IDENTIFICATION OF FUNGI BY THE FLUORESCENT
ANTIBODY TECHNIQUE

BY

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We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA
April, 1972
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Date April 28, 1972
ABSTRACT

The fluorescent antibody technique was investigated as a means of facilitating the recognition and identification of the fungal components of a western red cedar (Thuja plicata Donn) heartwood flora in situ.

Fungi isolated from the heartwood were grown in bulk and prepared for two different injection trials. In one trial the antigen was the particulate matter of the cell that could be centrifuged into a pellet after the hyphae were destroyed by a tissue grinder. In the second trial the hyphae were ground up and ultrasonically disintegrated. Only the cytoplasm and small wall fragments were retained for injection. After antisera collection the indirect staining method was employed. Unlabeled specific antiserum was layered over the antigen, allowed to incubate and washed off before fluorescent sheep anti-rabbit globulin was applied to form the final layer.

All attempts to detect specific antibodies to the fungal antigens failed. This was probably due to not using antigens rich enough in protein. Successful production of precipitating antibodies to fungal antigens has been shown by other workers to be more likely when the antigen contains greater than 10 milligrams of protein per milliliter of antigen solution. It has also been found that in some cases fresh antigen must be prepared for each diffusion test and injection as it can't be preserved even at -20°C. It is hoped that if fresh, high protein antigen were to be used this study could be successfully completed.
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ACKNOWLEDGEMENTS

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IDENTIFICATION OF FUNGI BY THE FLUORESCENT ANTIBODY TECHNIQUE

Introduction

Cross-sections of butts of old (approximately 200 years old) western red cedars (Thuja plicata Donn) commonly exhibit a central column of decayed material surrounded by several zones of variously stained heartwood (see Figure 1). The decay column is enclosed by a zone of brown stained, but sound wood. Outside of this lies a belt of red-brown or pink heartwood which in turn is surrounded by a straw colored zone.

Repeated isolation of fungi from the heartwood of Thuja plicata has demonstrated that the outer straw coloured heartwood may be considered sterile but that the red-brown and brown stained heartwood zones are colonized by characteristic fungi (van der Kamp, unpublished). Such isolation work is based on the assumption that the living organisms present in the heartwood will grow on the media used for isolation. Furthermore, in cases where two or more organisms occur together in the wood it is not uncommon for one of them to grow much faster on the isolation medium and in fact eliminate the other. Thus, the relative frequencies of two or more organisms in the heartwood cannot
be determined by the frequency of isolation. Furthermore, in the case of western red cedar heartwood it has proven impossible to separate the various fungal isolates by microscopic examination of variously stained sections of heartwood. Fluorescent antibody staining offered the best possibility to overcome both these problems.

The as yet unidentified fungi isolated from the stained heartwood of Thuja plicata may be viewed as forming stages in a succession of fungi leading to decay. Tests have shown that all the fungi commonly isolated from the red-brown and brown stained heartwood zones are able to break down thujaplicin, a potent fungicide naturally occurring in the heartwood of western red cedar. Naturally dark stained pieces of heartwood are much more susceptible to decay than light colored heartwood. Test blocks of straw colored heartwood inoculated with fungi isolated from the stained heartwood zone show a rapid decrease
in thujaplicin concentration. In this instance, however, there is no parallel decrease in decay resistance. This suggests that the breakdown product of thujaplicin may be as toxic as thujaplicin itself. This in turn could mean that the further steps in the breakdown of thujaplicin are mediated by organisms not commonly isolated. One or more of the occasional isolates could actually be common in the wood and essential for the loss of decay resistance. Fluorescent antibody staining, a technique used mainly in medicine, appeared to offer an effective means of approaching this problem. According to Nairn (1962), the fluorescent antibody technique (hereafter known as F.A.T.) "...is perhaps at its most useful when employed to determine the numerical or spatial distribution of microorganisms among a mixed population." Thus, the F.A.T. was investigated as a means of overcoming two specific problems.

The F.A.T. is based on the principle that specific antiserum can be precipitated when it contacts its homologous antigen. This fluorescent complex could be readily viewed under ultraviolet stimulation. It is important to note that the antigen(s) must be unique to one fungus if a specific stain is to be produced.

