

AN EVALUATION OF DELAYED HYPERSENSITIVITY IN GUINEA PIGS TO VARIOUS TRICHOPHYTIN PREPARATIONS

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Abstract. Guinea pigs immunized with mycelium from *Trichophyton mentagrophytes* in Freund's complete adjuvant were tested intradermally with the following trichophytin preparations: TrI extracted by ethylene glycol, TrII extracted by phenol and TrIII, a commercially obtained preparation from Sächsisches Serumverk KG. In our experience, TrI was found to be superior to the other preparations. It showed significant delayed skin reactions in all sensitized animals and none in the controls. Lymphocyte stimulation test with this preparation (TrI) gave positive results.

Key words: Lymphocyte transformation; Skin tests; Trichophytin

Trichophytin has been used since 1902 (11) as an aid in the demonstration of dermatophyte infections. Since then, numerous preparations of varied quality have been used. Trichophytin was usually obtained as a crude extract from cultures of one or several dermatophyte species grown on various media under different conditions (14). For these reasons, the usefulness of the trichophytin reaction for skin testing has been limited, due to the lack of either standardized or pure trichophytin. The first attempt to characterize the antigen was made by Bloch et al. (2) in 1925. They concluded that the allergen involved was an extractable carbohydrate protein complex. Cruickshank et al. (1, 4) assigned the antigenic properties to a chemically defined glycopeptide which could produce delayed hypersensitivity in humans exposed to dermatophytes. Subsequent investigations (7, 12) have essentially confirmed these findings.

The intensive development of in vitro test systems in demonstrating delayed hypersensitivity has increased the use of trichophytin as an antigen. Götz & Heitmann (5) showed that lymphocytes

from patients infected with dermatophytes were stimulated by commercial trichophytin in vitro. Later, Hanifin et al. (6) demonstrated lymphocyte stimulation with a purified trichophytin. Animal tests have not been performed, however.

The main purpose of this study was to compare different trichophytin preparations with respect to their biological activity as measured by intradermal test and by in vitro stimulation tests using lymphocytes from sensitized guinea pigs. At the same time it was possible to test the reproducibility of the methods used in the preparation of trichophytin and the sensitivity of the in vitro stimulation tests conducted with lymphocytes from guinea pigs. The determination of DNA-synthesis by measuring the incorporation of ^{14}C -thymidine in lymphocytes was the method chosen to study reactivity in vitro.

MATERIALS AND METHODS

Preparation of antigens

Organism. A recently isolated pathogenic strain of *Trichophyton mentagrophytes* from a patient with tinea corporis was used. Inoculates were cultured simultaneously on Sabouraud's glucose agar without added antibiotics, and on dermatophyte test medium (DTM) according to Taplin et al. (17). The strain was positive on DTM and was identified morphologically on Sabouraud's agar by its typical appearance, i.e. macroscopically, a creamy powdery thallus and a brown-tan underside and microscopically, numerous characteristic spherical microaleuriospores "en grappe".

Culture conditions. The growth medium contained 1% Panmede (Paines and Byrne Ltd.), 4% glucose and 2% casein hydrolysate (3). The pH was adjusted to 5.5 with 1 M HCl. Several 5 litre flasks containing 1 litre medium were inoculated with 1 ml concentrated mycelium suspension. The flasks were incubated at 30°C and shaken at 100 rpm to ensure proper aeration. After 4 days' growth the mycelium was separated from the fluid by filtration and

thereafter acetone dried. The dried mycelium was ground to a fine powder with a pestle and mortar.

Antigen I (Tr I) was extracted with ethylene glycol according to Cruickshank et al. 1960 (4) and Ottaviano et al. 1974 (13). Ethylene glycol 200 ml was added per gram dried powder. The suspension was stirred with a magnetic stirrer overnight at room temperature. After centrifugation at 15 000 g for 10 min, the supernatant was dialysed against distilled water at 4°C for 50 hours. The material remaining inside the dialysis tubing was concentrated $\times 15$ on a Diaflo PM 10 membrane (Amicon Ltd.). Four volumes of cold ethanol were added and the mixture was left overnight at 4°C. The precipitate formed was spun down by centrifugation at 20 000 g for 10 min and collected. The precipitate was dissolved in a minimal volume of 1% sodium borate (pH 8.5). Cetyltrimethylammonium bromide (BDH Chemicals Ltd.) 5% w/v was added until a precipitate was no longer formed. The pH was adjusted to 9.5 by careful addition of 1 M NaOH. After treatment at 4°C for 6 hours the suspension was centrifuged at 4 000 g for 10 min. The precipitate was washed twice with distilled water and dissolved in a minimal volume of 2 M acetic acid. The precipitate obtained by addition of 10 vol cold ethanol (95%) was spun down by centrifugation at 15 000 g for 10 min. The acetic acid-ethanol treatment was repeated once. The precipitate was washed with cold ethanol (99%) and centrifuged at 15 000 g for 15 min. The precipitate was then dissolved in distilled water, yielding a clear, slightly light brown solution. To ensure complete removal of cetyltrimethyl ammonium the solution was washed repeatedly with distilled water on a Diaflo PM 10 membrane.

