Rapid Visualization of Three Common Fungi Using Fluorescein-Conjugated Lectins

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The feasibility of using fluorescein-conjugated lectins to visualize and differentiate three fungi commonly involved in ophthalmic mycoses was evaluated. Using a panel of fluorescein-conjugated lectins, Candida albicans, Aspergillus fumigatus, and Fusarium solani were rapidly and reproducibly visualized in in vitro culture isolates, as well as in tissue samples and fixed histopathologic specimens taken from experimental mycoses. Additionally, Aspergillus and Fusarium were consistently differentiated from Candida. The binding affinities of the different lectins corresponded well with the individual sugar composition of the fungal cell walls. Invest Ophthalmol Vis Sci 27:500-506, 1986

The successful treatment of ophthalmic mycoses is predicated upon early diagnosis and institution of appropriate therapy.1 Diagnosis is frequently delayed, however, because the course of fungal keratitis or endophthalmitis is indolent, and the clinical signs may mimic those of other infectious agents. Furthermore, cultures of fungal specimens require a minimum of 48-72 hr, adding to the delay in diagnosis. Forster,2 therefore, has emphasized the importance of rapidly obtaining tissue scrapings or aspirates. The staining procedures used for the identification of fungal organisms from these scrapings or aspirates include: KOH wet mounts, Gram stain, Giemsa stain, periodic acid-Schiff stain, alcian blue, mucicarmine, and Grocott’s methenamine silver stain.3-9 The identification rate of fungal organisms using these stains, however, is frequently unsatisfactory; one series2 of culture-positive fungal keratitis cases yielded a 33% identification rate using KOH wet mounts, a 55% identification rate using Gram staining, a 66% identification rate using Giemsa staining, and an 85% identification rate using Grocott’s methenamine silver. In addition to variable identification rates, none of these techniques is able to differentiate species of fungi. Because fungal cell walls vary from species to species in their carbohydrate composition, we investigated the feasibility of using fluorescein-conjugated lectins in an attempt to rapidly visualize and differentiate three fungi that are commonly involved in ophthalmic mycoses.3,10-13

Materials and Methods

This study was divided into three phases. In the first phase, the binding of fluorescein-conjugated lectins to samples taken from laboratory fungal stock cultures was examined. In the second phase, using inocula from the same stock cultures, fungal keratitis and endophthalmitis was produced in rabbits; fluorescein-conjugated lectin binding to corneal scrapings and vitreous aspirates from the infected animals was then evaluated. In the final phase, the binding of fluorescein-conjugated lectins to fixed histopathologic sections of fungal keratitis was investigated.

Candida albicans, Aspergillus fumigatus, and Fusarium solani were used in this study. These organisms were obtained from human isolates and were maintained on Sabouraud’s dextrose agar at the Microbiology Laboratories of the Los Angeles County/U.S.C. Medical Center and Charity Hospital of New Orleans. The fluorescein-conjugated lectins used in this study were purchased from Vector Laboratories (Burlingame, CA); they were diluted with a solution of phosphate-buffered saline (PBS) and bovine serum albumin (BSA) to a final concentration of 0.03 mg/ml for all phases of this study.

Phase I (Lectin Binding to In Vitro Fungal Samples)

Isolates from stock laboratory cultures of Candida albicans, Aspergillus fumigatus, and Fusarium solani were used for this phase. The organisms were maintained on Sabouraud’s dextrose agar at room temperature. Using sterilized metal loops, samples from visible fungal colonies were dispersed into 5 ml of sterile saline.
One drop of the fungal suspension was then placed on glass microscope slides. These slides had been previously cleaned with 95% ethanol. The fungal samples were air-dried and then fixed by immersion in 95% ethanol for 5 min.

