Purified Trichophytin: Lymphocyte Reactivity in vitro and Trichophytin-induced Suppression

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Three trichophytin preparations from different strains of Trichophyton mentagrophytes were produced according to the ethylene glycol method and assessed for their lymphocyte stimulation activity in vitro (LST). These trichophytin preparations showed significant variations in their effects on cord lymphocyte reactivity. The preparations also varied in their stimulation of lymphocytes from patients with dermatophytosis. Conclusions could be drawn about the immunological specificity, sensitivity and lymphocyte toxicity, which is important for the standardization of antigens. Furthermore, it was demonstrated that trichophytin-preincubated lymphocytes mediated a suppression of lymphocyte reactivity to this antigen. *Key words: Dermatophytosis; Lymphocyte transformation.* (Received June 27, 1986.)

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Studies on antigenic extracts from dermatophytes, denominated trichophytin, started with the work of Plato in 1902. Since then numerous trichophytin preparations with varying quality with respect to biological activity have been tested. Several studies have demonstrated that trichophytin preparations purified according to the ethylene glycol method possess high immunological activity (1, 2).

We have applied the above mentioned purification method to the preparation of antigen from three different strains of Trichophyton mentagrophytes. The present study aimed at evaluating the antigenic and mitogenic activity of these three trichophytin preparations by means of lymphocyte stimulation test in vitro (LST), as a first step in the immunological standardization of trichophytin extracts. To that purpose lymphocytes were collected from adult subjects and newborn infants.

Due to the importance of cell-mediated immunity in the immune response to dermatophytes it was considered also to be of interest to investigate the suppressive activity in vitro of trichophytin on lymphocytes from patients with dermatophytosis.

MATERIAL AND METHODS

Antigen

Three different strains of zoophilic Trichophyton mentagrophytes were chosen for antigen production. Freshly isolated strains from clinically active skin lesions were used. The purified trichophytin preparations, numbered I, II and III, were obtained by identical procedures involving mild chemical extraction with ethylene glycol as described previously (3). All three trichophytin preparations were used in parallel in the lymphocyte stimulation tests. Results from suppression experiments with trichophytin I are reported.

Lymphocyte donors

Newborn infants and adult subjects including healthy controls and patients with mycologically verified dermatophytosis served as lymphocyte donors. The control subjects had no present signs or

past history of dermatophytosis. Patients with a duration of disease of more than one year were considered chronically infected. Cord blood was collected from newborn infants immediately after cutting the umbilical cord.

Separation of lymphocytes

Mononuclear cells were isolated from heparinized blood samples by density gradient centrifugation on Ficoll Hypaque.

Lymphocyte stimulation test (LST)

Triplicates of 10^6 lymphocytes were cultured in glass tubes containing 1 ml medium RPMI 1640 supplemented with 10% pooled, heat-inactived human serum, L-glutamin and antibiotics (penicillin and streptomycin). Lymphocytes were incubated at 37°C for 6 days with 10 and/or 100 μg/ml of trichophytin. Cord lymphocyte reactivity to Phytohaemagglutinin (PHA) was investigated to ensure cord lymphocyte viability. The lymphocyte responses to trichophytin were studied by means of determination of DNA synthesis, which was assayed by labelling cells with ^{14}C-thymidine (specific activity: 50–60mCi/mmole). 0.2 μCi of ^{14}C-thymidine was added to each culture 18 h prior to harvesting. The incorporated radioactivity was measured in a liquid scintillation counter.

Suppression of lymphocyte reactivity

Primary culture: 10^6/ml lymphocytes were cultured with 100 μg/ml trichophytin I at 37°C. Controls without trichophytin were included. After 7 days lymphocytes were treated with 25 μg/ml mitomycin for 20 min at 37°C, washed three times and counted.

Secondary culture: Fresh lymphocytes were collected from the same donors. Secondary cultures contained 10^6 fresh lymphocytes and 10^6 mitomycin treated trichophytin-primed or unprimed lymphocytes in 1 ml RPMI. Control cultures with 10^6/ml fresh lymphocytes and without pre-cultured cells were included. Secondary cultures in triplicates were stimulated with 100 μg/ml trichophytin I and incubated at 37°C for 6 days. The DNA synthesis was determined as described above.

