The Effect of Human Lymphokine on the Growth of *Trichophyton mentagrophytes*

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Lymphokine was tested for fungal growth inhibitory activity against the filamentous fungus *Trichophyton mentagrophytes*. Human peripheral blood lymphocytes from a donor exhibiting delayed type cutaneous hypersensitivity to a trichophytin skin test were cultured with trichophytin and PHA-P. Culture supernatants were assayed for lymphokine activity using the lymphotixin sensitive mouse L-929 alpha fibroblast. Lymphocyte activation to PHA-P and trichophytin was confirmed by monitoring "H-thymidine incorporation. Supernatants from 2-day PHA-P and 6-day trichophytin activated cultures were found to contain potent lymphokine activity. This activity was not diminished by the addition of ferric iron sufficient to saturate the contained transferrin. Supernatants from unstimulated control cultures contained no lymphokine activity. Undiluted lymphokine containing supernatants and nonlymphokine containing control supernatants were evaluated for fungal growth inhibitory activity using a sensitive radiometric growth assay. Iron supplemented supernatants retaining potent lymphokine activity did not inhibit fungal growth. Non-iron supplemented supernatants and fresh medium containing serum inhibited fungal growth. Our data suggest that lymphokine active against mammalian cells is not directly antagonistic to the growth of the filamentous fungus *T. mentagrophytes* but does not exclude the possibility that activated lymphocytes release a chelator such as transferrin that can inhibit fungal growth.

An acute inflammatory reaction correlates with the limitation of spread and eventual healing of experimentally induced dermatophyte infections in man [1] and guinea pigs [2,3]. This inflammation is thought to be cell mediated since a temporal correlation exists between the appearance of inflammation and the development of delayed type cutaneous hypersensitivity DH [1] and lymphocyte blastogenesis [3] elicited with trichophytin antigen. Additionally, the presence of DH to trichophytin was shown to correlate with the infection-free status of a large group of adult human males studied for evidence of dermatophytosis [4]. These observations suggest that cell mediated immunity, CMI, is involved in host defense against dermatophytes. The mechanism(s) whereby CMI may mediate host defense in this instance, however, remains unknown.

The dermatophytes colonize the dead upper keratinized layers of the epidermis [5]. These layers are essentially extracoronal and not normally exposed to the internal milieu of the host. That the cellular components of cutaneously expressed CMI reaction could come in contact with the fungus, much less survive in the environment of the skin surface and directly combat fungal growth, is very unlikely.

On the other hand lymphokines, the effector molecules of CMI which are soluble in body fluids, might be capable of diffusing into the stratum corneum and directly inhibiting fungal proliferation. A report that lymphokine containing supernatants inhibit the *in vitro* growth of the yeasts *Saccharomyces cerevisiae* and *Candida albicans* suggests that lymphokine can have a direct effect on fungi [6].

This study uses a sensitive radiometric microassay of fungal growth to determine whether a lymphokine, which inhibits the growth of mouse L-929 fibroblasts, will inhibit the growth of the filamentous fungus *T. mentagrophytes*.

**MATERIALS AND METHODS**

**Organism**

*Trichophyton mentagrophytes* var. granulare (American Type Culture Collection 18748 Rockville, Md.) was maintained on potato dextrose agar. A suspension of microaleuriospores (spores) for inoculum purposes was prepared [7].

**Peripheral Blood Lymphocytes**

Heparinized peripheral blood was obtained by venous puncture from a healthy human volunteer expressing cutaneous delayed hypersensitivity to a trichophytin antigen skin test. The blood was allowed to sediment at 37°C for 2 hr and the plasma layer was removed. Cells were concentrated by centrifugation, washed 3 times with Hanks balanced salt solution and resuspended in RPMI-1640 tissue culture medium supplemented with 10% heat inactivated AB positive serum and 4 mM 1-glutamine. The cell preparation was slowly filtered through a nylon wool column (10 cc syringe containing 0.8 g of nylon wool) saturated with supplemented RPMI-1640 and maintained at tissue culture conditions. The column was eluted 3 times with supplemented medium and the resulting cell preparation was concentrated by centrifugation. This preparation consisted of 98% small round mononucleated cells, that were 95% viable as estimated by the trypan blue dye test.