The glucosemannose content of carbohydrate of this preparation was 3:7 as determined by the method of McMurrough & Rose 1967 (9).

Antigen II (Tr II) was obtained by phenol extraction and purification according to Nozawa et al. (12). Dried mycelium 10 g was suspended in 160 ml water and mixed with 265 ml 75% w/v. phenol. The mixture was treated at 68°C for 30 min with occasional shaking and then centrifuged at 15 000 g for 10 min. The aqueous layer was collected and dialysed for 3 days against 0.15 M NaCl. The dialysate was concentrated by ultrafiltration on a Diaflo PM 10 membrane and added to a Sephadex G-100 column equilibrated with 0.15 M NaCl. The elution was followed by measurement of the UV-absorbance at 275 nm, and by the determination of sugar content of the 1 ml fractions as according to McMurrough & Rose (9). The fraction of the first peak corresponding to the GP₁ fraction of Nozawa et al. (12) was used as antigen Tr II in the subsequent experiments. The glucosemannose content of this fraction was 9:11 as determined by the method of McMurrough & Rose (9).

Antigen III (Tr III): Commercially obtained trichophyton. (Trichophyton-Vaccine, Batch no. 181171, Sächsisches Serumwerk KG, Dresden) (8). The glucosemannose content was 1:2 as determined by the method of McMurrough & Rose (9).

Biological tests

Immunization of animals. Guinea pigs (males approx. 400 g) were immunized by subcutaneous injection in the

nape of the neck with a mixture containing 0.2 ml mycelium-saline and 0.2 ml Freund's complete adjuvant. Each animal received 5 mg dried mycelium in this way.

Skin tests. After 7 weeks, guinea pigs were shaven and then injected intradermally into the abdominal wall with the trichophyton preparations. Each animal received 0.1 ml of two antigen preparations at two different concentrations. In addition the same volume of saline was given as a control.

Tr I was administered in the concentrations of 1 mg/ml and 0.1 mg/ml, Tr II at 5 mg/ml and 0.5 mg/ml. Tr III was used in a dilution of 1:20 and 1:200 of the stock solution. The skin of the animals was examined after 20 min and after 24, 48 and 72 hours. Perpendicular diameters of the indurated area were measured and the mean value calculated in each animal. Reactions with a mean diameter of at least 5 mm were considered to be positive.

Lymphocyte stimulation test

Spleen cells from 2 sensitized animals and one control animal were prepared approximately 2 months after the last injection, as indicated in the text. The animals were killed by a blow on the head. The spleens were excised under sterile conditions and immediately transferred to ice-cooled Petri dishes containing Tris-buffered Hank's balanced salt solution (pH 7.4). The spleens were perfused until free from blood, and then cut into small pieces. Cell suspensions were prepared by repeated pipetting. The suspensions were then transferred to ice-cooled centrifuge tubes in which larger pieces and debris remaining were allowed to settle to the bottom. The suspension of free cells was decanted. The cells were then suspended in Eagle's medium supplemented with glutamine, penicillin and streptomycin. The medium contained 10% heat-inactivated (90 min 56°C) foetal bovine serum. The cells were washed twice and centrifuged for 10 min at 350 g, and were then counted in a haemocytometer. During the preparation the cells were kept on ice. Two million lymphocytes and Tr I in an appropriate concentration were added to tissue culture tubes to a final volume of 1 ml. Tubes without antigen served as controls. After 5 days' incubation at 37°C in a continuous flow of a mixture of 5% CO₂ in air, 0.4 μ Ci of ¹⁴C-thymidine (spec. activity 54 mCi/mmol) was added to each tube. After a further 24 hours' incubation the cell suspensions were transferred onto membranes of a multiple sample collector (Type Millipore). The cells were washed first in saline and then in 5% trichloroacetic acid. The membranes were left to dry and then placed into vials containing 10 ml scintillation fluid. The radioactivity was measured with a Packard Tri-carb. liquid scintillation spectrometer. The results were recorded as the ratio between counts per minute obtained with and without antigen. This ratio was termed the lymphocyte stimulation index (LSI). A LSI of 2 was considered to be positive.

