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TRICHOPHYTIN CONTACT SENSITIVITY IN GUINEA PIGS WITH EXPERIMENTAL DERMATOPHYTOSIS INDUCED BY A NEW INOCULATION METHOD*

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ABSTRACT

Trichophytin contact sensitivity was demonstrated for the first time in guinea pigs infected with *Trichophyton mentagrophytes* using a new inoculation technique and a provocative patch test. The inoculation method utilizes an occlusive dressing to keep a high humidity in the inoculation site. With a zoophilic strain of *T. mentagrophytes*, secure occlusion of the inoculated site for 24 hr produced definite infection for several weeks.

Trichophytin contact sensitivity was detected by means of a provocative patch test with undiluted crude trichophytin from filtrates of broth medium cultured with the organism. The sensitivity was confirmed by a patch test with purified trichophytin consisting of polysaccharide with attached peptides. The incidence of contact sensitivity increased with the number of infections—11 out of 20 animals (55%) after the first infection, 12 of 15 (80%) after the second, and 11 of 12 (91.5%) after the third.

It is well known that guinea pigs inoculated with dermatophytes develop hypersensitivity, as indicated by the appearance of positive intradermal trichophytin tests, and acquire a resistance to subsequent infections [1]. However, little attention has been paid to contact sensitivity. Hypersensitivity to topically applied active antigenic material was demonstrated by Keeney and Huppert in an attempt to induce immunity to dermatophytes in guinea pigs [2]. Nonetheless, the development of trichophytin contact sensitivity has never been reported in experimental dermatophytosis. In a preliminary study, we observed that a small number of animals inoculated with dermatophytes by commonly used techniques [3, 4] clearly showed positive reactions to a diluted crude trichophytin solution applied by a conventional patch testing method and the histologic picture resembled that of allergic contact dermatitis. It was assumed at that time that this rare occurance of trichophytin contact sensitivity did not reflect the true incidence for the following reasons. (1) It is well known that the state of intradermal trichophytin hypersensitivity correlates well with the severity of the induced infection [1], and if this were also the case with trichophytin contact sensitivity, only a marginal state of sensitivity could be produced in the guinea pigs inoculated with dermatophytes by conventional techniques. (2) Active antigenic material of trichophytin consisting of a large molecular species presumably penetrates the intact horny layer poorly.

In this paper we describe an experimental study of the production of trichophytin contact sensitivity in guinea pigs and its demonstration by means of special techniques of patch testing. With regard to an inoculation technique, no method has been outstandingly successful in reproducibly yielding animal fungus infections; most of the techniques involve traumatization of the skin, and difficulties are encountered in repeating experiments without variability of results [3, 4]. In a recent review article, Knight mentioned an effective method developed by Reinhardt which allows production of a standard reproducible infection on human skin with a quantitated inoculum [5]. Similar experiments have been undertaken in our laboratory with guinea pigs and a simple experimental system which does not require prior injury of the animal's skin has been worked out. On the other hand, for the detection of contact sensitivity to a weak allergen with extremely limited capacity to penetrate the normal stratum corneum, Kligman described the SLS provocative patch test in humans [6]; the principle of the test depends on the effect of sodium lauryl sulfate (SLS) which assures penetration of allergens into the skin by damaging the stratum corneum barrier and initiating a mild inflammatory response. Using this technique we have succeeded in obtaining positive results.

MATERIALS AND METHODS

Preparation of the Fungal Inoculum

For the establishment of the standardized method, a strain of *T. mentagrophytes* was used predominantly because its sporulation enabled us to adjust easily the amount of inocula. The culture used for the study was a zoophilic granular strain of *T. mentagrophytes* obtained from patients with dermatophytosis. The organism was grown on Sabouraud's dextrose medium for 2 weeks at 28° C and suspensions were prepared by flooding the surface of the medium with sterile water and gently dislodging the colony with a wire loop. Clumps of fungus were ground with a mortar and pestle for 10 min; the suspensions were agitated vigorously for 10 min in a

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vortex mixer and filtered through 8 layers of gauze. The filtrates were washed with sterile water three times by centrifugation at 3,000 rpm for 10 min. In the case of *T. mentagrophytes*, the preparations were composed of numerous microconidia and a few hyphal fragments; the spores were counted using a hemocytometer and diluted to give the desired final densities. 10,000 spores per cm² skin area produced a definite infection in every animal within a few days and, therefore, the density of the spores was adjusted to this number.