**Lymphocyte Blastogenesis**

Lymphocytes, 1 x 10^6 in 200 μl of supplemented RPMI-1640, were delivered to wells of a Microtest-II culture plate (Falcon, Oxnard, Ca.). Blastogenesis was elicited with 3 μg of phytohemagglutinin-P (PHA-P) (Difco, Detroit, Mi) or 10 μg of trichophytin (Letterman Army Institute for Research, San Francisco, Ca.) per well. The plates were incubated at 37°C in an atmosphere adjusted to contain 3.5% CO₂ at a relative humidity approaching 100%. The cultures were pulsed 4 hr before termination with 0.5 μCi of "H-thymidine, (specific activity 25 μCi/mmol New England Nuclear, Boston, Mass.). The DNA from each culture was precipitated on glass filter paper (Reeve Angel Grade 934AH) following the procedure of Hartzman [8] using a multiple assay sample harvester (MASH) (Otto Heller Co., Madison, Wis.). The recovered filter paper planchets containing the precipitated labeled DNA were subjected to liquid scintillation counting.

**Lymphokine Production**

Nylon wool column purified peripheral blood lymphocytes were cultured in tissue culture tubes (Falcon 3033). Each tube contained 3 x 10^6 cells in 2.0 ml of supplemented RPMI-1640. Trichophytin (100 μg) or PHA-P (30 μg) in 100 μl of medium was prepared and added to the appropriate cultures. Control cultures received 100 μl of medium. Triplicate cultures were incubated at 37°C in an atmosphere adjusted to contain 3.5% CO₂ at a relative humidity approaching 100%. At 24 hr intervals stimulated and unstimulated control cultures were removed from the incubator and centrifuged at 150 g for 10 min. The supernatant fluids from replicate cultures were carefully harvested with a pasteur pipet, pooled and stored at −20°C.
Lymphokine Target Cells

Lymphotoxin sensitive mouse alpha L-929 cells were obtained from Dr. G. A. Granger (Scripps Clinic and Research Foundation, La Jolla, Ca.). The cells were maintained on supplemented RPMI-1640 and passed every 4 days. Cells obtained from 4-day-old maintenance cultures were used in all lymphokine assays.

Lymphokine Assay

The method for lymphokine assay was an adaptation from the lymphotoxin assay of Williams and Granger [9]. Two-fold serial dilutions of each lymphokine supernatant were prepared in supplemented RPMI-1640. Each test dilution was then added in a volume of 100 μl to replicate microtiter wells containing 3 x 10⁵ mouse alpha L-929 target fibroblasts in 100 μl of supplemented RPMI-1640. Where indicated 1 μg of ferric iron contained in 10 μl of ferric ammonium citrate solution was added. After 48 hr of incubation at tissue culture conditions, 165 μl of medium was withdrawn from the test well and replaced with 165 μl of PBS containing 0.01% trypsin and 10⁻⁵ M EDTA. After 3 min the contents of the well were tritiated and the cells enumerated in a hemocytometer. The results are reported as the percent of growth inhibition based on growth in nonsupernatant-containing supplemented medium.

Lymphokine Antifungal Assay

The effect of lymphokine on the growth of T. mentagrophytes was estimated visually and measured with a sensitive automated radiometric microgrowth assay. The radiometric assay correlates fungal growth with the incorporation of ¹⁴C (provided as ¹⁴C (μ) glucose) into accumulating trichloroacetic acid insoluble material [10,11]. The radiometric assay was adapted for this study as follows. The glucose content of each lymphokine supernatant was measured and adjusted so that it contained the same amount of glucose as the fresh supplemented RPMI-1640. This adjustment is necessary to assure that the final isotopic specific activity after the addition of ¹⁴C (μ) glucose is the same in each supernatant.