This technique has the advantages of high specificity, sensitivity and rapidity (once developed). The high degree of specificity is inherent in the antibody which is produced in response to the entry of a foreign, high molecular weight compound into an animal. Ideally this antibody globulin will complex only with the antigen (usually protein or polysaccharide) to which it has been prepared. Tagging

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antibody with some fluorochromes doesn't significantly affect its biological activity. In addition to being specific the F.A.T. is also sensitive. It is possible by this technique to detect as little as $1 \times 10^{-18}$ grams of dye (Eren and Pramer, 1966) and to identify a single bacterial cell that contains $5 \times 10^{-11}$ milligrams of nitrogen (Coons, 1956). Thomason et al. (1956) report no difficulty in locating specifically stained bacteria in mixtures containing ratios of contaminants to specific cells as high as $10^7:1$. Once the technique has been developed it can be used quickly to screen a large number of samples. Staining and microscopic examination can be completed in as little as one hour. Also, the presence of contaminants is of no concern if proper controls are maintained and the technique is equally useful for viable and nonviable organisms (Cherry et al., 1965).
Literature Review

Historical Perspective

In the period from 1930-42 several attempts were made to tag antibodies with azo-dyes (Reiner, 1930) and later with fluorochromes (Creech and Jones, 1941). The former resulted in low sensitivity while the latter complexes were deleterious to the antibody. Until the early 1940's most workers had been preoccupied with studying the effect of various radicals on the immunological activity of antibodies. Coons, Creech and Jones (1941) and Coons, Creech, Jones and Berliner (1942) were the first to study the tagged antibody from the point of view of using it as a tracer. They used fluorescein isocyanate-conjugated antibody to trace pneumococcus soluble polysaccharide antigen in tissue sections of mice infected with pneumococcus. In the period from 1950-51 a series of papers on the F.A.T.'s technical aspects were published by Coons and co-workers. The first (Coons and Kaplan, 1950) detailed the synthesis of the fluorochrome fluorescein isocyanate, and its conjugation to the antiserum along with instructions for microscope filtering systems, for tissue powder removal of nonspecific staining and for immunological proofs of specificity of staining. This publication along with four others (Coons, Leduc and Kaplan, 1951; Coons, Snyder, Cheever and Murray, 1950; Hill, Deane and Coons, 1950; Kaplan, Coons and Deane, 1950) established the technique's principles, basic mechanics and feasibility.
Until the mid-1950's F.A. work was being done exclusively by the direct staining method. In this case the fluorochrome is conjugated directly to the antibody (see Figure 2). This conjugate is able to

\[
\begin{align*}
\text{Antibody} & \quad \text{Fluorochrome} & \quad \text{Labeled antibody} \\
\text{Antigen} & \quad \text{Specific fluorescence} (+) \\
\text{Antigen} & \quad \text{Heterologous antibody} & \quad \text{Specific fluorescence} (-) \\
\text{Antigen} & \quad \text{Antibody} & \quad \text{Antigen-antibody complex} & \quad \text{Labeled antibody}
\end{align*}
\]

Figure 2. Direct Fluorescent Antibody Methods (Kawamura, 1969)
adsorb onto the homologous antigen to form a visible specific stain when excited by ultraviolet irradiation. Experience has shown the direct technique to be more specific than the indirect, though the reason for this is not known. Deacon et al. (1957) varied the procedure to indirectly detect antibodies in unlabeled test sera. In the indirect (also known as sandwich or antiglobulin) method the homologous antibody is not tagged. However, antibody to normal \( \gamma G \)-globulin (prepared in a different animal) is labeled and it is added to the homologous antigen-antibody complex resulting in specific fluorescence (see Figure 3). If there is specific fluorescence, one

\[
\text{Antibody to normal } \gamma G \text{-globulin} + \text{Fluorochrome} \rightarrow \text{Labeled antibody to normal } \gamma G \text{-globulin}
\]

\[
\text{Antigen} + \text{Antibody} \rightarrow \text{Antigen-antibody complex} \rightarrow \text{Labeled antibody to normal } \gamma G \text{-globulin} \rightarrow \text{Specific fluorescence (+)}
\]

**Figure 3** Indirect Fluorescent Antibody Methods
(Kawamura, 1969)

can identify the antigen when the nature of the primary antibody (the one that is not tagged) is known, or vice versa. See methods for details of the indirect technique.

