THE USE OF FLUORESCEIN-LABELLED LECTINS IN THE DETECTION AND IDENTIFICATION OF FUNGI PATHOGENIC FOR MAN: A PRELIMINARY STUDY

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PLATES XXIX-XXXIII

IN the deep-seated mycoses the existence of fungal infection is often unsuspected until yeasts or hyphae are seen in histological sections and at that stage there may be no unfixed material available for culture. Identification of the infecting organism then depends on the morphological and chemical characteristics of the fungus present in the tissue. However, problems of identification are frequently encountered when the genus involved lacks a distinctive form, or only a small amount of the fungus is present. Moreover, even if a fungus has been isolated there may be difficulty in deciding whether it is the same as that seen in the tissues, or is a contaminant. Uncertainty of this kind occurs especially with opportunistic infections, in which the infecting fungus is either common in the environment or endogenous in man. When fungi in tissue sections cannot be identified on morphological grounds, chemical criteria may be employed.

The lectins, which are proteins (mostly of plant origin) with specific carbohydrate-binding properties (Cook and Stoddart, 1973), afford a new approach to the problem of identifying fungi: by attaching fluorescent labels to the lectins, they can be used as specific histological reagents for several sugars commonly found in fungal walls. This paper describes the application of fluorescent lectins to the identification of six fungal species that can cause major systemic disease.

MATERIALS AND METHODS

Specimens of tissue containing fungi. Six different fungi were studied.

1 and 2. *Aspergillus fumigatus* and *Candida albicans*. Specimens were obtained *post mortem* from six organ-transplant patients who died with severe fungal infections. Three had disseminated aspergillosis and the other three had extensive candida lesions. The organs involved and examined by the FL method included lung, heart, kidney, thyroid and oesophagus. In each patient the infecting fungus was cultured from at least one affected organ and the cultural and histological assessments of the genus involved were consistent.

3. *Cryptococcus neoformans*. The only specimen available was a skin-biopsy sample from a renal transplant patient who had cryptococcal skin lesions; this patient later developed and recovered from cryptococcal meningitis. The organism was cultured from the skin-biopsy specimen and from the cerebrospinal fluid.

Received 2 Nov. 1977; accepted 17 Jan. 1978.

4. *Rhizopus oryzae*. A histological specimen of human rhinocerebral mucormycosis was given by Professor J. W. Rippon of the Pritzker School of Medicine, University of Chicago. The specimen consisted principally of adipose and connective tissue containing abundant hyphae.

5 and 6. *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis*. Blastomycosis specimens were obtained from laboratory animals infected with fungal strains originally isolated from human infections. The organisms were provided by the late Dr I. G. Murray, P.H.L.S., Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine, Keppel Street, London, and experimental infections were induced by Dr R. J. Olds of the Department of Pathology, University of Cambridge. Infection with *B. dermatitidis* was established in C57BL mice by intraperitoneal injection of a broth suspension prepared from a blood agar culture incubated at 37°C for 5 weeks. Infection with *P. brasiliensis* was established in guinea-pigs by intratesticular injection of a broth suspension prepared as described for *B. dermatitidis*. The mice and guinea-pigs were killed 3–4 weeks after infection.

Fixation and embedding. Most tissues were fixed in 10% formol-saline and after dehydration and clearing were embedded in paraffin wax; sections were cut at 5 µm. One part of the skin biopsy from the patient with cryptococcosis was fixed in "Susa" fixative, the results being identical with those obtained with the part fixed in formol-saline.

Sections of all the blocks of tissue were stained with haematoxylin and eosin and by the periodic acid-Schiff method and Gridley and Grocott methods (Drury and Wallington, 1967). They were also examined by the fluorescent lectin (FL) method.

FL reagents (table I). Wheat-germ Agglutinin was purchased as its fluorescein-labelled derivative from Miles Laboratories Ltd, PO Box 37, Stoke Poges, Slough SL2 4LY, England. Concanavalin A (crystallised three times) and Fucose-binding Protein were obtained unlabelled from Miles Laboratories. Agglutinin was isolated from seeds of *Ricinus communis zanzibariensis* by the method of Nicolson and Blaustein (1972) and the lectin of MW 120 000 daltons (specific for β -D-galactose only) was separated from the smaller lectin and the toxin. Soybean agglutinin was isolated from soybeans by the method of Lis and Sharon (1972).