Calculations

Geometric means of counts per minute (cpm) from each set of triplicates were used for expression of data. Lymphocyte stimulation index (LSI) was calculated as a ratio between cpm of antigen stimulated lymphocytes and cpm of non-stimulated lymphocytes. The results were analysed by Student's *t*-test for paired samples.

RESULTS

The reactivity of cord lymphocytes to the three trichophytin extracts, expressed in cpm, is presented in Table I. DNA synthesis of cord lymphocytes was not significantly affected by 100 μg/ml of trichophytin I. Mean cpm were close to those observed in non-stimulated lymphocytes. On the other hand, 100 μg/ml of trichophytin II had a depressing effect on DNA synthesis of cord lymphocytes which was statistically significant ($p<0.01$) when comparing cpm in the presence and absence of antigen respectively. In contrast to this 100 μg/ml trichophytin III stimulated cord lymphocytes ($p<0.05$), i.e. exerted a mitogenic effect on the unsensitized cord lymphocytes at the concentration tested. Cord lymphocyte reactivity to trichophytin II was significantly reduced in comparison to the reactivity to trichophytin I or III ($p<0.01$), when using the same concentration of the preparations.

The lymphocyte reactivity to trichophytin in adult subjects,expressed in cpm and LSI, is presented in Table II. All patient lymphocyte samples which in most cases were collected from chronic Trichophyton rubrum infections, were stimulated to a higher degree than control lymphocyte samples by all three trichophytin extracts. However, three of four control subjects responded positively to at least one of the three trichophytin extracts, having a LSI>2, which was arbitrarily chosen as indicative of positive reactivity. The three trichophytin extracts differed in their capacity to stimulate patient lymphocytes. The lymphocyte reactivity to 100 μg/ml trichophytin III was significantly higher than the reactivity to 100 μg/ml trichophytin II when comparing cpm ($p<0.05$). Patients' lympho-

Table I. *Cord blood lymphocyte reactivity in 7 donors to 100 μg/ml of trichophytin preparations*

CPM = counts per minute

	Trichophytin I (CPM)	Trichophytin II (CPM)	Non-stimulated Trichophytin III (CPM)	lymphocytes (CPM)
	5 089	2 059	3 658	3 296
	3 318	1 923	4 510	3 204
	4 402	2 088	6 395	4 265
	1 978	615	2 569	1 877
	4 382	3 764	10 900	5 482
	3 788	2 897	6 725	2 822
	3 019	2 258	5 600	2 992
Mean	3 711	2 229	5 765	3 420

cytes were stimulated approximately twice as much (cpm) by 100 μg/ml trichophytin than by 10 μg/ml irrespectively of the trichophytin extract used.

The suppressive effect of trichophytin-primed or unprimed pre-cultured lymphocytes is presented in Table III as cpm and LSI. Trichophytin-primed lymphocytes suppressed significantly trichophytin reactivity, expressed in cpm or LSI, in the second cultures as compared with unprimed pre-cultured lymphocytes ($p<0.05$). However, also these latter cells depressed trichophytin reactivity as compared with cultures without pre-cultured cells ($p<0.05$). The degree of suppression was approximately the same in patients with different duration of disease. In order to exclude the possibility of suppressive effect being caused by crowding of cells, the reactivity was examined in cultures containing 2×10^6 fresh lymphocytes. The mean trichophytin reactivity (cpm) in these cultures was 96.5% of that obtained with 1×10^6 fresh lymphocytes.