Numerous workers have investigated the techniques devised by Coons and co-workers and found them to be useful. Many minor and a few major refinements have been made. One important advancement in fluorescent antibody acceptability was made by Riggs et al. (1958) who
described the synthesis and use of isothiocyanate derivatives of fluorescein (yellow-green fluorescence) and tetraethylrhodamine B (orange-red). Previously most workers had used unstable fluorescein isocyanate whose preparation required the use of difficult-to-handle gaseous phosgene. The isothiocyanate derivative was prepared with liquid thiophosgene and was a stable product that could be stored for months and used to label proteins with no further chemical manipulations (unlike the isocyanate). Thus, with the development of protein-labeling dyes that were stable enough to be marketed commercially and easily handled, a major difficulty of F.A. technology was overcome.

F.A.T. Uses

Initially Coons et al. (1942) devised the F.A.T. to investigate medically-oriented problems. This continues to be the major use, especially in a diagnostic capacity. The technique has been used to identify and study viral, bacterial, protozoal, helminthic, fungal, amoebal, mycoplasmal and animal tissue antigens (Nairn, 1962). It has also been used for the microscopic identification of injected foreign antigens; intrinsic antigens, proteins, enzymes, and hormones, and specific antibodies (Mellors, 1959).
Some F.A. tests have proven so useful they are conducted regularly in diagnostic medical labs. Cherry and Moody (1965) have indicated that they consider the following to be the most important diagnostic applications of the F.A.T. in bacteriology: identification of Group A *Streptococci*, *Treponema pallidum* (Schaudinn and Hoffmann) Schaudinn, *Neisseria gonorrhoeae* (Zopf) Trevisan, *Cornebacterium diphtheriae* (Kruse) Lehmann and Neumann, *Bordetella pertussis* (Pribram) López, *Salmonella typhosa* White, serogrouping of enteropathogenic *Echerichia coli* (Migula) Castellani and Chalmers and *Shigella*. As a direct result of the success of researchers in medical bacteriology, workers in other fields are realizing the F.A.T.'s potential and are trying to adapt it to their particular needs. Several researchers have used F.A.T. to help overcome problems of working with soil microorganisms. F.A. staining aids recognition of the organism directly in its soil habitat. Different problems are being attacked with the F.A. as indicated by the following quotations:

Relationships between microorganisms in soil are revealed by indirect methods only, and it is seldom possible to easily differentiate organisms of particular interest from the myriad of others that comprise the microbial population of soil.  

Even though it may be shown that a particular species exists in a soil, cultural experiments cannot prove whether the organism is vegetative or dormant, whether it occurs as single cells or in colony form, or whether it is associated with any one type of particle, or microhabitat, in the soil.

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Schmidt et al. (1968) studied species of *Rhizobium* in the soil but had difficulty with nonspecific adsorption by soil particles. Paton (1960) showed the relationship between *Pseudomonas* and the soil free root surface of clover. F.A. studies of fungi in the soil have been able to detect *Aspergillus flavus* Link ex Fr. in mixed culture (Schmidt et al., 1962 and 1965). Eren and Pramer (1966) used F.A.T. to identify and measure the abundance in soil of *Arthrobotrys conioide* Drechs. a nematode-trapping fungus.

The F.A.T., when applied to fungi, has been mainly for the purpose of diagnosing and identifying pathogens of man (Kaplan and Kaufman, 1961). The technique's advantages for fungal identification include rapidity and sensitivity as compared with conventional cultural methods. Immuno-specific staining enabled Kaplan and Gonzales Ochoa (1960) to find fungus elements of *Sporotrichum schenckii* (Helet. and Perk.) de Beurm and Goug. in smears from a human lesion which proved negative by cultural techniques. This is one indication of its sensitivity though a study (Porter et al., 1965) of tissues infected with *Histoplasma capsulatum* Darling and *Blastomyces dermatitidis* Gilchrist and Stokes indicated that histopathological techniques were more effective than F.A.T., and both were better than culturing. This latter paper also points out the great diversity of results from studies involving different methods and organisms.

In other cases Gordon (1958) used F.A.T. to differentiate *Candida albicans* (Robin) Berkh. from other *Candida* species and from yeasts of other genera. Al-Doory and co-workers (1963) were able to distinguish between *Cladosporium carronii* Trejos and *C. bantianum* Borelli. *Histoplasma capsulatum* yeast cells have been selectively stained in the
presence of *Blastomyces dermatitidis* by Gordon (1959). Conjugated antisera to *Cryptococcus neoformans* (Sanfelice) Vuill. has been tested on 96 isolates of this organism and on a number (23) of heterologous species (Kase and Marshall, 1960). These workers reported specific staining of 95 isolates of *C. neoformans* and none of the other species.