Preparation of Antigenic Materials

Classic crude trichophytin was prepared from filtrates of Sabouraud's broth medium cultured with the granular strain of *T. mentagrophytes* at 28° C for 1 month. Phenol (0.5%) was added to the material for patch testing; for the intradermal trichophytin test, 0.1 ml of a 1:20 dilution of the original filtrates in saline containing 0.5% phenol was used.

Purified trichophytin was prepared from the mycelium of *T. mentagrophytes* according to the method of Watanabe [7]. The mycelia were removed from the medium, dehydrated, ground in a mortar to a fine powder, extracted with distilled water for 3 days, and made 1% with respect to trichloroacetic acid. After removal of the resulting precipitate, the pH of the solution was adjusted to 3.2 by the addition of 1 N NaOH. Three volumes of pure ethanol were added to obtain a precipitate which was dissolved in distilled water, dialyzed, and freeze dried. This material consisted of polysaccharide with attached peptides. The potency was assayed as described by Cruickshank et al [8] and it was demonstrated that 0.01% solution corresponded to 1:20 crude trichophytin solution. For patch testing, a 0.5% saline solution of this material was used.

Preparation of Experimental Animals and Inoculation Procedure

Guinea pigs (Hartley strain) weighing 350–500 gm were used. The skin of the midback was used as the inoculation site since occlusive dressings could be firmly held in place. The site was prepared by plucking and subsequently clipping any remaining short hairs; the presence of hair makes secure occlusion difficult and produces negative results. The traumatization induced by plucking did not affect the results.

0.01 ml of the spore suspension (containing 40,000 spores) was delivered to the inoculation site with a micropipette. The site was immediately covered with a 2 × 2 cm sheet of polyethylene film and the inoculum was spread by light pressure of the film, which was secured with an impermeable plastic tape (Blenderm®, Minnesota Mining & Manufacturing Co.) and held in place for 24 hr by elastic adhesive bandage 3.8 cm in width (Elatex®, Tokyo Eizai Co.). The occlusive dressing was left in place for 24 hr.

High Dose Inocula for Induction of Trichophytin Contact Sensitivity

Twenty animals were inoculated with 200,000 spores of *T. mentagrophytes* in a 2 × 2 cm area on the back, i.e., 50,000 spores per cm², under occlusion for 24 hr. Reinoculations with the same fungus were performed at 1-month intervals.

Detection of Trichophytin Contact Sensitivity

SLS provocative patch test with undiluted crude trichophytin. The procedure described by Kligman in humans [6] was modified according to the Guinea Pig Maximization Test of Magnusson and Kligman [9] as follows. A strip of skin lateral to the spinal groove was clipped and shaved with an electric razor. The area was pretreated with 10% sodium lauryl sulfate (SLS) in petrolatum without occlusion for 24 hr before the trichophytin patch was applied. 0.05 ml of undiluted crude trichophytin was applied to the skin on a 1-cm² cloth and covered by an overlapping impermeable plastic adhesive tape, which in turn was firmly secured by elastic adhesive bandage wound around the torso of the animal, and left for 24 hr. As a control, undiluted Sabouraud's broth was applied in the same way to the contralateral side. The sites were evaluated at 24 and 48 hr after removal of the patch. The result was recorded as positive if the trichophytin site showed more intense erythema than the control site. In a preliminary study none of 21 normal animals tested in this way showed positive reaction.

Patch test with purified trichophytin. The procedure was similar except that SLS pretreatment was omitted. 0.05 ml of 0.5% purified trichophytin and saline were applied under occlusion for 24 hr.

