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BLASTOMYCES DERMATITIDIS: CHARACTERIZATION OF THE A ANTIGEN AND PRODUCTION OF HYBRIDOMA-DERIVED ANTIBODIES SPECIFIC FOR A CYTOPLASMIC COMPONENT

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BLASTOMYCES DERMATITIDIS: CHARACTERIZATION OF THE A ANTIGEN AND PRODUCTION OF HYBRIDOMA-DERIVED ANTIBODIES SPECIFIC FOR A CYTOPLASMIC COMPONENT

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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degree of

DOCTOR OF PHILOSOPHY

BY

KEVIN DALE YOUNG

Norman, Oklahoma

1980

BLASTOMYCES DERMATITIDIS: CHARACTERIZATION OF THE A ANTIGEN AND PRODUCTION OF HYBRIDOMA-DERIVED ANTIBODIES SPECIFIC FOR A CYTOPLASMIC COMPONENT

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

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BLASTOMYCES DERMATITIDIS:

CHARACTERIZATION OF THE A ANTIGEN

AND PRODUCTION OF HYBRIDOMA-DERIVED ANTIBODIES

SPECIFIC FOR A CYTOPLASMIC COMPONENT

CHAPTER 1

ANTIGENS AND CHEMICAL COMPOSITION OF BLASTOMYCES DERMATITIDIS: A REVIEW

INTRODUCTION

ANTIGENS

- Blastomycin
  - Skin-test
  - Complement-fixation
  - Precipitation
  - Other tests
- Whole Yeast Cells
  - Skin-test
  - Complement-fixation
- Yeast Fractions
  - Ethanol-precipitates
  - Cytoplasmic
  - Exoantigen
  - The "A" antigen
  - ASWS

INTRODUCTION

Blastomyces dermatitidis causes disease in man with several possible presentations: subclinical-asymptomatic, acute pneumonia, chronic pulmonary, and/or a progressive systemic disease which may affect virtually any organ in the body. The disease was first described by Gilchrist in 1894 as being of protozoan origin (27), but he soon identified the agent as fungal (28). He and Stokes gave the causative organism its current name (29). Further information regarding the disease and its history can be found in recent reviews and texts (10, 25, 66).

The diagnosis of the disease is currently most reliably made by direct culture of the organism from clinical specimens. Serological and skin tests are available, but negative tests do not rule out the disease and positive tests do not confirm it (10, 25). This situation stems from the lack of a Blastomyces test antigen which displays sensitivity and selectivity, and this comprises the major serological problem in blastomycosis.

This has been compiled in the belief that the absence of a comprehensive treatment of the antigens of B. dermatitidis has discouraged a coherent study of these antigens. In the

hope of redressing this problem, the following four goals have been established: to provide a complete history of the work in this area; to identify and discuss the most promising antigens isolated to date; to encourage protracted, in-depth research of these antigens; and to stimulate research to isolate and identify new antigens of diagnostic and analytic interest.

Much of the information is arranged in tabular form to allow comparisons to be made more easily. This format also allows a quicker historical survey and evaluation of progress in each area. The major experimental data included in these tables are the sensitivity and specificity of the antigen. Sensitivity is expressed as the percentage of known cases accurately identified. Specificity is expressed as the percentage of cross-reactions which inaccurately identify other mycoses as blastomycosis. The optimum, then, is to have 100% sensitivity (all true cases identified) and 0% cross-reactions (no reactions occur between the antigen and heterologous cases). The effectiveness of a particular antigen as a diagnostic indicator, and its comparison with other antigens, can be rapidly assessed by determining how closely it approaches this optimum sensitivity-specificity combination.

Since the emphasis of this review is on the antigens themselves, many reports of the use of these antigens for epidemiologic, immunologic, and diagnostic purposes have been omitted. Included, for the most part, are only those

studies engaged primarily in the evaluation of an antigen or in the comparison of multiple antigens.

ANTIGENS

Blastomycin

Skin-test. A most popular early antigen of B. dermatitidis was blastomycin, the culture filtrate obtained after growing the organism in the mycelial phase for periods ranging from weeks to months. The interest in this fraction is understandable since histoplasmin, the corresponding antigen from Histoplasma capsulatum, showed such success as an epidemiologic tool in skin-testing surveys. The history of blastomycin as a skin-test agent is detailed in Table 1.

