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Temporal correlation of lymphocyte blastogenesis, skin test responses and erythema during dermatophyte infections

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SUMMARY

The development of cellular hypersensitivity, as measured by lymphocyte blastogenic and skin test assays, was correlated with intensification of lesion erythema in guinea-pigs infected with *Trichophyton mentagrophytes*. Development of lymphocyte blastogenic responses to trichophytin was assayed using forty infected animals and twenty controls; blastogenic conversion occurred between 9–11 days post spore application. The onset of cellular hypersensitivity was assayed *in vivo* in twenty infected guinea-pigs by skin testing at intervals after infection; positive responses occurred between 9–11 days post spore application which coincided with the time of blastogenic conversion. Clinical characteristics of lesions were followed in a group of twenty-four animals: lesions first appeared on day 7; erythema intensified until day 10; lesions reached maximum size by day 13; and alopecia began on day 17. Since cellular hypersensitivity (positive skin test and lymphocyte blastogenesis) develops at the same time as lesions reach maximum erythema, cellular hypersensitivity appears to be responsible for the intense inflammatory response associated with ringworm infections.

INTRODUCTION

Hosts infected with dermatophytes usually develop cellular hypersensitivity to fungal antigens (Bloch & Massini, 1906; Cruickshank, Trotter & Wood, 1960). Sulzberger (1950) hypothesized that the inflammatory appearance of the dermatophyte lesion occurred as a result of the hypersensitivity response rather than as a primary irritant reaction to the fungus. Positive temporal correlations between development of erythema and cellular hypersensitivity in humans (Jones, Reinhardt & Rinaldi, 1974) and in cattle (Lepper, 1972) have been reported; however, the descriptions do not clearly specify the closeness of the time relationships. The experimental lesions in humans may have been viewed as infrequently as once a week, and the intervals between skin tests were not stated. A close temporal correlation between onset of cellular hypersensitivity and acute intensification of erythema would indicate the importance of the hypersensitivity response in the inflammatory appearance of the lesion.

Studies relating clinical appearance and the development of cellular hypersensitivity have been hampered by the inability to produce a standard size lesion with a defined course of infection in a previously uninfected subject. We overcame these difficulties by using a recently developed quantitative model for *Trichophyton mentagrophytes* infections in guinea-pigs (Greenberg *et al.*, 1976a). The temporal relationship between development of cellular hypersensitivity and intensification of inflammation was investigated using both lymphocyte blastogenic and skin test assays. Lymphocyte blastogenesis, which may be a more sensitive assay for early detection of cellular hypersensitivity (Oppenheim, 1968), have not previously been investigated during development of ringworm infections, nor has the temporal correlation with skin test responses been shown.

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MATERIALS AND METHODS

Guinea-pig infections. Outbred Chase–Moen guinea-pigs of either sex, bred at our institute and weighing about 300 g, were infected with microconidiospores of *Trichophyton mentagrophytes* var. granulosum ATCC 18748. Infections were initiated by the method of Greenberg *et al.*, (1976a). This consisted of spreading 100 spores on a shaved 380-mm^2 area of unabraded skin, covering the infection site with a moistened gauze pad, spraying adhesive on the previously epilated area surrounding the infection site, and then pressing teflon onto the adhesive, thus occluding the infection site. The animals were then wrapped with medical adhesive and tape. The occlusive materials were removed at the end of 3 days.

Scoring of infection. Animals were examined daily for time of appearance of the lesion, degree of erythema, lesion size, amount of scaling, amount of crusting, and appearance of alopecia. The following criteria were used for scoring erythema (rated from 0–3): barely visible = 0·5, pale pink = 1, red = 2, reddish purple = 3. Lesions were measured in two dimensions at 90° angles from each other. Sizes were calculated from these measurements using the formula for an elipse. Scaling and crusting (scored in units from 0–4) were evaluated as follows: visible, but 30% or less of the lesion involved = 1; 30–60% of area involved = 2; 60–90% of lesion affected = 3; 90–100% of area affected = 4. The first day that smooth skin was noted in any area of the lesion was considered as the beginning of alopecia.