Two hundred microliter aliquots of each supernatant were pipetted into microtiter wells. Into each well was pipetted 10 μl of ¹⁴C (μ) glucose solution (0.10 μCi, 4.06 mCi/mmmole, New England Nuclear) and where indicated 1 μg ferric iron contained in 10 μl of ferric ammonium citrate solution. Each well was inoculated with 1 x 10⁷ spores of T. mentagrophytes in a 10 μl volume.

After 36 hr the fungal content of each well was collected on filter paper (Grade 934 AH, Reeves Angel) and washed 10 times with 300 μl volumes of distilled water, 10 times with 300 μl volumes of 5% trichloroacetic acid and 10 times with 300 μl volumes of absolute methanol using a multiple automated sample harvester. Filter paper planchets containing the washed fungus were subjected to liquid scintillation counting to determine the amount of fungal growth.

RESULTS

Lymphocyte Reactivity

Purified peripheral blood lymphocytes were obtained from a human donor with documented acquired immunity to infection with T. mentagrophytes and who responded to a trichophytin skin test with a strong cutaneous delayed hypersensitivity reaction. These cells were tested for their capacity to undergo a blastogenic response to PHA-P and trichophytin and were used to produce lymphokine. Blastogenesis was monitored by the incorporation of °H-thymidine (Table I). The response to trichophytin increased through the 7th and last day of culture. These results establish the T-cell mitogen PHA-P and fungal antigen reactivity of the donor lymphocytes used for generating lymphokine.

Lymphokine Activity

Lymphokine activity was assayed by monitoring the capacity to inhibit the growth of lymphotoxin sensitive L-929 fibroblasts. The Figure demonstrates that the supernatants from day 2 PHA-P and day 6 trichophytin-stimulated lymphocyte cultures contain potent activity. The activity of these supernatants decreased linearly with dilution. An early (day 2) trichophytin-stimulated culture supernatant had much less lymphokine activity which was not diminished by dilution. Maximum inhibi-

### Table I. The incorporation of °H-thymidine by human peripheral blood lymphocytes selected for the preparation of lymphokine after stimulation with PHA-P or trichophytin a,b

<table>
<thead>
<tr>
<th>Time in culture (days)</th>
<th>°H-Thymidine incorporation (CPM)b</th>
<th>PHA-P</th>
<th>Trichophytin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>250 ± 10</td>
<td>31878 ± 1011</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>404 ± 18</td>
<td>45293 ± 1461</td>
<td>3367 ± 136</td>
</tr>
<tr>
<td>4</td>
<td>485 ± 35</td>
<td>93599 ± 2423</td>
<td>8009 ± 533</td>
</tr>
<tr>
<td>5</td>
<td>322 ± 24</td>
<td>57549 ± 1071</td>
<td>29274 ± 875</td>
</tr>
<tr>
<td>6</td>
<td>256 ± 21</td>
<td>43631 ± 1019</td>
<td>39209 ± 1274</td>
</tr>
<tr>
<td>7</td>
<td>270 ± 19</td>
<td>ND</td>
<td>51498 ± 1758</td>
</tr>
</tbody>
</table>

* 1 x 10⁵ nylon wool column purified lymphocytes were cultured in 200 μl volumes and harvested with a MASH on the indicated day after initiation of the culture.

* Each value is the mean of 6 replicate cultures plus or minus the standard deviation. ND means not determined.

![Fig 1. Lymphokine activity of supernatants tested for antifungal activity. Lymphokine was assayed by the capacity to inhibit the growth of lymphotoxin sensitive mouse L-929 fibroblasts. Each point is the mean of 4 replicate assays each of which varies no more than 5% from the mean.](image-url)
Lymphokines were prepared by stimulating lymphocytes with PHA-P and the fungal antigen trichophytin. This lymphokine although not purified or concentrated, had potent mammalian cell growth inhibitory activity when assayed against mouse L-929 fibroblasts (Figure). Lymphokine containing supernatants and fresh tissue culture medium supplemented with serum inhibited the growth of T. mentagrophytes (Table IV). This inhibition of fungal growth was negated by the addition of ferric iron which, however, did not diminish the lymphokine activity for mouse L-929 fibroblasts (Table III). Since both the lymphokine containing supernatants and the freshly prepared serum supplemented medium inhibits fungal growth, the activity is most likely accounted for by the presence of iron unsaturated transferrin from the serum supplement. Neither iron saturated nonlymphokine containing control or lymphokine containing supernatants inhibited fungal growth indicating that lymphokine active against L-929 fibroblasts does not inhibit the growth of the filamentous fungus T. mentagrophytes (Table II).