Trichophytin Skin Test

The test was performed by intradermally injecting 0.1 ml of 1:20 crude trichophytin into the skin of the lower back and observing the response 24 hr after the injection.

Histologic Procedures

Skin biopsy specimens were obtained from lesions at various intervals after inoculation and from patch test sites. Tissues fixed in 10% formalin were sectioned and stained with hematoxylin and eosin and PAS.

RESULTS

Lesions. The onset, initial extent, and severity of the lesions were dependent upon the amount of inocula. With 10,000 spores per cm², faint erythema was occasionally present even 24 hr after inoculation. Unequivocal skin infection usually became recognizable on the 2nd or 3rd postinfective day by the formation of papuloerythematous lesions confined to the inoculation site, the surface of which became scaly in a few days. Although the lesions spread peripherally, they hardly ever reached a size twice as large as the inoculation area (Fig. 1). The extent of the lesions was recorded every day on a 1 to 3 scale—1 representing a few tiny spots, 2 being used for reactions in which the inoculation site showed several scattered erythematous patches, and 3 representing an entirely diffuse, erythematous plaque. The most intense stage was between the 6th and 12th postinfective day, when the infiltrated erythematous base was covered by thick silvery scales with areas of crusting and oozing. The lesions began to resolve by the third week and by the fourth week they completely regressed to leave an alopecic scar. Positive skin scrapings for fungal elements and isolation of the fungus were possible from the scales during infection.

Dose-response relationships. Four groups of 7 animals were inoculated with four levels of inocula of *T. mentagrophytes:* 80, 400, 2,000, and 10,000 spores per cm² skin area with 24-hr occlusion. All

the animals inoculated with 10,000 spores developed definite infection with a lesion covering the entire inoculation site (a score 3 lesion), whereas the remaining animals inoculated with a smaller number of spores showed minimal or no lesions (Fig. 2). The initiation of the lesions with lower doses was occasionally delayed until the 5th to 7th postinfective day.

Influence of occlusion period on response. Three groups of 2 animals were inoculated with a threshold amount of inoculum, i.e., 2,000 spores of *T. mentagrophytes*. The occlusive dressings were removed from 2 guinea pigs of each group on days 1, 2, or 4 postinoculation. As is evident from Figure 3, the incidence and severity of lesions increased after 2 days of occlusion.

Repetition of infections with a high dose of inoculum (50,000 spores/cm². As is well known [3], the initial signs of infection developed earlier in reinfected animals and the inflammatory states that followed were much less severe and subsided faster than with the first infection. Twenty-four hr after initial infection erythema was observed in only 10 out of 20 animals, whereas 13 of 16 in the second infection and 10 of 13 in the third developed erythema at 24 hr. In regard to the duration of infection, it took at least 3 weeks for regression after the first inoculation, while the second and third infections subsided within 10 days in all animals.

Trichophytin contact sensitivity in repeatedly infected animals. The SLS provocative patch test with undiluted crude trichophytin provoked positive reactions in 11 of 20 animals (55%) after the first infection. The reactions consisted of a diffuse, slightly swollen erythema. The incidence of trichophytin contact sensitivity increased with the number of infections; 12 of 15 (80%) after the second infection and 10 of 12 (82.5%) surviving the third infection demonstrated the contact sensitivity (Table). Since the experiments involved rather drastic procedures for animals, i.e., repeated fungal infections, SLS irritation, and, particularly, application and removal of the elastic adhesive bandage, there was a high loss of experimental animals. In regard to the 9 animals which did not show trichophytin contact sensitivity after the first infection, 3 were lost, while 5 animals showed positive reactions later: 3 after the second infection and 2 after the third. However, 1 of the latter 2 animals revealed its trichophytin contact sensitivity only to patch testing with purified trichophytin. Therefore, when the 12 surviving animals after the third infection were patch tested with 0.5% purified trichophytin, 11 of 12 (91.5%) showed positive reactions consisting of a slightly swollen erythema, occasionally associated with vesicopustules or crusty scales. As a result, excluding the 3 animals which died during the second infection, there was only one animal which did not show a positive reaction even after the third infection.