RESULTS

Intradermal tests

Tr I: Reactions which were present upon examination after 24 hours were essentially unchanged after

Table I. Delayed cutaneous sensitivity to trichophytin antigens

Antigen...	Mean diameter in mm at 24 hrs				Mean diameter in mm at 48 hrs			
	Tr I		Tr II		Tr I		Tr II	
	1 mg/ml	0.1 mg/ml	5 mg/ml	1 : 20	1 mg/ml	0.1 mg/ml	5 mg/ml	1 : 20
Guinea pig No.								
1	10.5	6	11		10	2	10	
2	17.5	0	0		14	5.5	5	
3	17.5	11	0		16	9.5	0	
4	7.5	6	0		7.5	3.5	0	
5	11.5	8	0		10.5	5.5	2	
6	18.5	8.5	0		14	8	2.5	
7	7	0		0	6	0		0
8	14.5	3		3	14.5	4.5		0
9	5	2.5		0	5	4		0
10	12	3		0	9.5	0		0
11	12	4.5		11	11	0		10
12	17.5	6		16.5	17	3.5		12
13	17.5	2.5		4	15	0		0

48 hours, but diminished significantly at the 72 hour reading. As shown in Table I, all tests performed with the preparation containing 1 mg/ml were positive upon examination at both 24 and 48 hours. The results obtained at a concentration of 0.1 mg/ml were less consistent, whereby 5 of 13 tests were positive. Eight non-sensitized animals were tested and none of them reacted with a positive skin test.

Tr II: Only one animal of the 6 tested showed a positive reaction after 24 hours at an antigen concentration of 5 mg/ml (Table I). After 48 hours another positive reaction appeared. No distinct reactions could be seen when 0.5 mg/ml of antigen was used. None of the non-sensitized animals showed a positive reaction.

Tr III: Two of the 7 seven animals tested reacted positively at the dilution 1 : 20 after both 24 and 48 hours (Table I). No positive reactions whatsoever were observed in animals receiving the 1 : 200 dilution. None of the 3 control animals reacted positively when tested with the antigen.

Furthermore, neither the sensitized animals nor the controls reacted when injected with saline. No reactions of any kind appeared after 20 min.

Lymphocyte stimulation test

Tr I stimulated increased DNA-synthesis in lymphocytes from 2 immunized guinea pigs (which also reacted with positive skin tests to Tr I) as compared to lymphocytes from the non-immunized guinea pig. The complete protocol of one stimulation experi-

ment is shown in Table II. It can be seen from this table that all three concentrations of antigen, which were used in this experiment, had a clear stimulatory effect on lymphocytes from one immunized animal. The other sensitized animal reacted in the same way. No such effect could be observed on lymphocytes obtained from the control guinea pig.

DISCUSSION

There is evidence to show that the age of the strain affects its antigenic properties (15). Moreover laboratory strains might lose some of their patho-

Table II. Incorporation of ^{14}C -thymidine into DNA in lymphocytes in the presence of trichophytin antigen (Tr I)

Antigen concentration $\mu\text{g/ml}$	CPM	LSI ^a
<i>Immunized guinea pig</i>		
10	5 233	7.2
1	5 437	7.5
0.1	3 942	5.4
	728	
<i>Control guinea pig</i>		
10	501	0.8
1	801	1.4
0.1	1 014	1.8

^a LSI=lymphocyte stimulation index

$\frac{\text{cpm in the presence of trichophytin}}{\text{cpm in control tubes without trichophytin}}$

genicity through several transfers which could thus alter the antigenic nature of the strains. Consequently a recently isolated pathogenic strain of *Trichophyton mentagrophytes* was used for the preparation of the antigens in this study.

It is well-known (16) that the degree of cutaneous sensitivity is lower in guinea pigs than in man. Since we lacked information regarding the optimal concentration of the antigens TrII and TrIII in guinea pig experiments, we used not only concentrations normally given in human experiments but also a 10-fold more concentrated test solution. This relationship is already well documented in the case of TrI (4).

As shown in Table I, TrI is superior to the other antigens tested. TrI at a concentration of 1 mg/ml gives positive reactions in all guinea pigs after 48 hours. As might be expected, 0.1 mg/ml (i.e. the concentration used in man) gave less consistent results. The reactions are essentially unchanged after 48 hours, which indicates that immunological and not toxic reactions are involved.