All the lectins were given fluorescent labels by a modification of the method of Smith and Hollers (1970) in which 0.5M final concentrations of the appropriate sugars were added, to avoid excessive injury to the binding sites of the lectins. Because of solubility problems, the labelling of the ricinus agglutinin was approximately one-third of that of the other lectins.

Aprotinin was purchased from Bayer Pharmaceuticals, Haywards Heath, W. Sussex, England and was labelled by the method of Kiernan and Stoddart (1973).

FL-staining and examination of sections. After removal of wax by means of xylene, the sections were re-hydrated by passage through graded alcohols to water. Autofluorescence was quenched by exposure to osmic acid 0.5% (w/v) for 1 min. and the sections were then washed in running tap water for 4 h and finally in glass-distilled water. Drops of a solution of the appropriate fluorescent stain were placed on the sections and left for 1–2 min.; excess stain was then removed for re-use and the slides were washed in three changes of glass-distilled water. The stained preparations were dehydrated through graded alcohols to xylene and mounted in DePeX mountant.

Slide cultures of *A. fumigatus* and smears of *Cand. albicans* were also prepared, fixed with ethanol and examined by the FL method.

Sections were examined and photographed by means of a Zeiss fluorescence microscope with trans-illumination. Excitation was maximal near 400 nm and emission was observed between 500 and 530 nm.

RESULTS

Aspergillus fumigatus

The pattern of hyphal staining was identical in organisms in tissue sections and in those derived from cultures. Both the hyphae and the septa were

DEMONSTRATION OF FUNGI BY FLUORESCENT LECTINS

FIG. 1.—*Aspergillus fumigatus* invading a thrombus in a pulmonary blood vessel. The FL-SBA stain demonstrates N-acetylgalactosamine. $\times 450$.

FIG. 2.—*Candida albicans* in debris over an esophageal ulcer. Yeasts cells are present. The FL-ConA stain demonstrates mannosyl residues. $\times 2250$.

DEMONSTRATION OF FUNGI BY FLUORESCENT LECTINS

FIG. 3.—*Cryptococcus neoformans* in a biopsy of skin and subcutaneous tissue, stained with five different reagents. (a) FL-ConA reagent: most of the fungal material is in macrophages and multinucleate giant cells; it is virtually unstained but there is some fluorescence of the cytoplasm of the phagocytes. $\times 450$. (b) FL-RCA reagent: the result is similar to that produced by FL-ConA, but the staining of the cytoplasm of the macrophages and giant cells is rather more pronounced. $\times 450$. (c) FL-SBA reagent: the organism is virtually unstained. $\times 450$. Continued on plate XXXI.

DEMONSTRATION OF FUNGI BY FLUORESCENT LECTINS

FIG. 3.—Continued from plate XXX. (d) FL-A reagent: there is intense staining of yeast cells, many of which are in macrophages and giant cells; the large dark spaces are adipose tissue cells. $\times 450$. (e) FL-A reagent: intracellular yeast cells of varying maturity are intensely stained. $\times 2250$.

DEMONSTRATION OF FUNGI BY FLUORESCENT LECTINS

FIG. 4.—*Rhizopus oryzae* in adipose and connective tissue. Stained with FL-WGA. $\times 710$.

DEMONSTRATION OF FUNGI BY FLUORESCENT LECTINS

FIG. 5.—*Blastomyces dermatitidis* in the liver of a mouse. Stained with FL-A. $\times 450$.

FIG. 6.—*Paracoccidioides brasiliensis* in testis of guinea-pig. Stained with FL-A. $\times 450$.

TABLE I

The sources and chemical specificities of five lectins and of aprotinin

Lectin used to prepare fluorescent lectin reagent	Fluorescent lectin reagent	Specificity of reagent	Source of lectin
Concanavalin A	FL-ConA	α -D-Mannopyranose } terminal or α -D-Glucopyranose } 1,2 linked α -D-N-Acetylglucosamine terminal only	Jack bean (<i>Canavalia ensiformis</i>) seeds
<i>Ricinus communis</i> agglutinin	FL-RCA	β -D-Galactopyranose terminals	Castor bean (<i>Ricinus communis zanzibariensis</i>) seeds
Soybean agglutinin	FL-SBA	β -D-N-Acetylglactosamine terminal (β -D-Galactosyl terminals, weakly)	Soybean (<i>Glycine max</i>) seeds
Wheat germ agglutinin	FL-WGA	β -D-N-Acetylglucosamine terminals	Wheat (<i>Triticum vulgare</i>) germ-lipase fraction
Fucose-binding protein	FL-FBP	α -L-Fucopyranose terminals	Asparagus pea (<i>Lotus tetragonolobus</i>) seeds
Protein			
Aprotinin (not a true lectin)	FL-A	Sialic and uronic acids	Bovine lung