FIG. 1: Score 3 reaction on the 5th postinfective day. The entire inoculated area (marked on the edge) is covered by an erythematous, scaling lesion.

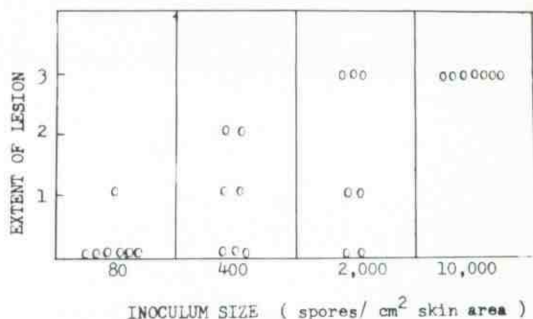

FIG. 2: Dose–response relationships. The extent of the lesions was recorded on the 7th postinfective day on a 1 to 3 scale.

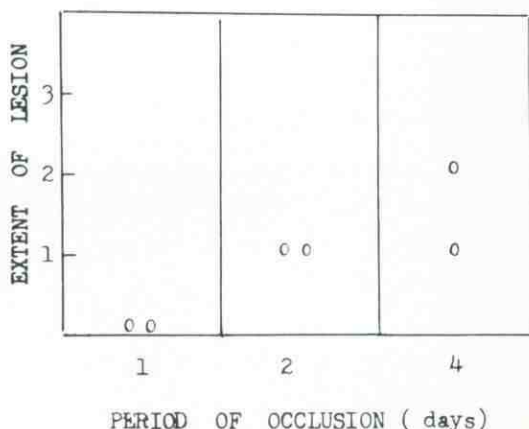

FIG. 3: The effect of the period of occlusion on inoculability of fungus. A threshold amount of inocula was applied under occlusion for 1, 2, or 4 days.

The patch test with 0.5% purified trichophytin was repeated at various intervals in 5 guinea pigs newly inoculated with *T. mentagrophytes* in order to investigate the latent period between infection and the development of trichophytin contact sensitivity. A weak, doubtful reaction was first noted at the patch test site on the 9th postinfective day in one animal. The patch test performed on the 10th postinfective day provoked definitely positive reaction in 2 of 5 animals.

The persistence of the contact sensitivity was

TABLE

Incidence of trichophytin contact sensitivity and average diameter of intradermal trichophytin tests

	Number of past infections		
	1	2	3
SLS provocative patch test with crude trichophytin	11/20 (55.5%)	12/15 (80.0%)	10/12 (82.5%)
Patch test with 0.5% purified trichophytin			11/12 (91.5%)
Mean delayed intradermal reaction (mm) ± SE	9.9 ± 0.46		9.0 ± 0.49

followed until 6 months after the last infection, when 7 out of 10 surviving animals still showed positive responses to 0.5% purified trichophytin.

Trichophytin skin test in repeatedly infected animals. Intradermal trichophytin reactions at 24 hr became demonstrable 7–9 days after the first inoculation of *T. mentagrophytes* in all the animals. However, no significant increase in intradermal trichophytin reactivity occurred even after repeated infections (Table).

Histopathology. The histologic features of the induced lesions were similar to those produced by other methods [3]. At the peak of infection, there was a massive cellular infiltrate composed of neutrophils, eosinophils, and mononuclear cells in the upper dermis, and exocytosis of these cells was noted in the acanthotic epidermis. The stratum corneum was parakeratotic and markedly thickened due to accumulation of dense cellular debris and plasma. PAS stain revealed scattered mycelia in the horny layer of the lesions from the 4th to 12th postinfective day.