E. R. Long is the first on record to use the term "blastomycin" (48). His work with guinea pigs showed blastomycin to be of no use in skin-testing but 100% effective in identifying infected animals in testes-testing. He conducted no cross-reaction studies, however, so that the major shortcoming of blastomycin was not documented until the work of Emmons et al. (26).

Howell's evaluation of blastomycin remains probably the most extensive conducted (34). He documented the effectiveness of several lots of antigen, dilutions, and groups of infected guinea pigs. Skin-test reactivity varied widely with all three. Undiluted antigen elicited reactions

TABLE 1

BLASTOMYCIN: SKIN-TEST[1]

DATE-REFERENCE	SUBJECT	SENSITIVITY	CROSS-RXS	REMARKS
1926 Long (48)	g-pig[2]	100%	-[3]	testes-test reactions
		0%	-	skin-test reactions
1945 Emmons + (26)[5]	g-pig	88% (7/8)[4]	100% (8/8)	
	patients	-	97% (34/35)	
1947 Howell (34)	g-pig	88% (29/33)	12% (5/40)	best of a range, see text
1963 Balows (4)	patients	4% (1/25)	-	
1964 VA Study (74)	patients	50%	-	
1974 Cox/Larsh (13)	g-pig	37% (20/54)	40% (12/30)	commercial blastomycin
		65% (36/55)	74% (23/31)	isolate "KCB" preparation
1976 Lancaster + (45)	g-pig	100% (16/16)	0% (0/16)	F fraction / isotachophoresis
		55%	0%	B fraction fractions from
		0%	25%	G fraction BPD

1) Unless otherwise stated, all antigen preparations were crude culture filtrates from mycelial cultures
2) guinea-pig
3) - = not reported or not done
4) indicates: number positive/number tested.
5) "+" indicates "et al." in this and subsequent tables

in 90% (9 of 10) normal animals, but this disappeared when dilutions greater than 1:10 were injected. Above this dilution factor, sensitivity ranged from 6-88% and specificity from 0-84%. He concluded that yeast phase antigens were superior to the filtrate preparations in both respects.

In 1950, Martin reported that of 972 patients tested, none of whom were known to have blastomycosis, 15 were skin-test positive to blastomycin but that 13 of these reacted with histoplasmin as well (in: 53). He later mentioned that, due mainly to this extensive cross-reactivity, blastomycin was used primarily as a control antigen in studies of histoplasmosis and coccidiodomycosis (53).

The reports of Balows and the VA study regarding the insensitive nature of blastomycin seemed to put to rest hopes of ever using this fraction diagnostically (4, 74). Cox and Larsh followed with the conclusion that the yeast-phase antigens were more specific (13). However, Lancaster and Sprouse fractionated B. dermatitidis KCB blastomycin by an unpublished gel chromatography procedure to give what they termed BPD -- blastomycin protein derivative (45).

Isoelectric focusing and isotachophoresis revealed BPD to be composed of 8 and 6 components, respectively. Nine fractions were isolated from BPD by isotachophoresis, three of which are recorded in Table 1. Fractions F and G were intermediate-migrating proteins and fraction B migrated rapidly. It is surprising that the F and B fractions elicited skin test reactions only in infected

animals, though with different sensitivities (100% and 55%, respectively). The G fraction reacted only with H. capsulatum-infected animals and so may be one of the culprits in the cross-reactive nature of blastomycin, especially with histoplasmosis sera. The excellent results obtained with the F fraction have not been followed by additional studies, and its possibilities and nature remain unknown.

Complement fixation. Blastomycin was found to be as poor an antigen in complement-fixation tests as in skin tests. The studies evaluating this role are given in Table 2. Overall, the best sensitivity that can be expected is 50%. Virtually no studies have been reported for the antigen's cross-reaction rate, though its low sensitivity is enough to disqualify it for diagnostic use.

Precipitation. The advantage in using precipitin tests, and especially the Ouchterlony method, for testing suspect sera is that not only does a positive or negative reaction occur, but precipitin reactions can often be differentiated by the location of the resulting bands. The sensitivity of blastomycin in precipitin tests is outlined in Table 3, and shows a slight advantage over skin-testing or complement-fixation.