A panel of 14 commercially available lectins was used for this phase of the study. These included: Concanavalin A (Con A), wheat germ agglutinin (WGA), peanut agglutinin (PNA), Ricinus communis agglutinin, soybean agglutinin (SBA), Dolichos bifloris agglutinin (DBA), Ulex europus agglutinin (UEA), Lens culinaris agglutinin (LCA), Pisum sativum agglutinin (PSA), Bandiera simplicifolia agglutinin 1 (BSL), succinylated wheat germ agglutinin (SWGA), Sophora japonica agglutinin (SJA), Phaseolus vulgaris leucoagglutinin (PHA-L), and Phaseolus vulgaris erythroagglutinin (PHA-E).

The technique utilized for lectin binding was modified from that of Hatten and co-workers.14 After drying and fixing the slides, they were incubated for 15 min in 10 mM PBS at a pH of 7.2; this solution also contained 1 mM CaCl₂, 1 mM MgCl₂, and 1 mg/ml BSA. After incubation in the PBS-BSA mixture, the slides were immersed for 10 min in PBS without BSA. After draining them of excess buffer and placing two drops of the fluorescein-conjugated lectins onto the samples, the slides were incubated in humidified chambers for 30 min in the dark at room temperature. The slides were rinsed in PBS and glass coverslips were mounted with an aqueous, non-fluorescing mounting medium (Immuno-Mount, Shandon, Inc.; Sewickley, PA). The slides were examined using a Zeiss epifluorescence microscope (Carl Zeiss, Inc.; Oberkochen, West Germany); photographs were made with Kodak (Rochester, NY) Ektachrome 400 film (exposure index 800).

Phase II (Lectin Binding to Fungi in Experimental Mycoses)

Fungal suspension were created by diluting inocula from the stock cultures of Candida albicans, Aspergillus fumigatus, and Fusarium solani in sterile distilled water. Suspension concentrations, as determined by a hemocytometer, were 50,000 spores/ml for both Aspergillus and Fusarium; for Candida, the concentration of blastospores was 8 x 10⁸/ml.

Animals were housed and cared for in accordance with the ARVO Resolution on the Use of Animals in Research. Four adult (2–3 kg) New Zealand white (NZW) rabbits were used. Both 48 hr prior to, and on the day of inoculation, methylprednisolone 20 mg was injected subconjunctivally in the right eye of each animal. Immediately prior to inoculation, each animal was anesthetized with intramuscular ketamine 0.6 ml and xylazine 0.1 ml. In order to facilitate the establishment of corneal infections (Kaufman HE: personal communication), cryotherapy was applied to the central cornea of each animal's right eye until a visible ice ball was formed. Under the operating microscope, the right cornea of each animal was inoculated with intrastromal injections of 0.15 ml of the fungal suspension; for this, a 27-gauge needle attached to a tuberculin syringe was used. This technique of intrastromal injection has been previously shown to induce experimental fungal keratitis.15-17 Two rabbits were inoculated with Candida albicans; one was inoculated with Fusarium solani. Daily biomicroscopic examinations of the animals were performed. The right eye of each animal was treated every other day with subconjunctival injections of gentamicin 8 mg and methylprednisolone 20 mg; daily topical applications of both prednisolone phosphate 1% and neomycin–bacitracin ophthalmic solutions were also given.

When corneal infiltrations were clinically evident, the animals were anesthetized as before and scrapings were taken of the infected tissue using a #64 Beaver blade. The scrapings were placed onto previously cleaned glass slides, air dried, and fixed in 95% ethanol for 5 min. Five of the fluorescein-conjugated lectins (Con A, WGA, UEA, LCA, and SWGA) were used to stain these specimens. These lectins were specifically chosen because they had consistently produced positive staining in the in vitro phase (Phase I). The lectin staining procedure was identical to that described for the in vitro stock fungal suspensions (Phase I), except that the fluorescein-conjugated lectins were diluted to 0.008 mg/ml. This concentration, we had previously determined, resulted in minimal background staining of the corneal stroma and vitreous, yet still permitted visualization of the fungi. The presence of infectious mycotic agents was confirmed by staining smears with periodic acid–Schiff stain and also by culturing the corneal scrapings on Sabouraud's dextrose agar.