Biopsy specimens obtained from positive SLS provocative patch test reaction sites and those from positive purified trichophytin patch test sites revealed similar changes. The epidermis showed acanthosis, areas of spongiosis, and exocytosis. A dense cellular infiltrate consisting of mononuclear cells, eosinophils, and neutrophils was noted in the dermis.

DISCUSSION

We have previously encountered difficulties in obtaining reproducible results with generally used inoculation techniques for dermatophytes. They depend upon traumatization of the skin, but also are not quantitative and the inoculum does not remain in place [3, 4]. These problems have been solved by utilizing the occlusive dressing technique, a simple device to maintain high humidity at inoculation sites. In experimental human fungus infections, successful inoculation without prior scarification has been achieved, based on the principle of producing high humidity in the skin [10, 11]. This is also the case with guinea pigs [12]. In the guinea pig, a tight occlusive state can be easily established by placing a piece of polyethylene film over the inoculum and sealing it with elastic adhesive tape as long as the skin is free of hair. Haphazard clipping alone often produces a false negative result and clean shaving with an electric razor or depilation are necessary.

In this study, 100% infectivity of a strain of *T. mentagrophytes* with an intense inflammatory reaction occupying the inoculated site was achieved using 10,000 spores per cm^2 skin area under occlusion for 24 hr. The degree of infection was related to the dose of fungus material, to the period of occlusion, and to the size of the inoculated area. Using this method, Soh and Fuyuki† compared the pathogenicity of species of *Nannizzia* and confirmed that the plus strains were nonpathogenic, while among the minus strains the *M. gypseum complex* (*N. incurvata* and *N. gypsea*) was more pathogenic than *N. fulva*. In the past no difference had been noted in the severity in experimental guinea pig infections between the mating types of *M. gypseum complex* [13]. The technique has many advantages over those previously used and should find a wide application in the field of experimental mycology.

In 1930, Sulzberger and Lewis demonstrated that the skin of certain persons reacted to the application of trichophytin, by means of patch test, with an eruption of an eczematous type [14]. Later, this type of hypersensitivity was extensively investigated by Ninomiya [15]. He noted that trichophytin patch tests with pretreatment of the skin by means of a physical insult, i.e., rubbing with glass powder, were positive in 73.5 percent of patients with dermatophytosis, even when conventional patch testing with trichophytin was negative. Further studies on this problem were carried out by Watanabe and Fujisawa [16]. They reported a high incidence of trichophytin contact sensitivity among patients with tinea pedis associated with trichophytid. On the other hand, trichophytin contact sensitivity following experimental dermatophytosis has not been demonstrated in guinea pigs.

The present study clearly demonstrated that contact sensitivity to crude trichophytin was noted in half of the guinea pigs inoculated only once with *T. mentagrophytes*. The percentage of contact-sensitive animals increased with the number of infections, and after the third infection most of the surviving animals showed positive reactions, although the intradermal trichophytin hypersensitivity was hardly influenced by repeated infections. This was confirmed by patch testing with purified trichophytin which did not require the SLS pretreatment of the skin because of its high potency.

Although our study presents evidence that guinea pigs inoculated with *T. mentagrophytes* develop trichophytin contact sensitivity, its role in the pathogenesis of experimental dermatophytosis

† Soh Y, Fuyuki S: Personal communication.

is still left unsolved. Keeney and Huppert noted that topical application of antigenic material prepared from *T. mentagrophytes* induced the development of hypersensitivity in guinea pigs as well as an increased resistance to infection with the homologous fungus [2]. In our experiments we observed that in repeatedly infected animals the inflammatory lesions developed earlier and the entire process followed a shortened course. It is of interest to speculate, on the basis of these findings, that trichophytin contact sensitivity is related to the early development of inflammatory changes, which lead to an unfavorable condition for the proliferation of the fungus and result in a rapid regression of the lesions. The inflammatory changes presumably give rise to an increase in turnover rate of the epidermis and the production of parakeratosis, on the one hand, and produce exudation of serum on the other. As a result, the fungus is not only shed rapidly but also inhibited in its later proliferation by antifungal factors in the serum [17, 18].

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