In 1962 May used F.A.S. to study and identify the sites of cell wall extension of *Schizosaccharomyces pombe* Lindner. Two years later Goos and Summers used a similar technique to study the morphogenesis of two fungi. They found that wall material of the *Candida albicans* parent cell is incorporated into the wall of daughter cells or hyphal walls. Conidia of *Fusarium oxysporum* f. *cubense* (E.F. Smith) Snyder and Hansen stained intensely but germ tubes emerging after staining did not. In 1964 Paton made a significant contribution to aid plant pathologists with use of the F.A.T. He described the preparation of plant tissue for examination and included valuable information on control of host autofluorescence and nonspecific staining. In spite of this aid F.A. studies of plant pathogenic and saprophytic fungi are not common. Beno and Allen (1964) used immuno-fluorescent staining to rapidly identify a specific line of germinating *Puccinia sorghi* (Schw.) uredospores. The presence of *Polyporus tomentosus* in mixed cultures, soil, and infected pine roots was detected by F.A. (Kumar and Patton, 1964). In 1965 some factors affecting the antigenicity of the mycelia of 3 species of *Phytophthora* Fr. were reported by Burrell *et al*. They found the type and concentration of nitrogen source, the amount of inoculum, and the age of mycelium greatly affected the antigenic efficacy of the preparations. Amos and Burrell the following year found the F.A.T. to be the most useful technique (in comparison with agglutination and gel diffusion) in differentiating among eight
species of Ceratocysis. In another study a fluorescent antibody reagent for Botrytis cinerea Pers ex Fr. was able to distinguish spores and mycelium of that organism from those of three others (Preece and Cooper, 1969). Price (1970) was unable to produce a fluorescent antibody reagent specific for Sphaerothecia pannosa conidia. A large number of his comparative test species fluoresced along with S. pannosa while other species did not.

Problems Encountered with the F.A.T.

The problems that can and do arise with the F.A.T. are manifold but most can be controlled to some extent. One problem is auto-fluorescence of the organism being studied, of other contaminating members and of the organism's substrate or host. Tissue auto-fluorescence in particular masks the low intensity immune-specific reactions. Autofluorescence can be minimized by prompt use of cut sections and is partly removable by filtering and counterstaining. Counterstains are useful if brilliantly fluorescent and if they have an emission maximum well separated from that of the fluorescent conjugate (Hall and Hansen, 1961). Another problem is nonspecific fluorescence which may result from the unreacted fluorochrome substance, from antibody with too strong a negative charge, from improper tissue fixation, or from letting the specimen dry out during the staining procedure (Kawamura, 1969). Nonspecific staining may be reduced or controlled by purification and fractionation of antigenic components, dilution of antiserum, improved tissue
preparation techniques, removal of unconjugated fluorochrome and adsorption with tissue powders or homogenates. Thirdly, it is possible to obtain false positive staining reactions due to the antibody already being present in experimental animals after natural infection. This will be identifiable if controls are taken unless animals are naturally infected after normal serum is drawn. Fourthly, the same antigens in heterologous organisms may result in cross-staining. Fifthly, fluorescent artifacts may be produced by manipulating (fixing, etc.) the section to be observed. Once stained, conditions can be altered such that the antigen–antibody complex can be broken down. In addition Gooding (1966) points out that in most cases it is necessary to work with fungal extracts which consist of multiple antigen systems which are difficult to analyze. He successfully isolated and used a crude DNA fraction from Fomes annosus Fr. in double diffusion tests. Finally, as Kaplan and Kaufman (1961) point out, there is a need for standardization of reagents, procedures and equipment so results will be more comparable. Undoubtedly many anomalies and discrepancies to date are due to variation in immunizing strain, immunization schedule, the fluorochrome used in labeling, host reactivity, antibody titers, optical equipment used, etc.
Methods and Materials

Growth of Fungi in Bulk

Three fungi commonly isolated from western red cedar heartwood were chosen to be studied. They were a *Kirschsteiniella*-like species, a *Verticillium*-like species and another unidentified species, each respectively known in the U.B.C. Forest Pathology Collection as C4D4, VIQQ and IVIB.