strongly stained with FL soybean agglutinin (FL-SBA) and this made the hyphae very clearly visible in sections of infiltrated tissues (fig. 1 and table II). Staining was virtually confined to the cell walls, the hyphal contents remaining almost unstained.

TABLE II

Staining of hyphae of three fungal species by fluorescent-lectin reagents

Fungal species	Fungal structure	Staining reaction with					
		FL-ConA (α -D-Mannose, α -D-glucose, α -D-N-acetyl glucosamine)*	FL-RCA (α -D-Galactose)	FL-SBA (β -D-N-Acetyl galactosamine, β -D-galactose [weak])	FL-WGA (N-Acetyl glucosamine)	FL-FBP (α -L-Fucose)	FL-A (Probably glucuronic acid)
<i>Aspergillus fumigatus</i>	Hyphae	++ to +	+	+++	+	+	+
	Septa	++ to +	+	+++	+	—	+
<i>Candida albicans</i>	Hyphae	+++	±	±	+	+	++
<i>Rhizopus oryzae</i>	Hyphae	±	++	+	+++	+	+
	Septa	±	++	+	+++	+	+

* The sugars stained are given in parenthesis.
The degrees of staining are indicated as follows: — = nil; ± = faint, erratic; + = weak; ++ = moderate; +++ = strong; ++++ = intense.

Candida albicans

The characteristic budding blastospores were strongly stained with FL concanavalin A (FL-ConA) so that they were easily seen in sections, even in the presence of collagenous tissue, in which glucosyl groups are attached to the collagen (fig. 2). Hyphae observed in sections showed a staining reaction similar to that of the blastospores. No differences were seen between the yeast forms in culture and in infected tissues (tables II and III).

Cryptococcus neoformans

Brilliant staining of the spherical, encapsulated yeast cells of this organism was seen with FL aprotinin (FL-A) but not with other lectins (fig. 3 and table III). Most of the staining with FL-A appeared to be confined to the fungal cell walls. In contrast the cytoplasm and nuclei of the enveloping macrophages were weakly fluorescent with FL-A but strongly stained with FL-ConA and FL *Ricinus communis* agglutinin (FL-RCA). No differences in the cell walls were seen at the sites of buds.

Rhizopus oryzae

This organism was stained strongly with FL wheat germ agglutinin (FL-WGA) (fig. IV), and with FL-RCA it was stained more strongly than was *A. fumigatus*. Its reaction with FL-SBA was much less than was that of *A. fumigatus*. The other reagents stained relatively weakly (table II).

Blastomyces dermatitidis

The walls of the yeast cells of this organism were stained much less intensely by FL-A than were those of *Crypt. neoformans* (fig. 5 and table III), but the

TABLE III
Staining of yeast cells of four fungal species by fluorescent-lectin reagents

Fungal species	Staining reaction with					
	FL-ConA	FL-RCA	FL-SBA	FL-WGA	FL-FBP	FL-A
<i>Candida albicans</i> (yeast form)	+++	±	±	+	+	++
<i>Cryptococcus neoformans</i>	—	±	—	±	—	++++
<i>Blastomyces dermatitidis</i>	++	++	+	++	++ to ±	++
<i>Paracoccidioides brasiliensis</i>	+ to ±	+	++	+	+ to —	+

The degrees of staining are as indicated in table II.

fluorescence with FL-SBA was considerable. FL-ConA and FL-RCA were quite strongly bound. The cellular contents of the yeasts were weakly fluorescent with FL-A and FL-ConA. Staining of the cell walls with FL-WGA was much more striking than in *P. brasiliensis* or *Crypt. neoformans*. Larger cells stained well with FL fucose-binding protein (FL-FBP). Some organisms showed a double layer to their walls and some showed single buds, but no obvious chemical properties were associated with them.