Using the same fungal suspensions, exogenous mycotic endophthalmitides were established in four additional adult female NZW rabbits. Forty-eight hours prior to inoculation, as well as on the day of inoculation, each of these rabbits received a subconjunctival injection of methylprednisolone 20 mg in the right eye. Immediately prior to inoculation, each animal was anesthetized with intramuscular ketamine 0.6 ml and xylazine 0.1 ml. Inoculations of the fungal suspensions were performed in the right eye. Under the operating microscope, an 18-gauge needle was placed through the pars plana and into the central vitreous cavity. After aspiration of 0.3 ml of liquid vitreous, an equal volume of the fungal suspension was injected. Two of the rabbits were inoculated with Candida albicans, one was inoculated with Aspergillus fumigatus, and one was inoculated with Fusarium solani. Daily indirect
Table 1. Lectin binding to in vitro fungi (Phase I)

<table>
<thead>
<tr>
<th>Fungus</th>
<th>ConA</th>
<th>WGA</th>
<th>PNA</th>
<th>DBA</th>
<th>SBA</th>
<th>PSA</th>
<th>LCA</th>
<th>PHAE</th>
<th>PHAL</th>
<th>SWGA</th>
<th>UEA</th>
<th>BSL</th>
<th>SJA</th>
<th>RCA</th>
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<tr>
<td>Candida albicans</td>
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<td></td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Fusarium solani</td>
<td>+</td>
<td>+</td>
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* + indicates moderate to bright staining of fungus with fluorescein-conjugated lectin.
† - indicates no more than background staining of fungus with fluorescein-conjugated lectin.
‡ ± indicates variable staining of fungus with fluorescein-conjugated lectin.

ophthalmoscopic examinations were performed. Each animal’s right eye was treated every other day with subconjunctival gentamicin 8 mg and methylprednisolone 20 mg, as well as daily topical applications of prednisolone phosphate 1% and neomycin–bacitracin ophthalmic solutions.

When ophthalmoscopic examination revealed the presence of vitreous clouding, the animals were anesthetized as before and pars plana vitreous aspirations were performed using an 18-gauge needle. The vitreous aspirates were placed on previously cleaned glass slides, air dried, and fixed in 95% ethanol for 5 min. Fluorescein-conjugated Con A, WGA, UEA, LCA, and SWGA were again used. The lectin binding procedure was identical to that described for the in vitro phase (Phase I). The presence of infectious mycotic agents was confirmed by culturing samples of the vitreous aspirates on Sabouraud’s dextrose agar, as well as staining smears with periodic acid–Schiff stain.

Phase III (Lectin Binding to Fixed Histopathologic Specimens)

After the appropriate tissue samples were removed from the animals with experimental mycotic keratitis, they were euthanized with intracardiac injections of pentobarbital. The corneas were removed, fixed in 10% buffered formalin, embedded in paraffin, sectioned and placed onto glass slides.

Tissue sections were deparaffinized by first immersing them in xylene for 10 min. This was followed by rapid rehydration in a series of graded alcohols (absolute, 95%, 70%, 50%, 30%). The slides were rinsed for several minutes in distilled water and immersed in PBS–BSA solution for 15 min. The remainder of the lectin staining procedure was identical to that described for the in vitro stock fungal suspensions.

Binding Inhibition Studies

In order to determine the specificity of lectin staining, various sugars were employed to block the lectin binding to cell walls of the fungi utilized in the above experiments. These sugars (Sigma; St. Louis, MO) included 0.5 M methyl-alpha-D-mannopyranoside, N-acetyl-D-glucosamine, 1-O-methyl-beta-D-galactopyranoside, and N-acetyl-D-galactosamine.