These fungi were transferred from storage slants to petri plates containing malt agar where they were allowed to grow until approximately two-thirds of the plate was covered with an actively growing culture. Then the agar embedded mycelial mat was transferred into a Waring Blender with 150 milliliters of sterile water and ground up for 15 seconds. Ten milliliters of this slurry were added to each of ten 250 milliliter shaker flasks containing 50 milliliters of culture medium. This was repeated for each of the three fungi. The growth medium was as follows:
0.75 g of MgSO$_4$$\cdot$7H$_2$O
0.75 g of KH$_2$PO$_4$
10.0 g of yeast extract
20.0 g of glucose
1000.0 ml of water

The two filamentous fungi (C4D4 and IVIB) grew for 9 to 16
days until sufficient mycelium had been obtained. VIQQ, unlike the
other two fungi, grew slowly in a yeast-like fashion in liquid culture
and as a result required 27 days to produce enough fungal material
to harvest. The fungi were then centrifuged (Sorvall SS-1 Angle
Centrifuge) and washed three times in phosphate buffered saline (here-
after known as P.B.S.) (Cherry and Moody, 1965).

Antigen Preparation

Numerous attempts were made to disrupt the fungal cells to expose
antigenic material while at the same time minimizing denaturation.
First a Pyrex tissue grinder with a tolerance of 0.005 to 0.007 inches
was used, sometimes in combination with glass chips. It did not prove
satisfactory as only about 20 percent of the mycelial cells and
significantly less of the yeast-like cells were broken up. The second
attempt involved freezing the organism in liquid nitrogen and grinding
with mortar and pestle. In a similar case the fungi were frozen
in liquid nitrogen and struck repeatedly in a chilled compression
cylinder. The latter attempts weren't any more successful than the
first method and were much more difficult to complete. The fourth
time, the hyphal fungi were ground up by the Pyrex tissue grinder and
then subjected to ultrasonic disintegration (Bronwill Biosonik III) in short (12 second) bursts for a total time of one minute while being cooled by an ice-water bath. Microscopic observation of the treated tissue showed the hyphal fungi to be macerated but such was not the case with VIQQ until it had been subjected to a further one minute of treatment. This final and most successful treatment ruptured at least 50 percent of the cells.

In the first injection sequence (trial one) the antigen used was basically the particulate matter of the cell. Any cell component that could be centrifuged into a pellet at a R.C.F. of 6500 in 20 minutes was used. The fungus was macerated with the tissue grinder only. Fungal particulates were suspended at five milligrams per milliliter in P.B.S. and frozen (-20°C) in three milliliter lots. To this suspension an equal amount of Freund's complete adjuvant was added at the time of injection to enhance antigenicity.

In the second trial the antigen was prepared by first grinding the fungus and then subjecting it to ultrasonic disintegration. The heavy cell wall components were centrifuged and discarded. The supernatant containing cytoplasm and very small wall fragments were diluted to five milligrams per milliliter. An equal volume of Freund's complete adjuvant was added and mixed vigorously just before injection. Prepared antigen, in P.B.S., was stored at -20°C in small vials.
Injection

Young, three to four pound rabbits were injected subcutaneously on the scapular region of the back using sterile disposable syringes. The syringes had a capacity of three milliliters with one and one-half inch long, 21 gauge needles. Each of the three fungi were injected into two rabbits for the first trial and into three rabbits for the second trial. On injection day each rabbit received a total of one milliliter of emulsion. For the first trial each rabbit received a single injection once a week while the rabbits in the second received half a milliliter in each of two places every two weeks. Each trial had four injection days. When two injections were given to each rabbit on one day they were at least two inches apart. The Freund's sometimes caused open wounds which were quickly treated with Cicatrin\(^4\) antibiotic powder and the subsequent injection did not contain the adjuvant.

Bleeding and Antiserum

Two weeks after the last injection the rabbits were bled from the midvein of the ear. Fifteen to 20 milliliters of blood were collected from each animal and left overnight in a cooler 4°C for the clot to form. Antiserum was decanted from the clot and centrifuged to remove any large debris. A small amount (1.5 milliliters) of raw antiserum was immediately frozen to -20°C. The remainder of the antiserum was cross-adsorbed with heterologous antigens to remove

\(^4\) Cicatrin, Calmic Limited, Toronto
nonspecific antibody. Then the remainder was divided into small vials and frozen (-20°C) until used.

Antibody Detection

Visible precipitation or clumping of particles occurs with most antigens as a result of the multivalent (many bonding points for antibody) antigen and bivalent antibody forming aggregates or lattices of antigen and antibody. The two basic antigen-antibody reactions are the precipitin if the antigen is in a soluble form and agglutination if the antigen is particulate. These reactions allow detection and quantitative estimation of antigens or antibodies in solution and tissues (Kabat, 1968).