These findings differ from those of Pearsall, Sundsmo, and Weiser [6] who reported that supernatants from cultures of allogenic mouse spleen cells stimulated with PHA-P for 2 days inhibits the growth of the yeasts S. cerevisiae and C. albicans. This apparent discrepancy may be explained in several ways. First, their supernatants and ours contained transferrin from a 10% serum supplement and concentrated before testing for antifungal activity that is an iron chelator similar to transferrin or lactoferrin is secreted by activated lymphocytes. A chelator released from activated lymphocytes could account for the difference in activity of the control compared to lymphokine supernatant in the study of Pearsall, Sundsmo, and Weiser [6]. Consistent with this idea is the report that human lymphocytes can synthesize transferrin [14]. Whether lymphocytes produce and secrete transferrin capable of inhibiting fungal growth is not known.

Second, lymphokine may inhibit the growth of yeasts but not filamentous fungi due to the biochemical and structural differences between yeasts and filamentous fungi.

Although the present study does not provide support for the antifungal activity of lymphokine, additional studies in which lymphokine is purified and concentrated before testing for antifungal activity are needed. That the lymphokine content of our supernatants is significantly less than that achieved in vivo at the site of a cutaneous CMI inflammatory reaction is also possible. We have, however, inoculated microcultures of both PHA-P and trichophytin activated human lymphocytes with the spores of T. mentagrophytes, supplemented these cultures with ferric iron, and microscopically monitored fungal growth. Under these conditions the antifungal effect of lymphocytes or their nonchelator products should be maximal. We found that there was no microscopically detectable inhibition of fungal growth.

It is our contention that lymphocytes or their products (lymophokine) other than chelators do not act directly on the fungus but may act indirectly against some host component, possibly epidermal cells, during a dermatophyte infection [1]. The inhibition of L-929 fibroblast growth by trichophytin elicited lymphokine is consistent with this theory as is the report of increased epidermal proliferation at the site of a cutaneous delayed hypersensitivity reaction elicited by fungal antigen [15].

**DISCUSSION**

**TABLE II. The effect of lymphokine on the growth of T. mentagrophytes monitored by the uptake of 14C into new mycelia.**

<table>
<thead>
<tr>
<th>Supernatant collected at time (days)</th>
<th>Fungal growth (CPM 14C)</th>
<th>Supernatant collected at time (days)</th>
<th>Fungal growth (CPM 14C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PHA-P</td>
<td>Trichophytin</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>---------</td>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>1/4</td>
<td>9644 ± 718</td>
<td>9669 ± 294</td>
<td>9498 ± 549</td>
</tr>
<tr>
<td>2</td>
<td>8011 ± 1473</td>
<td>ND</td>
<td>7622 ± 1320</td>
</tr>
</tbody>
</table>

"All supernatants were adjusted to contain 1.63 mg/ml of glucose. 200 ml of the indicated supernatant was placed into wells of a microtiter plate, supplemented with 0.1 μCi of 14C(μ) glucose, 1.0 μg of ferric iron and inoculated with 1 × 10⁴ spores of T. mentagrophytes."

"Each value is the mean of 4 replicate cultures plus or minus the standard deviation. ND means not determined.