TrII, which in its chemical composition is similar to TrI, produced only two positive reactions at the higher concentration, and none at the lower, despite the fact that all animals reacted to TrI. The results might indicate that we have used too weak a concentration of antigen. It is also possible, however, that this discrepancy might be due to a species variation, since no guinea pig experiments have been reported with antigen prepared in the manner as described for TrII.

It is interesting that TrIII, the commercially obtained antigen, gave inconsistent results. Only two positive tests were observed in the 7 animals tested. The results indicate that only the strongly immunized animals responded to this antigen.

Antigen-induced stimulation of lymphocytes has been demonstrated to be closely correlated to delayed hypersensitivity (10).

Our observations confirm earlier findings that this also applies to the immune response against trichophytin antigens (5). The results of stimulation tests indicate the great sensitivity of this method, which makes it suitable for determining the potency of various antigen preparations.

Our experiments showed in the comparison of the biological activity in guinea pigs of different trichophytin preparations, that the purified trichophytin TrI gave the most consistent results. It appeared to be sensitive with respect to delayed hypersensitivity

in guinea pigs immunized with mycelium and showed no tendency to produce toxic reactions.

Thus, it seems that this purified trichophytin is suitable for further studies in man.

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REFERENCES

1. Barker, S. A., Cruickshank, N. D., Morris, J. M. & Wood, S. R.: The isolation of Trichophytin glycopeptide and its structure in relation to the immediate and delayed reactions. *Immunology* 5: 627, 1962.
2. Bloch, B., Labouchère, A. & Schaaf, F.: Versuche einer chemischen Charakterisierung und Reindarstellung des Trichophytins (des aktiven, antigenen Prinzips pathogener Hautpilze). *Arch Derm Syph* 148: 413, 1925.
3. Codner, R. C., Cruickshank, C. N. D., Trotter, M. D. & Wood, S. R.: The production of Trichophytin antigen in submerged culture of *Trichophyton mentagrophytes*. *Sabouraudia* 1: 116, 1961.
4. Cruickshank, C. N. D., Trotter, M. D. & Wood, S. R.: Studies on Trichophytin sensitivity. *J Invest Dermatol* 35: 219, 1960.
5. Götz, H. & Heitmann, H. J.: Über in-vitro Reaktionen von Lymphozyten in Gegenwart von Trichophytin. *Hautarzt* 18: 23, 1967.
6. Hanifin, J. M., Ray, L. F. & Lobitz Jr, W. C.: Immunological reactivity in dermatophytosis. *Br J Dermatol* 90: 1, 1974.
7. Ito, Y.: On the immunologically-active substances of the dermatophytes. II. *J Invest Dermatol* 45: 285, 1965.
8. Jung, H.-D.: Über die Brauchbarkeit neuentwickelter Pilzkulturextrakte (Trichophytin und Trichophyton). *Z Aertzl Fortbild (Jena)* 47: 685, 1953.
9. McMurrough, I. & Rose, A. H.: Effect on growth rate of substrate limitation on the composition and structure of the cell wall of *Saccharomyces cerevisiae*. *Biochem J* 105: 189, 1967.
10. Mills, J. A.: The immunologic significance of antigen induced lymphocyte transformation in vitro. *J Immunol* 97: 239, 1966.
11. Neisser, A.: Plato's Versuche über die Herstellung und Verwendung von "Trichophytin". *Arch Derm Syph* 60: 63, 1902.
12. Nozawa, Y., Noguchi, T., Ito, Y., Sudo, N. & Watanabe, S.: Immunochemical studies on Trichophyton mentagrophytes. *Sabouraudia* 9: 129, 1971.
13. Ottaviano, P. J., Jones, H. E., Jaeger, J., King, R. D. & Bibel, D.: Trichophytin extraction: biological comparison of Trichophytin extracted from *Trichophyton mentagrophytes* grown in a complex medium and a defined medium. *Appl Microbiol* 28: 271, 1974.
14. Seelinger, H. P. R.: Standardization and assay of skin test antigens for mycotic diseases. *Prog Immunobiol Stand* 2: 154, 1965.

15. Stuka, A. J. & Burrell, R.: Factors affecting the antigenicity of *Trichophyton rubrum*. *J Bacteriol* 94: 914, 1967.
16. Sutter, E.: Weitere Beiträge zur Lehre von der Immunität und Überempfindlichkeit bei Trichophytieerkrankungen. *Dermatol Z* 24: 65, 1917.
17. Taplin, D., Allen, A. M. & Mertz, P. M.: Experience with a new indicator medium (DTM) for the isolation of dermatophyte fungi. *Proc Internat Symp Mycoses*. Washington, D.C., Pan American Health Organization, Publ. no. 205, 1970.

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