Factors Affecting the Antigenicity of Trichophyton rubrum

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Nitrogen determinations, performed upon the mycelia of Trichophyton rubrum, indicated that both the total nitrogen to mycelial weight ratio and the protein nitrogen to mycelial weight ratio decreased as the age of the mycelia increased. An increase in nitrogen concentration in the medium produced an increase in the total nitrogen to mycelia weight ratio, but did not necessarily increase the protein nitrogen to mycelia weight ratio. The optimal nitrogen source concentration which produced the highest protein nitrogen to mycelium ratio was found to be considerably less than that recommended in most standard Sabouraud medium formulations. Antisera to antigen preparations, grown on low concentrations of Multipeptone, produced more lines in the gel diffusion reaction than did antisera to antigens grown on standard concentrations of Multipeptone. Antisera to antigenic preparations from 2-week-old mycelia exhibited better and sometimes more lines than those of antigens prepared from 1- or 3-week-old mycelia, regardless of the nitrogen concentration in the medium. Dialysis and storage of the antigen produced no change in the quality of the precipitin lines, even though both processes involved considerable loss of Lowry protein. Immunofluorescence studies showed that young mycelia were more antigenic than the old mycelia, since a substantial degree of cell wall fluorescence was exhibited by the young mycelia, especially at the hyphal tips. Older mycelia lacked this fluorescence. An extracellular antigen was also found to be associated with the young mycelia, but cytoplasmic fluorescence was not observed.

Production of sensitive, specific antigens from filamentous fungi for immunization purposes and in vitro studies has been difficult. Much of the work has led to poor serological responses to such fungal antigens. Few reports have been concerned with the study of the basic biological properties of the fungi that may account for antigenicity (1, 2, 6, 7). In such a study on three species of the phycomycete genus Phytophthora (2), it was learned that the age of the mycelium, the nitrogen concentration of the medium, and the amount of inoculum into the growth medium all had an effect on the efficiency of the antigenic preparations. Results from these studies together with immunofluorescence experiments showed that the antigen was located in the young growing tips of the hyphae, and it was this portion that had to be used for the production of sensitive antisera. It was also shown that these antigens were extremely labile to ordinary storage procedures, thus adding to the difficulty.

The purpose of this paper is to determine whether the factors which effect the antigenicity of the genus Phytophthora can be applied equally as well to fungi of medical importance, as represented by the dermatophyte Trichophyton rubrum.

MATERIALS AND METHODS

Antigens. A stock culture of T. rubrum CDC 25 (typicus) was grown in a medium containing Multipeptone (Fisher Scientific Co., Fair Lawn, N.J.) and dextrose. Four variations of this medium were made using Multipeptone at 10.0, 7.5, 5.0, and 2.5 g/liter concentrations. For ease of expression, these Multipeptone concentrations will be referred to as 100, 75, 50, and 25% respectively, based on the assumption that 10.0 g/liter is equivalent to 100% Multipeptone. The dextrose concentration was 40.0 g/liter in each instance. The media were dispensed in 25-ml quantities into 125-ml Erlenmeyer flasks and were autoclaved. Each was inoculated with 1 ml of a starter culture, which was prepared by mincing six mats of 2-week-old fungal mycelia in a sterile blender with 100 ml of sterile saline.

Stationary cultures, 1, 2, and 3 weeks old, grown in different concentrations of Multipeptone at 25°C, were harvested on filter paper, washed with distilled water, and dried at 37°C for 24 hr. Dried fungal mats, obtained from separate flasks, were weighed and averaged. A mat of fresh mycelia equal to the dry
weight equivalent was suspended in phosphate-buffered saline (PBS), pH 7.2, to effect a final concentration of 10.0 mg/ml, was homogenized in a Waring Blender for 5 min, and was disrupted in a Branson model LS75 Sonifier (9 amp/5 min).

Immunization of animals. A 1.0-ml sample of each antigen was mixed with an equal amount of sodium alginate-calcium gluconate adjuvant (Colab Laboratories, Inc., Chicago Heights, Ill.). Of this mixture, 2 ml was injected subcutaneously into each of 2- to 3-kg albino rabbits (two rabbits per antigen) weekly for 6 weeks. The sera were harvested 2 weeks after the last injection.

Nitrogen determinations. Total nitrogen determinations were performed on triplicate samples of wet mycelia, which had been dried for 72 hr at 37 °C, by conventional micro-Kjeldahl methods (11). The results were recorded as a ratio in milligrams of total nitrogen per gram of mycelia.