Antigens. PHA-M (Grand Island Biologicals) was reconstituted to 20 ml with RPMI containing 1% guinea-pig serum. Lymphocytes were cultured in a further 1:20 dilution of PHA in medium. These cultures served as positive internal controls.

Trichophytin was produced by growing *Trichophyton mentagrophytes* in a static culture and processed by the procedure described by Ottaviano *et al.* (1974). This antigen was added to appropriate lymphocyte cultures to yield a final concentration of 50 μg/ml.

Skin tests. Guinea-pigs were injected i.d. on a shaved area of their backs with 100 μg trichophytin in saline. The sites were examined to determine size and degree of erythema, and degree of induration at 24, 48, and 72 hr. With this antigen, erythema was never demonstrable in control animals.

Lymphocyte culture medium. RPMI 1640 medium (Grand Island Biologicals) was used which contained 15% heat-inactivated guinea-pig serum, 10 mM hepes buffer, 100 μg/ml streptomycin, 100 u/ml penicillin, 0·29 mg/ml L-glutamine, and 0·15% sodium bicarbonate.

Lymphocyte blastogenesis. Lymphocytes from axillary and inguinal lymph nodes of guinea-pigs were collected by rubbing the organs on 60 gauge mesh screens. Cells were washed three times, and cultured in microtitre plates (Cook Engineering) at 5×10^5 cells per well in 0·2 ml of medium alone (controls), medium plus PHA, or medium plus trichophytin. Each set consisted of nine replicates. After 2 days incubation in 5% CO_2 at 37°C, tritiated thymidine (0·5 μc per well) was added and the microtitre plates incubated for an additional 24 hr, following which the cultures were harvested.

Harvesting was accomplished by depositing the lymphocytes on glass fibre filters (Reeve Angil) using a multiple sample collector (Microbiologicals Associates). Cells were rinsed with saline, fixed with 10% TCA and rinsed with water. Portions of filter strips corresponding to individual wells were placed in vials containing 10 ml scintillation fluid. Vials were counted in a liquid scintillation spectrophotometer for 2 min.

Statistical analysis. Counts per minute from individual vials (minus background) were transformed into natural logarithms, and the mean and variance for the nine replicates in each set were subsequently determined. The mean values of the transformed data were used to calculate lymphocyte blastogenic ratios (mean of experimental/mean of control). Scheffe's critical value was determined from the variances of both the control and the trichophytin sets of data from experimental animals only. This value was used to determine if a given lymphocyte blastogenic ratio indicated a positive or negative response to trichophytin. Since two batches of tritiated thymidine were used to label cells, Scheffe's critical value was determined twice. The critical value for the first series of days (7, 9, and 11) was 2·4, and for the second series of days (13 and 15) was 3·0.

RESULTS

Specificity of trichophytin induced blastogenesis

Lymphocytes from eight guinea-pigs infected with *T. mentagrophytes* spores and from eight non-infected animals were assayed for blastogenic stimulation with trichophytin. Lymphocyte cultures from non-infected animals did not undergo blastogenic stimulation in the presence of trichophytin, whereas blastogenesis was noted in all cultures from previously infected animals. Lymphocyte blastogenic ratios ranged from 20–150 (mean 48·7$\pm$42 s.d.) in infected animals, but only from 0·6–1·9 (mean 1·3$\pm$0·4 s.d.) in the control animals.