Results

Phase I (Lectin Binding to In Vitro Fungal Samples)

Incubation of diluted samples from stock cultures of Candida albicans, Aspergillus fumigatus, and Fusarium solani with 14 fluorescein-conjugated lectins resulted in a definitive and reproducible binding pattern (Table 1). Con A brightly stained all three fungi (Fig. 1). Similarly, SWGA stained all three fungi. Two of the lectins, WGA and UEA, stained Aspergillus and Fusarium (Fig. 2), but did not stain Candida. Conversely, one other lectin, LCA, stained Candida, but did not stain Aspergillus or Fusarium. The remaining seven lectins did not consistently stain any of the fungi.
Phase II (Lectin Binding to Fungi in Experimental Mycoses)

All four animals injected intrastromally developed corneal infections by the eighth day after inoculation. Positive cultures from the corneal scrapings confirmed the presence of mycotic infections. Of the four animals inoculated through the pars plana, only three developed endophthalmitis; the animal receiving *Fusarium solani* did not develop an infection. All three cases of endophthalmitis developed within 14 days after inoculation. Cultures of the vitreous aspirates in these animals did grow out the appropriate fungus.

A reproducible pattern of lectin staining was noted for the fungi obtained from the corneal scrapings and vitreous aspirates (Table 2). Con A brightly stained all three fungi (Figs. 3–6). Although there was some background staining, the fungi were easily discerned. WGA and UEA stained Aspergillus (Fig. 7) and Fusarium, but did not stain Candida. LCA moderately stained Candida, but did not stain either Aspergillus or Fusarium. SWGA brightly stained all fungi.

Phase III (Lectin Binding to Fixed Histopathologic Specimens)

A reproducible pattern of fluorescein-conjugated lectin binding to fixed histopathologic specimens of Candida and Aspergillus keratitis was noted (Table 2).

### Table 2. Summary of lectin binding to fungi in experimental mycoses (Phases II and III)

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Con A</th>
<th>WGA</th>
<th>UEA</th>
<th>LCA</th>
<th>SWGA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em></td>
<td>+*</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Fusarium solani</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* + indicates moderate to bright staining of fungus with fluorescein-conjugated lectin.
† - indicates degree of fungal staining with fluorescein-conjugated lectin less than or equal to background staining.

Con A and SWGA stained both Aspergillus and Candida (Fig. 8), while WGA stained Aspergillus but not Candida. Conversely, LCA moderately stained Candida and did not stain Aspergillus.

### Binding Inhibition Studies

Blocking experiments with the various sugars revealed that the Con A-positive staining of Candida, Aspergillus and Fusarium was blocked by methyl-beta-D-manno.pyranoside and that the WGA staining of Aspergillus and Fusarium was blocked by N-acetyl-D-glucosamine.

### Discussion

Lectins are ubiquitous proteins that have the unique property of binding specifically to carbohydrate groups,
especially oligosaccharides and polysaccharides. Al-
though originally thought to be present only in plant
seeds, lectins have been found in animals and even
microbes. Most lectins have more than one carbo-
hydrate-combining site; they were, therefore, used ini-
tially for agglutination studies. Lectins have also been
used in the stimulation of lymphocyte mitogenesis, in
the identification of malignantly transformed cells, and
in the biochemical characterization of cell surfaces.
In ophthalmology, investigators have used
lectins to identify migrating corneal epithelial cells,
to enhance liposome attachment to surface epithelial
cells, to assess adherence properties of bacteria to
corneal epithelial cells, to identify sugar residues in
macular corneal dystrophy, and to characterize retinal
photoreceptor cells biochemically.

Lectins have also played a role in microbiologic
studies, especially in the characterization of cell wall
glycoproteins. More recently, they have been used in
attempts to identify microbial agents in both in vitro
cultures and in vivo tissue samples. Fungi are partic-
ularly well-suited for lectin investigation, because the
majority of their cell wall macromolecules consist of
oligosaccharides. In addition, fungal cell walls have
other sugar residues capable of interacting with lectins,
such as chitin and glucans. These carbohydrate cell
wall components vary among fungal species, forming
the basis for taxonomic classification of fungi. Two
recent, detailed reviews have documented the many
efforts to identify individual fungi by agglutinating
them with various lectins.