DISCUSSION

The present knowledge of immunological mechanisms in dermatophytosis derives to a great extent from studies on trichophytin, in which intradermal application, in vitro lymphocyte stimulation and serological reactivity have been used. Trichophytin is also used in clinical work to indicate sensitization to dermatophytes, e.g. in diagnosing derma-

Table II. *Lymphocyte reactivity in healthy controls and patients with dermatophytosis to 100 μg/ml of trichophytin preparations (means ± SD)*

CPM = counts per minute, LSI = lymphocyte stimulation index

Subjects	Trichophytin I	Trichophytin II	Trichophytin III
Controls, $n=4$			
CPM	3 743±2 140	3 361±1 076	3 075±1 447
LSI	1.87±0.49	1.81±0.28	1.84±1.47
Patients, $n=7$			
CPM	16 337±5 651	12 085±5 576	18 104±6 092
LSI	8.26±2.65	6.29±2.72	10.13±5.50

Table III. *Suppressive effects of precultured lymphocytes on lymphocyte reactivity to 100 µg/ml trichophytin in 9 patients with dermatophytosis*

CPM = counts per minute, LSI=lymphocyte stimulation index

	Fresh lymphocytes + autologous precultured trichophytin-primed lymphocytes		Fresh lymphocytes + autologous precultured unprimed lymphocytes		Fresh lymphocytes	
Dermatophytosis	CPM	LSI	CPM	LSI	CPM	LSI
Non-chronic						
E.floccosum	3 002	2.26	5 503	4.76	11 006	8.85
T. mentagrophytes	703	1.23	1 570	1.79	3 972	4.39
T.mentagrophytes	5 106	2.16	13 405	6.07	32 740	19.57
Chronic						
T.mentagrophytes	1 818	2.15	5 425	6.12	7 074	9.07
T.rubrum	1 966	0.94	281	0.84	19 700	18.31
T.rubrum	1 965	0.51	4 879	1.09	11 176	6.32
T.rubrum	1 277	1.39	4 775	5.50	9 252	7.76
T.rubrum	3 868	1.42	5 025	2.77	5 539	2.51
T.rubrum	1 614	1.04	1 001	0.96	16 692	8.02
Mean	2 369	1.46	4 652	3.32	13 017	9.42

tophytid reactions (4). Furthermore trichophytin is sometimes included in batteries of so-called recall antigens employed in screening for immunological defects. Despite this wide use of trichophytin there is no general agreement on the standardization criteria of dermatophyte antigens. A standardization procedure of a given preparation usually includes investigation of various immunological and chemical properties. An attempt was made in this study to define some aspects of immunological reactivity to trichophytin extracts. Specificity, sensitivity and lymphocyte toxicity were studied by the application of LST in vitro. Information on specificity, i.e. the probability of a negative trichophytin reactivity in a non-sensitized individual, was obtained in LST with lymphocytes from adult, healthy controls and cord blood samples. These latter samples are to be preferred as sensitization to dermatophytes might be present in adult healthy controls. One of the tested trichophytin preparations was shown to have mitogenic effects on cord lymphocytes, thus possessing a low specificity in the concentration tested. On the other hand, another trichophytin preparation had probably toxic, inhibitory effects on the DNA synthesis of these lymphocytes. LST carried out with a lower concentration of the same trichophytin resulted in a reduced stimulation of patient lymphocytes which makes this preparation less suitable for use. The sensitivity, i.e. the probability of a positive LST in a sensitized individual, was estimated in experiments with lymphocytes from patients with dermatophytosis. These patients responded positively (LSI>2) to all trichophytin extracts, but there was a considerable variation in response to different trichophytin extracts. In conclusion, one trichophytin preparation combined a high sensitivity with a high specificity without lymphocyte toxicity in the concentration tested. Experimentally sensitized guinea pigs have previously been used in the standardization of trichophytin (3). However, the handling of animals and immunization procedures are laborious and there always is a risk of misinterpretation of results due to the possibility of earlier sensitization

in animals and of intercurrent infections. LST with lymphocytes from selected donors, i.e. patients with dermatophytosis and newborn infants constitutes an alternative and seems to be a more convenient tool to measure and standardize the antigenic properties of trichophytin extracts.

The DNA synthesis of trichophytin stimulated lymphocytes was markedly suppressed when they were co-cultured with trichophytin-preincubated lymphocytes. Previously a similar generation of suppressor cells resulting from activation of lymphocytes in vitro has been shown to occur with e.g. mycobacterial antigens (5, 6) and this capacity for inducing cells that suppress lymphocyte response may be a general property of microbial antigens. The demonstrated in vitro suppression in dermatophytosis does not seem to be dependent on the duration of disease and therefore probably not related with the mechanisms responsible for the chronicity of disease.

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