Protein nitrogen determinations were made on soluble extracts from these dried mycelia. The extracts were prepared by homogenizing PBS suspensions of dried mycelia in a Waring Blender and a Branson Sonifier. After centrifuging the homogenates at 20,000 × g for 10 min, 10 volumes of 10% trichloroacetic acid were slowly added at 4 °C to each supernatant fluid in an attempt to precipitate the protein. After 15 min, the precipitates were recovered by centrifugation. Micro-Kjeldahl determinations were then performed, and the results were recorded as before.

When increased sensitivity was needed, as when low nitrogen values were expected, the Lowry procedure (10) was used for protein determinations.

Serological studies. Immunofluorescent procedures used were essentially those of Cherry et al. (3). Slide cultures were prepared by use of 6 mm² agar squares. After 4 days, the agar square was removed. Mycelia which had adhered to the slide were fixed in a mixture of 95% ethyl alcohol-ethyl ether (1:1) for 10 min at 25 °C, followed by 20 min of fixation in 95% ethyl alcohol at 37 °C. Specific antisera were then placed on the fixed mycelia and allowed to react for 30 min. The indirect method of staining was employed with sheep anti-rabbit globulin conjugated with fluorescein isothiocyanate (FITC; Nutritional Biochemicals Corp., Cleveland, Ohio). A stock solution of fluorescein orange (Nutritional Biochemicals Corp., Cleveland, Ohio) was prepared, and the mycelia were treated with a 1:8 dilution of the stock solution for 30 sec, rinsed with PBS (pH 7.6), and immersed in PBS for 1 min (9). The slide was mounted in FA Mounting Fluid (Difco) and was viewed on a Leitz Ortholux ultraviolet microscope. The field was illuminated with HBO 200 light source and was observed through a 3 mm BG-12 filter and the Leitz EEUQM barrier filter.

Immunodiffusion analyses were performed according to the methods of Crowle (3), with homogenized mycelial suspensions as antigen.

RESULTS

Two factors were studied with respect to their effects on antigenicity: the age of the mycelium and the nitrogen concentration of the medium. With the assumption that the major antigens were protein in nature, an estimation of antigenicity was first compared with the ratio of total nitrogen to mycelia. Mycelial mats obtained from the various media were removed, washed three times with distilled water in a Büchner funnel, and dried for 72 hr at 37 °C. Samples of 100 mg of dried mycelia were subjected to micro-Kjeldahl analysis. Average results from a series of three determinations are presented in Table 1. The total nitrogen to mycelial weight ratio decreased as the mycelia progressed in age, but increased as the concentration of Multipeptone was increased.

To correlate total nitrogen with protein nitrogen values, micro-Kjeldahl analyses were performed upon mycelial proteins. The averages of three micro-Kjeldahl determinations for each specimen, obtained from the precipitated proteins, are found in Table 2. An increase in age was found to produce a decrease in the protein nitrogen to mycelial weight ratio, the greatest ratio being obtained with a Multipeptone concentration of 5.0 g/liter.

Since it had been stated in previous work (2)...

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**Table 1. Effect of age of mycelium and nitrogen concentration of medium on total nitrogen in mycelia of Trichophyton rubrum**

<table>
<thead>
<tr>
<th>Conc. of Multipeptone in medium (g/liter)</th>
<th>Age of mycelia (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2.5</td>
<td>67.6a</td>
</tr>
<tr>
<td>5.0</td>
<td>70.3</td>
</tr>
<tr>
<td>7.5</td>
<td>72.3</td>
</tr>
<tr>
<td>10.0</td>
<td>74.4</td>
</tr>
</tbody>
</table>

*Results expressed as total nitrogen in milligrams per gram of mycelia (averages of triplicate samples). An asparagine standard showed an efficiency of 98%.

**Table 2. Effect of age of mycelium and nitrogen concentration of medium on protein nitrogen in mycelia of Trichophyton rubrum**

<table>
<thead>
<tr>
<th>Conc. of Multipeptone in medium (g/liter)</th>
<th>Age of mycelia (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2.5</td>
<td>9.72a</td>
</tr>
<tr>
<td>5.0</td>
<td>17.24</td>
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<tr>
<td>7.5</td>
<td>16.37</td>
</tr>
<tr>
<td>10.0</td>
<td>16.45</td>
</tr>
</tbody>
</table>

*Results expressed as protein nitrogen in milligrams per gram of mycelia (averages of triplicate samples).*
that antigenic instability presented problems in working with fungal mycelia, the stability of the antigen was investigated. Lowry protein determinations (10), performed on the soluble extracts at various periods of time, were used as indicators of antigenic stability. The extract was centrifuged at 20,000 × g for 10 min prior to the Lowry protein determination to rid it of accumulated particulate material. A fraction of this antigen was dialyzed against PBS for 72 hr at 4 C, and was also tested for loss of soluble protein in this manner. The results of the Lowry determinations (Fig. 1) show a decrease of soluble protein with an increase of time. It should be noted that dialysis for 72 hr at 4 C greatly decreased the amount of Lowry protein.