Because the cell wall sugars differ among fungal spe-
cies, we wondered whether three fungi commonly in-
Fig. 4. Photomicrograph of corneal scraping from rabbit infected
with Fusarium solani. Scraping had been incubated with fluorescein-
conjugated Con A. Compared to background corneal stroma, the
hyphal cell walls are easily visible (arrow) (X400).

Fig. 5. Photomicrograph of vitreous aspirate from rabbit infected
intracoarally with Candida albicans. Aspirate had been incubated
with fluorescein-conjugated Con A. Note the walls of both the
yeast and its bud stain brightly (X630).

Fig. 6. Photomicrograph of vitreous aspirate from rabbit infected
intracoarally with Aspergillus fumigatus. Aspirate had been incubated
with fluorescein-conjugated Con A. Note the tangle of hyphae with
typical branching patterns (arrow). Again, the lectin staining is con-
centrated in the cell walls (X630).
involved in ophthalmic mycoses (Candida albicans, Aspergillus fumigatus and Fusarium solani) could be visualized and differentiated with lectins that had been conjugated with fluorescein. Using a panel of fluorescein-conjugated lectins, the patterns of staining that we noted corresponded well to the cell wall chemistries of these fungi. Fluorescein-conjugated Con A consistently stained all three fungi in all phases of the study. Con A, derived from jack bean seeds (Canavalia ensiformis), selectively binds to carbohydrate residues containing alpha-D-mannose (Man) or alpha-D-glucose (Glc) terminal groups.19,30,32 The results of our binding inhibition experiments confirm the observation of other investigators31-32 that the three fungi studied contain the common sugar alpha-D-mannose.

We were also able, in all three phases of this study, to differentiate the two filamentous fungi Aspergillus fumigatus and Fusarium solani from the yeast Candida albicans. Fluorescein-conjugated WGA and UEA stained Aspergillus and Fusarium, but not Candida, in all three phases of this study. WGA binds primarily to N-acetyl-D-glucosamine (GlcNAc) terminal groups and sialic acid residues; these are prominent components of both Aspergillus and Fusarium, but are not present in Candida.31 We were unable, however, to differentiate between the two filamentous fungi; this was not surprising, as their basic cell wall carbohydrate compositions are similar.31 In this study, we observed that fluorescein-conjugated LCA, unlike Con A, stained Candida albicans only to a moderate degree and did not stain the two filamentous fungi at all. This is in spite of the fact that, similar to Con A, LCA binds to alpha-D-mannose and alpha-D-glucose. One possible explanation may be that the binding affinity of LCA for these sugars is markedly less than that of Con A.33 We are presently investigating this by repeating the experiments with markedly greater concentrations of LCA. Another possibility may be that LCA is known to bind to alpha-D-mannose and alpha-D-glucose only if fucose residues are present in the core chitobiose region of the complex N-linked oligosaccharide.34

In conclusion, this study demonstrated that the use of fluorescein-conjugated lectins permits rapid visualization of three common fungi in pure culture isolates, tissue samples and fixed histopathologic specimens. Also, using a panel of lectins, we noted that the two filamentous fungi can be differentiated from the yeast Candida. Clinically, this may eventually prove useful in identifying the pseudohyphae of Candida which may be morphologically similar to the hyphae of filamentous fungi. We believe, therefore, that fluorescein-conjugated lectin staining may eventually have an adjunctive place in the diagnostic evaluation for suspected ophthalmic mycoses. Further studies are planned, including comparing the sensitivity of this technique to established fungal stains, expanding the number of fungi and lectins tested, and applying this technique to human ophthalmic mycoses.
Key words: ophthalmic infections, mycoses, lectins, cornea, vitreous

References


