphocyte reactivity to different antigens in dermatophytosis has been studied by many investigators and the reported results differ (1, 5, 7, 13). It appeared from the present study that the patients with positive skin test also show positive LST to purified trichophytin, in the majority of cases. The patients with negative skin tests on the other hand showed lymphocyte reactivity in about half of the cases (Fig. 1). As these findings do not tally with earlier reports (1, 5) it might be of interest to analyse in greater detail these two groups of patients—skin-positive and skin-negative. The skin-positive group consisted mainly of patients with *T. mentagrophytes* and *E. floccosum* infections. *T. mentagrophytes* is well known to give a high frequency of delayed skin reactions and so was the case in this study. *E. floccosum*, which is generally regarded as a weak sensitizing agent, gave in most cases in our investigation a positive reaction, thus confirming our earlier observations (11). As expected, the correlation between skin test and lymphocyte reactivity was good in both these groups of patients, all skin-positive patients with *T. mentagrophytes* and *E. floccosum* infections also being positive in LST.

The majority of the patients with *T. rubrum* infections, on the other hand, were skin test negative, which is in agreement with earlier reports on skin anergy in *T. rubrum* patients (5, 6, 8, 16). It is of interest, however, that 6 of 10 skin-negative patients had positive LST, thus demonstrating a discrepancy between *in vitro* and *in vivo* reactivity. Hanifin et al. (5) who like us employed the same purified trichophytin antigen for skin and lymphocyte tests, reported a very close correlation between *in vitro*

and *in vivo* test. Lymphocytes from *T. rubrum* infected patients with negative skin test were found to be non-responsive *in vitro* too. However, in other studies in which only LST was employed, positive reactions were demonstrated in such patients (7). Finally, Svejgaard et al. (13) were unable to show any difference in lymphocyte reactivity between chronic and non-chronic patients. It is not clear what is the cause of the conflicting evidence on lymphocyte reactivity in dermatophytosis. It may be due to some extent to differences in the methodology and the interpretation of clinical status.

Another question of interest is why the antigen fails to elicit skin reactivity in certain patients whose lymphocytes respond *in vitro*. A serum factor which inhibits cellular reactivity *in vitro* has been described (15) and this finding could suggest blocking antibodies as one explanation.

Another possibility is that LST is a more sensitive method than skin test in measuring mycotic sensitization in dermatophytosis and that positive skin reactions are only demonstrable in highly reactive cases. This, however, is less likely as there was no correlation between the respective degrees of lymphocyte and skin responsiveness. The discrepancy between skin and lymphocyte reactivity has also been described in chronic mucocutaneous candidiasis (3), where it is considered to reflect some degree of immunodeficiency. Furthermore, in the present cases it was mostly seen in patients with chronic infection. All these patients had a positive tuberculin reaction, which excludes some general defect in their cell-mediated reactivity, either on the level of activation of memory cells or in the complex series of steps leading from the introduction of antigen, to the measurement of skin induration, such as processing of antigen, release of lymphokines, function of inflammatory cells etc. The negative skin reaction may be due to some specific partial defect in the above-mentioned series of reactions. If this is the case, it remains to be proved on which level, and also whether it results from deficiency or suppression.

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