A series of five experiments was performed by use of gel diffusion techniques. The first experiment was performed to determine the effect of mycelial age and medium nitrogen concentration on the immune response. Sera, obtained from rabbits immunized with 1-, 2-, and 3-week-old mycelial preparations, each from 25, 50, 75, and 100%; Multipeptone media, were reacted with one antigen (2-week-old mycelia from 50% Multipeptone). Although two variables were compared simultaneously, rabbits immunized with mycelia from 25% Multipeptone media gave the sharpest and most abundant lines compared to when Multipeptone was used alone; however, regardless of Multipeptone concentration, sera from rabbits immunized with 2-week-old mycelia gave the best reactions. No serum reacted with medium alone.

Sera, known to give precipitin reactions, were reacted with three lots of 2-week-old 50% Multipeptone grown antigen. Even though the protein concentrations of the antigens ranged from 2,200 to 4,200 μg of protein per ml, the lines produced by the three lots appeared to be the same. There did not appear to be any correlation between quality of lines and concentration of protein, at least not in this range. In addition, antigen preparations stored at 4 C for as long as 2 weeks did not lose their immunodiffusion reactivity, although there was a decrease in soluble protein. Finally, dialysis of the antigen for 72 hr at 4 C against PBS had no effect on the quality of the immunodiffusion lines, although this treatment drastically lowered the Lowry protein nitrogen (from 4,200 to 900 μg of protein per ml).

The indirect fluorescent antibody technique was utilized to determine the location of the mycelial antigens. Experiments were performed to determine the best method of fixation. Mycelia were exposed to three fixation techniques: fixation with 95% ethyl alcohol and ethyl ether (1:1) for 10 min at room temperature, followed by 95% ethyl alcohol for 20 min at 37 C, conventional heat fixation, and fixation by air drying. Heat fixation and fixation by air drying were found to be inferior means of fixation. The mycelia treated by these means often failed to stain with the FITC, because of the failure of the antiserum and FITC to "wet" the mycelia, as revealed by the appearance of highly refractile hydrophobic areas surrounding each hypha. The alcohol-ether fixation removed this hydrophobic area, allowing the stain to "wet" and thus react with the mycelia. The fluorescence of the mycelia was found to be confined to the cell walls of the hyphae with the most intense fluorescence appearing at the hyphal tips. In addition, an antigen, distinct from the cell wall fluorescence, also was detected exuding centrifugally from the hyphae. Both cell wall and extracellular antigens were present primarily in the young hyphae, and seemed to disappear as the mycelia aged.

Slide cultures 4 to 8 and 12 days old were subjected to the FA technique to determine whether or not the amount of fluorescence was influenced by the age of the culture. Little difference in fluorescence was detected from day to day. How-

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\text{Fresh antigen (4,200 μg of protein per ml)} \quad \text{Dialyzed 72 hr at 4 C (900 μg of protein per ml)}
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FIG. 1. Decrease in soluble protein as exhibited by Lowry protein tests.
DISCUSSION

Although the data obtained from these studies tend to confirm the findings of Burrell et al. (2) in their work with Phytophthora, certain exceptions were noted. The nitrogen data obtained from the Trichophyton cultures were similar to those of the Phytophthora (4) in that there was a definite decrease in the ratio of protein nitrogen to mycelial weight as the fungus aged. However, although the total nitrogen content did not necessarily decrease with age (it depended on the concentrations of nitrogen source in the medium) in the Phytophthora cultures, there was always a decrease with Trichophyton. Regardless of the growth rate of each fungus, it was concluded that the decrease in ratio of protein nitrogen to mycelial weight with age was a reflection of the relative proportion of the young growing hyphal tips to older mycelia. The advantage of using a young culture in serological studies to obtain a maximal ratio of protein nitrogen to mycelial weight is self-evident.