Onset of cellular hypersensitivity to trichophytin

Lymphocyte blastogenesis. Infections in forty guinea-pigs were initiated with *T. mentagrophytes* spores, and the time of conversion to positive lymphocyte blastogenic responses to trichophytin determined on sets of eight animals at intervals of 7, 9, 11, 13, or 15 days post spore application. At each interval tested,

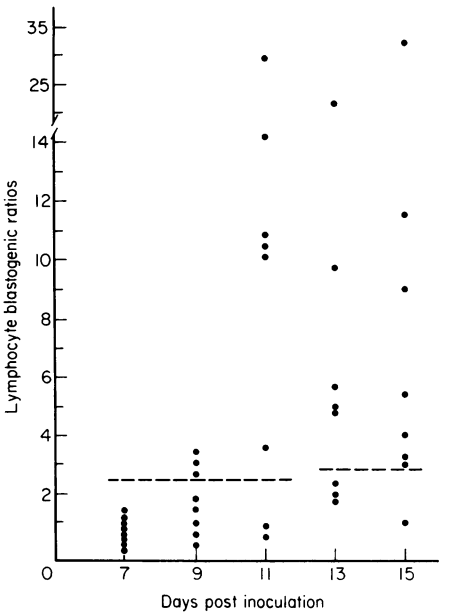

FIG. 1. Trichophytin stimulation of lymphocytes at intervals after infection of guinea-pigs with *T. mentagrophytes*. Each dot represents the ratio of ct/min experimental: ct/min control for one animal. The dotted horizontal lines indicate Scheffe's critical values, and therefore ratios above this line signify positive responses.

two negative control animals (non-infected) and two positive control animals (previously infected) were assayed for blastogenic stimulation with trichophytin; results were as expected (ratios ranged from 0·6–1·9 in negative controls and from 10–160 in positive controls). PHA, the internal control, stimulated lymphocyte cultures from all animals (ratios ranged from 10–853). Fig. 1 shows the blastogenic ratios to trichophytin from individual infected experimental animals at various times after infection. Blastogenic ratios above 2·54 (for days 7, 9, and 11) or above 3·0 (for days 13 and 15) indicated positive responses, thus demonstrating that the conversion to positive blastogenic responses to trichophytin was between the 9th and 11th day. Although three of eight animals had statistically significant responses on day 9, these ratios were very low and close to the critical value, whereas by day 11 the majority of animals had high blastogenic ratios to trichophytin.

Skin tests. The conversion to positive trichophytin skin rest responses was determined using sets of five animals infected with *T. mentagrophytes*. The onset of cellular hypersensitivity as measured by skin testing was between the 9th and 11th day, which coincided with the period of conversion to detectable lymphocyte blastogenic responses (Table 1). A higher proportion of animals had positive blastogenic responses than skin test responses on day 9 while the opposite was true for day 11, but the

TABLE 1. Skin test and lymphocyte blastogenic responses to trichophytin at intervals after infection with *T. mentagrophytes*

Days post infection	No. positive lymphocyte blastogenic responses	No. positive skin tests
7	0/8	0/5
9	3/8	1/5
11	6/8	5/5
13	5/8	5/5
15	7/8	n.t.*

* n.t. = Not tested.

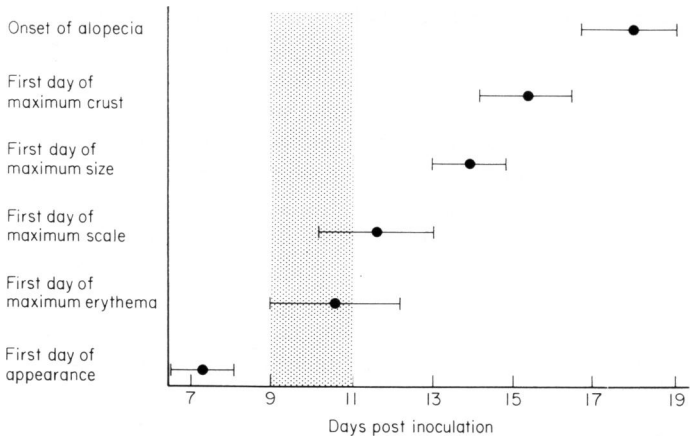

FIG. 2. Clinical characteristics of *T. mentagrophytes* lesions in twenty-four infected guinea-pigs. The mean plus or minus one s.d. is shown. Shaded area indicates time of onset of positive skin test responses to trichophytin.

differences were not statistically significant ($P = 0{\cdot}49$ (day 9); $P = 0{\cdot}36$ (day 11), Fisher's exact test). Hence, both assays detected positive responses on days 9 and 11, but there is no evidence that one assay was more sensitive than the other.