Paracoccidioides brasiliensis

This fungus differed in lectin staining pattern from *Crypt. neoformans* and *B. dermatitidis*, despite the close resemblance of the yeast-cells of the three organisms. FL-SBA was more strongly bound than in *B. dermatitidis* and FL-A and FL-WGA less so (fig. 6 and table III). However, there was little staining with FL-ConA, FL-RCA or FL-FBP. As with *B. dermatitidis*, some cells showed a double-layered wall but, unlike *B. dermatitidis*, *P. brasiliensis* sometimes showed multiple buds; no peculiarities in the staining of the walls were associated with these buds.

DISCUSSION

With the exception of concanavalin, all the lectins used in this study are capable of binding only to residues at the non-reducing terminals of saccharide chains and not to internal sugars. Concanavalin will interact, theoretically, with 1,2-linked, internal, α -D-mannosyl or α -D-glucosyl groups, as well as with terminal residues, but in most instances steric hindrance will prevent such interactions. Aprotinin may well be able to attach to a wide range of uronosyl groups. The patterns of staining with the reagents used in this work are not a reflection of the gross composition of the fungal walls, but depend in most instances upon the terminal sugars. At the same time they may not detect the minor antigenic differences that sometimes depend upon variations in a small number of terminal or near-terminal saccharides.

The patterns of staining found here are consistent with what is known of the chemistry of the walls of the fungal species studied. *Cand. albicans*, like many other yeasts, has a wall rich in mannose, and both terminal α -D-mannosyl and sub-terminal 2-substituted α -D-mannosyl groups occur (Suzuki and Sunayama, 1968 and 1969; Sunayama, 1970; Sunayama and Suzuki, 1970; Phaff, 1971; Ballou, 1974). The strong binding of FL-ConA observed was therefore to be expected. The slight staining with FL-WGA, FL-FBP and FL-A may have reflected the presence of small numbers of residues of N-acetylglucosamine, fucose and uronic acid. The first is widespread in fungi, and fucose and uronic acid are both known in other species of *Candida* (Gorin and Spencer, 1968).

In *Crypt. neoformans*, the mannan has no internal residues to which concanavalin can bind and its terminal groups are all blocked by xylose or glucuronic acid (Miyazaki, 1961*a*, *b* and *c*; Blandamer and Danishefsky, 1966; Phaff, 1971). The lack of staining with FL-ConA and the intense reaction with

FL-A are consistent with this structure. The serological type of the organism used here was not known. In types A and B a little galactose is present (Drouhet, Segretain and Aubert, 1950; Evans and Kessel, 1951; Evans and Mehl, 1951; Evans and Theriault, 1953; Rebers *et al.*, 1958; Blandamer and Danishefsky, 1966), but if it were not terminal it would not react with FL-RCA; this might explain the lack of staining with FL-RCA.

B. dermatitidis and *P. brasiliensis* are closely related species (Andrieu *et al.*, 1969) and resemble *Crypt. neoformans*; however, in this study their pattern of staining differed from that of *Crypt. neoformans*. In *Paracoccidioides* spp. glucans and chitin have been described (Kanetsuna *et al.*, 1969; Moreno, Kanetsuna and Carbonell, 1969; Carbonell, Kanetsuna and Gil, 1970; Kanetsuna and Carbonell, 1970) and San-Blas, San-Blas and Cova (1976) obtained a mutant in which a mannan replaced the glucan. The glucans and chitin of *B. dermatitidis* are similar to those of *P. brasiliensis* (Domer, 1971; Kanetsuna and Carbonell, 1971; Cox and Best, 1972). Both species contain galactomannans (Azuma *et al.*, 1974), but little is known of their detailed structure except that they possess an α -1,6-linked backbone. It is not clear how far the glucans contribute to the interaction of *B. dermatitidis* and *P. brasiliensis* with FL-ConA, but it is possible that their mannans are less heavily substituted than are those of *Crypt. neoformans* and that some branches or 1,2 links occur. The binding of FL-WGA was probably due to accessible chitin, while the other reagents probably stained components of the galactomannans of the fungal walls.

Several species of *Aspergillus* have been shown to contain galactomannans, in which both 1,2-linked internal-mannosyl groups and terminal galactosyl groups can occur (Sakaguchi, Yokota and Suzuki, 1969; Azuma *et al.*, 1971; Bardalaye, Nordin and Corson, 1977). In some instances, at least a large part of the galactose is in a furanose configuration; this would preclude its interaction with FL-RCA or FL-SBA. The very weak staining with FL-RCA and the strong staining with FL-SBA, would suggest that the latter was binding to terminal residues of N-acetylgalactosamine and not to galactose.