An increase in nitrogen source (Multipeptone) was found to increase the total nitrogen to mycelial weight ratio, but not the protein nitrogen to mycelial weight ratio. Maximal protein ratios were observed with a Multipeptone concentration of 5.0 g/liter, which indicates that the optimal concentration of Multipeptone for protein production is not necessarily the highest concentration. In other words, the Multipeptone concentration in most standard Sabouraud media formulations might be considered too high for antigen production purposes.

With the assumption that protein is the chief antigenic component of fungal mycelia, this important finding should be kept in mind when immunizing animals with filamentous fungi. Of the amount of mycelia being injected into the animal, only a relatively small percentage of it is protein. Thus, the concentration of protein being injected would be a more important factor to be considered than the concentration of mycelia.

Results of the gel diffusion analyses indicated that some antigens elicited a different immune response than others, depending upon the age of mycelia and concentration of Multipeptone used to grow the mycelia. Antigen from mycelia grown on 25% Multipeptone for 2 weeks produced the most lines, even though this antigen contained the least total and protein nitrogen to mycelia weight ratios. The remaining antigens produced two or three lines in all instances. This seemed to indicate that growth of mycelia on a limited concentration of Multipeptone results in the production of a greater number of antigens than growth in high Multipeptone concentrations.

Antigens prepared from 2-week-old mycelia gave better and sometimes more lines, as is the case with antigens obtained from mycelia grown on 25 and 50% Multipeptone concentrations. It is possible that, although a greater ratio of total and protein nitrogen to weight of mycelia existed during the 1st week, some antigens (25 and 50% Multipeptone grown antigens) need time to develop before they can be detected by the Ouchterlony method. These antigens were developed enough 2 weeks after inoculation, but could not be detected in the 3rd week, possibly owing to their being incorporated into other compounds which constitute the hyphal walls, a possibility originally advanced by Fultz and Sussman (6). This may account for the disappearance of these lines during the 3rd week.

The production of lines of equal quality in the Ouchterlony gel diffusion test by antigens of either high or low protein concentration with antisera of known precipitin ability was an interesting point. Even though dialyzing the antigen greatly decreased the Lowry protein values, the lines produced were of quality equal to those of the fresh antigen. Since the Lowry method measures tyrosine radicals which are assumed to be integrated into protein and peptide structures, the loss of Lowry-demonstrable material could be attributable to the dialysis of free tyrosine or of low molecular weight peptides. Antigen, which was stored at 4°C for 2 weeks, also exhibited a decrease in soluble protein, but produced lines of quality equal to those of fresh antigen. Hence, present methods of antigenic preparation seemed to result in the incorporation of much nonspecific material, which might have interfered with optimal antibody production.

This project was originally based on the assumption that the major antigens were protein. The possibility that the major antigens were not protein, but rather polysaccharides, must be considered. Even though there was a drastic reduction in the nitrogen content of the antigens after dialysis or after centrifugation of the precipitate formed after storage, there was no decrease in serological activity. Although the proteins were unstable, it is possible that the gel diffusion reactions were due to polysaccharide antigens. This
does not detract from the observations that the best serological responses were obtained with mycelia grown on 25% Multipeptone and from 2-week-old cultures. Polysaccharide antigens for various species of dermatophytes have been reported by Grappel et al. (8).

The value of using young mycelial preparations in the fluorescent antibody technique can be confirmed by the data obtained. The young mycelia were observed fluorescing intensely in the cell wall regions, with the strongest fluorescence being observed at the hyphal tips. This indicated the high degree of antigenicity exhibited by young mycelia. An extracellular fluorescence similar to that reported by Al-Doory and Gordon (1) was also observed exuding from the hyphae of young mycelia. Unlike Phytophthora, no cytoplasmic fluorescence was noted. A possible explanation for the lack of fluorescence by the old mycelia might be that antigenic sites that are present in young mycelia are hidden in the old mycelia owing to their incorporation into other cell wall components. Fultz and Sussman (6), studying antigenic differences in the surfaces of hyphae and rhizoids in Allomyces by these techniques, have reported that there are antigenic changes in the surface of these structures with age. They proposed that a continuous structure seems to synthesie different superficial compounds. This would seem to be the case with Trichophyton, since in a single filament, one can observe intense fluorescence at the tip which gradually decreases with distance from this tip, and, hence, with age. There is also a possibility that the antigenic material has "leaked out" of the mycelia, since many observations have been made on what appeared to be antigenic material exuding from hyphae. This extracellular appearance of antigen also decreases with age.

**Literature Cited**