Course of infection. The course of infection in twenty-four animals infected with *T. mentagrophytes* is summarized in Fig. 2, which shows the first day that each sign reached its maximum intensity. On the average, erythema intensified until day 10·6, which correlated with the conversion to positive cellular hypersensitivity responses.

DISCUSSION

This report indicates that during an acute dermatophyte infection, the development of cellular hypersensitivity coincides in time with the development of maximum erythema in the lesion. These results provide substantiating data for previous reports (Jones, Reinhardt & Rinaldi, 1974; Lepper, 1972) which suggested that this occurred. Temporal associations such as these cannot prove a cause and effect relationship between one phenomenon and another, and therefore these data do not prove that the disease is mainly an allergic response to the fungus; however, these data are in agreement with this conclusion and few strong alternative hypotheses are apparent since it is unlikely that a primary irritant response would occur as late as 11 days after spore inoculation.

The above conclusion, that the intense erythema seen in acute dermatophyte infections is caused by the host's immunologic response, is also supported by the association between erythema and cellular hypersensitivity in chronic (as opposed to acute) dermatophyte infections. Chronically infected patients with highly inflamed lesions usually demonstrate positive skin test responses to trichophytin, whereas patients with mildly inflamed lesions are usually skin test negative (W. Lobitz, and J. Hanifin, written communication). An additional indication that the inflammatory response is a result of the host's cellular hypersensitivity is seen during secondary dermatophyte infections where lesions are maximally inflamed almost at their onset, rather than requiring several days to develop (Greenberg *et al.*, 1976a; Jones, Reinhart & Rinaldi, 1974).

Defining the cause of the acute inflammation in dermatophyte infections is important in evaluating and proposing methods of therapy. Desensitization and steroid treatment would be reasonable approaches to limiting the severe manifestations of inflammatory dermatophytosis if the inflammation is immunologically induced. Alternatively, systemic steroid therapy or desensitization could prove harmful if the immunologic response is important in not only causing the inflammation but also in clearing the dermatophyte infection. The use of systemic steroids has produced conflicting results (Kanof, 1958; Kligman

et al., 1951; Fisher & Sher, 1972; Goss *et al.*, 1963) and although desensitization appears to be beneficial to some patients, experiments have not included adequate control groups (Sulzberger & Wise, 1932; Kerr, Pascher & Schulzberger, 1934; Traub & Tolmach, 1935; Bazyka, Kashkin & Siluyanova, 1966; Tager *et al.*, 1973). While the efficacy of systemic steroid treatments and desensitization therapy is still unresolved, it is possible that the use of topical steroids may prove to be a safer choice of therapy.

This study shows that trichophytin skin test and lymphocyte blastogenic assays both convert to positive responses at the same time period after infection. This is consistent with the hypothesis that they are *in vivo* and *in vitro* assays of the same phenomena. Since the development of positive responses for both assays occurs so closely in time, there is no statistical evidence that one assay is more sensitive than the other.

Previous studies addressing temporal correlations of classical delayed type skin tests and lymphocyte blastogenesis have not found close associations (Oppenheim, 1968; Chaparas, Good & Janicki, 1975; Fulton *et al.*, 1975), indicating either differences in test sensitivities in early stages of conversion, or that the assays are not measures of the same event. Differences between their results and ours could be attributed to different model systems, and particularly to the fact that trichophytin skin tests are of the cutaneous basophilic hypersensitivity type (Greenberg *et al.*, 1976b) and not of the classical delayed-type hypersensitivity. Alternatively, further investigation of lymphocyte blastogenic responses using other lymphoid organs or antigenic preparations in our model system might reveal temporal differences. Confirmatory experiments are needed to distinguish between these alternatives.

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