The agglutination reaction was used to test for antibody prepared to the particulate antigen of the first trial. A prerequisite for the agglutination test is a homogeneous suspension. Therefore only the small wall fragments of the hyphal fungi could be used. After low speed centrifugation to eliminate the larger wall pieces the smaller ones were driven into a pellet and washed several times in P.B.S. This was the antigen used in the passive haemagglutination test as described by Campbell et al. (1964). In this procedure fresh sheep red blood cells were washed and then tanned with tannic acid. Then they were coated with the antigen by mixing together and suspended in normal serum and saline. Finally the coated red blood cells were added to serial dilutions of the serum, shaken and allowed to stand at room temperature for three to four hours. A positive reaction was indicated by a compact granular agglutination or a diffuse film of agglutinated cells covering the bottom of the tube.
while a negative one appeared as a heavy ring of cells or discrete smooth button of cells in the center of the tube.

The yeast phase of the dimorphic fungus, VIQQ, was studied by the tube agglutination method of Cozad (1958). To serial dilutions of serum (0.5 milliliters) in P.B.S. was added 0.5 milliliters of the antigen suspension and the tubes were shaken vigorously. The tubes were immediately refrigerated (4°C) and left overnight. The degree of agglutination was indicated by the size of cell aggregates and turbidity of supernatant.

Ring and gel double diffusion tests were used to detect antibody to the cytoplasmic and fine fragments of fungi used in the second trial. The ring or interfacial test involved carefully overlaying antiserum with a solution of antigen such that a sharp liquid interface was formed. Antibody can be detected in amounts as small as one microgram of protein. An example of the test including controls is given in Figure 4. A positive result (as in

<table>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td>Top Layer</td>
<td>B</td>
<td>B</td>
<td>Ag</td>
<td>B</td>
<td>Ag</td>
</tr>
<tr>
<td>Bottom Layer</td>
<td>Ab</td>
<td>Ag</td>
<td>N</td>
<td>N</td>
<td>Ab</td>
</tr>
<tr>
<td>Observed Reaction</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</table>

Key

- B = buffer
- Ab = antibody
- Ag = antigen
- N = normal serum

Figure 4 Typical Ring Precipitin Test With Controls

Figure 4) is indicated by a fine line or ring of precipitated particles at the interface of the two complementary solutions while a negative one has no ring.

The gel diffusion test was also used to detect antibody in the second trial. In this case concentrations of antigen and antibody
diffuse toward each other and if optimal they form visible bands of precipitation in the semisolid medium (Difco Noble Agar). Ouchterlony's double diffusion method (as described by Campbell et al., 1964) was used. This test resolves components in mixtures. The number of bands indicates the minimal number of antigen-antibody systems present. Figure 5 shows the pattern of a diffusion plate.

Figure 5  Diffusion Plate Pattern

Indirect Staining Method

The indirect method was selected rather than the direct for several reasons. According to Kawamura (1969) the indirect is five to ten times as sensitive. One explanation for the differences in sensitivity is the additional combining sites offered by the antibody molecule sandwiched between the antigen and tagged anti-rabbit serum when using the indirect method (Nairn, 1962). In Figure 6 only three
sites in the antigen are available for antibody and these three would be the total number possible if using the direct method. In the indirect method though the second layer is unconjugated antibody which behaves as an antigen for the conjugated antibody. Thus, in Nairn's two dimensional model the reactive sites have increased fourfold. Also, the indirect method was chosen as the result of a personal communication with Dr. Yasu Hiratsuka who indicated he was having difficulty with the sensitivity of staining from the direct method and he was switching to the indirect F.A.T. in hopes of improvement. He found that autofluorescence was masking some of his weak immune-specific reactions. Moreover, it is possible to buy labelled anti-rabbit globulin commercially so if one uses the indirect F.A.T. the conjugation of fluorescein isothiocyanate is unnecessary.
See Figure 7 for a comparison of the direct and indirect methods.

Staining was initiated using each fungus fixed to a slide. Fungi fixed with ethanol and with heat were dislodged during washing so Haupt's adhesive with phenol was used. Eventually it was planned to study the fungus in wood so each fungus, in pure culture, was started growing on western red cedar heartwood. The inoculated samples would allow a more realistic trial of the staining technique, yet with the fungus known. At the same time it would provide a place where the "bugs" (for example, autofluorescence of wood) could be worked out.