**TABLE III. The effect of ferric iron on the activity of lymphokine to inhibit the growth of mouse L-929 fibroblasts**

<table>
<thead>
<tr>
<th>Supernatant dilution</th>
<th>Day 2 PHA-P</th>
<th>Day 6 trichophytin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Fe⁺³</td>
<td>1.0 μg Fe⁺³/ml</td>
</tr>
<tr>
<td></td>
<td>No Fe⁺³</td>
<td>1.0 μg Fe⁺³/ml</td>
</tr>
<tr>
<td>1:2</td>
<td>86±9</td>
<td>78±74</td>
</tr>
<tr>
<td>1:4</td>
<td>72±8</td>
<td>69±62</td>
</tr>
<tr>
<td>1:8</td>
<td>69±5</td>
<td>51±54</td>
</tr>
<tr>
<td>1:16</td>
<td>68±5</td>
<td>42±44</td>
</tr>
<tr>
<td>1:32</td>
<td>45±38</td>
<td>25±26</td>
</tr>
<tr>
<td>1:64</td>
<td>34±18</td>
<td>17±14</td>
</tr>
<tr>
<td>1:128</td>
<td>28±15</td>
<td>16±8</td>
</tr>
</tbody>
</table>

"Lymphokine activity is reported as the percent growth inhibition based on growth in fresh media. Each value is the mean of 3 replicate assays each of which varied no more than 5% from the mean.

**TABLE IV. The effect of ferric iron on the capacity of lymphokine containing supernatants to inhibit the growth of T. mentagrophytes.**

<table>
<thead>
<tr>
<th>Supernatant</th>
<th>Fungal growth (CPM 14C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA-P Day 2</td>
<td>183 ± 42</td>
</tr>
<tr>
<td>Trichophytin Day 6</td>
<td>275 ± 11</td>
</tr>
<tr>
<td>Fresh media</td>
<td>158 ± 12</td>
</tr>
</tbody>
</table>

"All supernatants were adjusted to contain 1.63 mg/ml of glucose. 200 ml of the indicated supernatant or media supplemented with 10% human serum was placed into wells of a microtiter plate, supplemented with 0.1 μCi of 14C(μ) glucose, iron as indicated and inoculated with 1 × 10⁴ spores of T. mentagrophytes."

"Each value is the mean of 4 replicate cultures plus or minus the standard deviation.

These data indicate that lymphokine capable of inhibiting the growth of mouse L-929 fibroblasts does not inhibit the growth of the filamentous fungus T. mentagrophytes.
Announcement: Program Research Interests in Immune Mechanisms and Cutaneous Disorders (immunodermatology), National Institute of Allergy and Infectious Diseases

The National Institute of Allergy and Infectious Diseases is interested in expanding research activities of the Immunology, Allergic, and Immunologic Diseases Program concerned with immune mechanisms and hypersensitivity reactions in diseases of the skin. The purpose of this announcement is to encourage the interaction of researchers in allergy, dermatology, and immunology in order to advance progress in the prevention, diagnosis, and treatment of immune-mediated skin diseases. Some areas encompassed by the scope of this program include investigations designed to study allergic phenomena and immune mechanisms in the following conditions:

1. studies to differentiate allergic skin disorders arising as a result of IgE related mechanisms; cell-mediated immunity/delayed hypersensitivity, and inflammation emerging from activation of the complement cascade and the effects of chemical mediators;
2. atopic dermatitis: the definition of possible interacting etiologies that influence the development and course of allergic eczema as a multifactorial disorder;
3. urticaria and angioedema: investigations to detect and define the multiple allergic, neurogenic, chemical, and microcirculatory factors that result in heterogeneous disorders with identical presentation;
4. contact hypersensitivity: evaluation of the nature of normal skin cell components converted to antigenic determinants as a result of interaction with sensitizing agents;
5. infection: immune responses to both pathogenic and saprophytic flora serving as microbial antigens in immune and hypersensitivity reactions.

For further information investigators are encouraged to contact:

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Telephone: (301) 496-7104

In order to alert the Skin Diseases Program of the NIAMDD to the submission of proposals with primary thrust directed to dermatology, you may wish to communicate with:

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Director, Skin Diseases Program
Extramural Programs
National Institute of Arthritis, Metabolism, and Digestive Diseases
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REFERENCES

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