Little is known of the detailed chemistry of the walls of *Rhizopus* spp., though there is information on other members of the Mucorales. Among the neutral sugars that have been identified are L-fucose, D-galactose, D-glucose and D-mannose (Miyazaki and Irino, 1970 and 1972); *Mucor* spp. are reported to lack galactosamine and to differ from *Aspergillus* spp. in containing considerably more glucosamine (Wai, 1970). However, Miyazaki and Irino (1972) reported N-acetylgalactosamine from *Mucor mucedo*. Glucuronic acid is widespread in the Mucorales (Miyazaki and Irino, 1970 and 1971) and Bartnicki-Garcia and Lindberg (1972) have shown that the uronic acid forms a heteropolymer with mannose in *Mucor rouxii*. A similar polysaccharide in *Rhizopus nigricans* contains galactose and large amounts of fucose, as well as mannose and uronic acid. Thus the pattern of staining found here for *R. oryzae* is generally consistent with the chemical features of its class.

These interpretations are based upon the specificities of lectins for substrates of low molecular weight. Lectins might behave differently and more selectively

if used upon polysaccharides of high molecular weight, such as those in fungal walls.

The results suggest a practical application. In tissue sections fungal morphology is not always sufficiently characteristic to allow identification and methods capable of detecting differences in fungal composition may then be essential. Conventional histochemical staining techniques may occasionally help, and the value of the fluorescent antibody method is well established. The fungi that can be demonstrated by this method include *B. dermatitidis* (Kaplan and Kraft, 1969), *Cand. albicans* (Gordon, 1958; Berge and Kaplan, 1967), *Coccidioides immitis* (Kaplan and Clifford, 1964; Kaplan and Kraft, 1969), *Crypt. neoformans* (Kase and Marshall, 1960), *Histoplasma capsulatum* (Yamaguchi, Adriano and Braunstein, 1963), *P. brasiliensis* (Kaplan, 1972) and *Sporotrichum schenkii* (Kaplan and Kraft, 1969).

The fluorescent antibody technique and the method described in this paper have obvious close analogies. In both, the specific reactive molecules in the reagents are labelled with the same or a similar marker and are recognised by their fluorescence. However, in the context of fungal identification, the significant common factor between the two techniques is the nature of the groups in the fungal wall with which the reagents bind. The fluorescent lectins combine with well-defined terminals on exposed saccharide chains, and it is probable that most of the epitopes with which the fluorescent antibody molecules combine are also carbohydrate in nature. The results suggest that the fluorescent lectin technique will assist in identifying fungi in tissue sections to the level of genus. For example, *Aspergillus* and *Rhizopus* spp. in our specimens were readily distinguished by their different reactions with soybean and wheat germ agglutinins. The reproducibility of the method, its simplicity and its established chemical basis, together with the relative robustness of the reagents and their comparatively low cost are attractive features. However, the number of fungi examined in this initial study was small and a broader and more detailed investigation is being undertaken.

SUMMARY

Fluorescein-labelled lectins of known specificities for different sugars were used in an attempt to identify fungi in paraffin sections of surgical and post-mortem material. *Aspergillus fumigatus*, *Blastomyces dermatitidis*, *Candida albicans*, *Cryptococcus neoformans*, *Paracoccidioides brasiliensis* and *Rhizopus oryzae* have been studied with five fluorescein-labelled lectins and with fluorescein-labelled aprotinin. The fungi were readily distinguishable on the basis of differences in their reactions with these stains. The results accord well with what is known of the chemistry of the organisms and the method offers promise to practising histopathologists.

We thank Dr R. J. Olds, Department of Pathology, University of Cambridge, for the material from experimental blastomycoses; Professor J. W. Rippon, Pritzker School of Medicine, University of Chicago, for the specimen of mucormycosis; Mrs B. King and Mrs P. M. Stoddart, Strangeways Laboratory, Cambridge, and the technical staffs of the Clinical Laboratory, Addenbrookes Hospital, Cambridge, and Department of Pathology, University

of Cambridge, for their assistance. Special thanks are due to Dr W. Jacobson, Strangeways Laboratory, Cambridge, for his encouragement and assistance. One of us (R. W. S.) held a grant from the Cancer Research Campaign during this study.

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