Once the tissue was fixed to the slide the staining procedure of Goldman (1968) was followed as indicated below:

Step I  
(a) A few drops of unlabeled specific (against each fungus) antiserum were layered over the antigen.
(b) Slides were incubated in a moist chamber for 60 minutes at 25°C.
(c) Antiserum drops were shaken off.
(d) Slides were immersed in a saline, with occasional agitation for 10 minutes.
(e) Slides were rinsed in tap water and dried.

Step II  
(f) Fluorescent sheep anti-rabbit globulin was applied to the antigen for 60 minutes in moist chamber.
(g) Slides immersed in P.B.S. and agitated occasionally for 10 minutes.
(h) Slides were rinsed with water and mounted with buffered glycerol (P.B.S.: glycerol = 1:9).

<table>
<thead>
<tr>
<th>Direct F.A.T.</th>
<th>Indirect F.A.T.</th>
</tr>
</thead>
<tbody>
<tr>
<td>- simpler procedure</td>
<td>- more complex procedure</td>
</tr>
<tr>
<td>- more specific</td>
<td>- more sensitive</td>
</tr>
<tr>
<td>- each antiserum must be labeled</td>
<td>- only one antiserum must be labeled</td>
</tr>
<tr>
<td>- uses greater quantities of specific antiserum</td>
<td>- uses less specific antiserum</td>
</tr>
</tbody>
</table>

Figure 7  Comparison of Direct and Indirect Methods
Microscopy

Prepared slides were observed with a Leitz Ortholux microscope equipped with a high-pressure mercury vapor lamp. The filter system included a BG12 (4 mm) and UG1 (1 mm) filter as exciting filters and a K430 mÅ barrier filter (Culling, 1963).

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Reagent for Step I</th>
<th>Reagent for Step II</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homologous</td>
<td>- P.B.S.</td>
<td>- labeled antiglobulin</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>- normal serum</td>
<td>- labeled</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>- adsorbed specific antiserum</td>
<td>- labeled</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>- specific anti-serum to C4D4</td>
<td>- labeled</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>- specific anti-serum to C4D4</td>
<td>- labeled normal serum</td>
<td>-</td>
</tr>
<tr>
<td>Heterologous  (VI QQ)</td>
<td>- specific anti-serum to C4D4</td>
<td>- labeled antiglobulin</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 8 Typical Staining Trial With Controls For One Fungus

Results

It was not possible by any of the three methods nor for either of the trials to demonstrate the production of antibodies. In addition there was no fluorescent staining when the procedure of Goldman (1968) was carried out.

Discussion

Researchers pioneering the serology of fungi were often frustrated
by the extreme variation in the immunizing capacity of fungal antigens. For example, Coons and Strong (1928) and Nelson (1933) were not able to produce antisera, to several species of *Fusarium*, that was of sufficiently high titer for precipitin tests. However, other *Fusarium* species produced a high titer easily. Seeliger (1960) reported that no reliable method of immunization had been worked out, as of 1960, for the medically important fungal genus, *Cryptococcus*.

Many experimenters working with fungi have worked with yeast or yeast-like forms or with spore or conidial suspensions which can be treated with techniques similar to those used for bacteria. It is the hyphal fungi that have proven most difficult to handle. Unfortunately it was not possible in this study to produce antisera to the hyphal or yeast-like fungi.

At the time the first trial was being attempted the author had only a very cursory understanding of the principles and techniques of serology. As such, two papers (Preece and Cooper, 1969; Eren and Pramer, 1966) successfully applying F.A.T. to fungi were blindly followed with some minor modifications. Cell wall fragments were used as the antigen because it was the wall of an intact hypha that would be contacted by the conjugated antibody. Gordon stated that "the cell wall is the exclusive site of reactivity in intact cells of most fungus species."\(^5\) In spite of not being able to detect antibodies in the first trial the indirect staining procedure was attempted because of the following statement by Paton:\(^6\)

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On occasion, a serum has been produced which showed no agglutination titer but proved to be of excellent value for this technique. The only valid criterion of usefulness is the staining reaction itself. No staining was observed however so experienced aid was solicited. Dr. R.J. Bandoni suggested that the cell walls of the fungi being studied might be so similar that each fungus would cross-adsorb the antibodies for the other antigen or, if not adsorbed, would form a nonspecific stain. Since only the cross-adsorbed antisera had been studied it was possible that antibodies might be present in the raw antisera. This proved negative also.

The plan and results of trial one were taken to Dr. J.J. Stock of the U.B.C. Department of Microbiology. He had no experience with F.A.T. but had attempted some fungal serology. He suggested using the cytoplasmic fraction as antigen (Hook et al., 1967) and injecting it every two weeks in two places on the rabbit's back. Antibodies to the soluble antigen would be easier to detect than they had been in the first trial. However, once again no antibodies were detected and no staining observed.

Since the second trial an important paper has come to the author's attention. Burrell, Clayton, Gallegly and Lilly (1965) studied the factors affecting the antigenicity of the mycelium of three species of Phytophthora. They subcutaneously injected soluble mycelial suspensions of fourteen-day-old cultures into rabbits and failed to demonstrate the presence of precipitating antibodies. They hypothesized that the preparations were low in protein and therefore antibody production wouldn't be stimulated. Burrell et al. determined the factors that influence the nitrogen content of mycelium and found that the protein...
content per unit weight of mycelium was greatest after three days of incubation when ammonium sulfate was used at two grams per liter as nitrogen source. Rabbits immunized subcutaneously by this high-protein, soluble antigen yielded a good precipitin production.

If time had permitted the author would have prepared a cytoplasmic antigen containing ten milligrams per milliliter of protein. Protein concentrations could be measured by the Biuret reaction or by micro-Kjeldahl analysis (Campbell et al., 1964; Kabat et al., 1961). One milliliter of antigen would be subcutaneously injected in the form of a solution containing 50 percent Freund's adjuvant and 50 percent of the antigen (20 mg of protein/ml) in P.B.S. Young rabbits would be injected once a week for five weeks (Pepys et al., 1967) with trial bleedings conducted ten days after the last injection and weekly thereafter until a sufficiently high titer was obtained. The antisera would be treated as previously described and specific antibodies detected on gel double diffusion plates.

If the findings of Burrell et al. (1965) are generally applicable it may be necessary to prepare a fresh antigen for each injection and each diffusion test. These workers were not even able to preserve antigens frozen at -20°C. There was a loss of precipitating activity within a week. It would be hoped that the use of a soluble high protein antigen and fresh antigen for each treatment might result in specific antibody production. However, the antibody formed may not be specific in which case it would be necessary to fractionate the antigen complex until a specific antigen would produce a specific antibody.
Though the results of this project were not encouraging it is felt that new attempts, keeping Burrell et al.'s (1965) studies in mind, could be successful. Many years of medical mycology research have shown the technique's feasibility with pathogenic fungi of mammals. The basic methodology is available and could be extremely useful in plant pathology. In addition to the uses envisioned in this study the F.A.T. could be used in such identification situations as for spores on spore traps and for mycorrhizae. Other possibilities include using the technique to study disease development, for example in structures like cankers.
agglutination reaction - a reaction involving the clumping or aggregating of large particulate antigens (e.g., bacterial cells) by specific antisera; in this case the particles are large enough to be seen under the microscope.

antibody - a humoral globulin produced in response to the parenteral introduction of an antigen into an animal; this globulin will react with its homologous antigen.

antigen - any substance which, when introduced parenterally into an animal will cause the production of antibodies by that animal and which will react specifically with those antibodies.

conjugated - a state in which the antibody is coupled to fluorochrome; synonymous with "tagged".

fluorochrome - a substance which fluoresces under ultraviolet stimulation and can be conjugated to antibody.

F.A. - fluorescent antibody abbreviation; indicates antibody conjugated with fluorochrome.

F.A.T., F.A. staining, immuno-specific staining, immuno-fluorescent staining - terms used interchangeably to denote a staining method using fluorochrome-labeled antibody as specific staining agents for antigen.

gel diffusion - antigen and antibody are brought together, through diffusion in a semisolid, where they precipitate to form visible bands.

parenteral - other than by way of the intestines.

precipitin reaction - a reaction occurring between large antigen molecules and antibody molecules resulting in the formation of an antibody-antigen precipitate.

tissue powder - a preparation used to eliminate nonspecific fluorescence. The powder is usually from the tissue to be stained and is produced by acetone precipitation of the tissue homogenate.

titer - the highest dilution of an antiserum that will show detectable agglutination or precipitin reaction; concentration or activity or potency of a serologically active substance.
REFERENCES