Keeney and Eriksen prepared an ammonium sulfate-precipitated fraction of blastomycin and reported precipitin bands against 1:2 or 1:4 dilutions of infected guinea pig sera (43). Pates followed the course of precipitin formation

TABLE 2

BLASTOMYCIN: COMPLEMENT-FIXATION

DATE-REFERENCE	SUBJECT	SENSITIVITY	CROSS-RXS	REMARKS
1948 Tenenberg + (73)	g-pig	46% (6/13)	0% (0/3)	
1953 Campbell + (9)	patients	-	-	cross-rxs with heterologous sera higher than homologous
1964 VA Study (74)	patients	49% (51/105) 41% (56/136)	- -	
1967 Busey/Hinton (7)	patients	50%	-	

TABLE 3

BLASTOMYCIN: PRECIPITATION TESTS

DATE-REFERENCE	SUBJECT	SENSITIVITY	CROSS-RXS	REMARKS
1948 Pates (58)	g-pig	low 56% 100%	84% 84% 0%	2 weeks after infection 3 - 5 weeks after infection 7 - 9 weeks after infection
1961 Abernathy + (1)	patients	77%	-	all cross-rxs distinguishable by banding pattern
1967 Busey/Hinton (7)	patients	50%	-	9% of patients were positive to mycelial filtrate only

in guinea pigs over a nine-week period (58). Blastomycin had extremely low sensitivity and high cross-reactivity in the early stages of the infection. This situation was reversed in later time periods to the point that the antigen was 100% accurate in identifying infected animals. These results complement Howell's observations (34) that the sensitivity of blastomycin in skin-testing varies with the sensitivity of the animal. This situation may make blastomycin of some use in following the course of the disease but of little use in rapid or accurate diagnosis.

The report by Abernathy and Heiner points out the advantage of the precipitin test since, in an undisclosed number of cross-reactions, false positive reactions could be distinguished by the banding pattern (1). That blastomycin may still be of some, though not ideal, use as a precipitin antigen was documented by Busey and Hinton's finding that 9% of the patients studied were identified only with this preparation to have blastomycosis (7).

The use of immunoelectrophoresis to separate the components of blastomycin into a greater number of well-defined precipitin bands has allowed a more detailed comparison of its immunologic relations with other fungal antigens. Andrieu et al. identified 18 separate antigens in a blastomycin preparation, 13 of which were held in common with a similar preparation of H. capsulatum (2). One of these common precipitin bands showed catalase activity. In a similar study, Kashkin et al. identified

6 separate antigens by immunoelectrophoresis, all of which were common to culture filtrates of either H. capsulatum or Coccidiodes immitis (39). Gel electrophoresis of their blastomycin revealed 6 proteins, 4 glycoproteins and 1 nucleoprotein, out of which only one protein was absent in antigen preparations of the other two fungi.

Huppert et al. have recently tested blastomycin against an anti-coccidiodin reference serum (35). This group has found that blastomycin shares 12 antigens with coccidiodin, 3 with spherulin, 6 with an alkali-soluble water-soluble (ASWS) extract of C. immitis, 9-10 with histoplasmin, and 6 with ASWS from H. capsulatum. These numbers are obviously minimums since another reference serum, or sera raised to antigens other than coccidiodin, could show additional common components.

The extensive similarities between the mycelial cluture filtrates of all these fungi make it somewhat surprising that they should exhibit anything less than 100% cross-reactivity with heterologous sera. The crudity of the preparations is of course the complicating factor. A purified fraction of blastomycin has shown 100% sensitivity with no cross-reactions in skin-testing guinea pigs (45), but no purified preparations have been used in the precipitin test.

Other tests. Attempts to use blastomycin in agglutination tests have uniformly failed. The earliest record of such studies is that of Hektoen (in: 62, p. 502).

He tested sera from immunized dogs and found that undiluted sera agglutinated whole organisms, but only after several hours. Sera from other mycotic cases, though, also showed slight agglutinating ability. In rabbits only faint agglutination was observed.

Saslaw and Campbell attached the antigen to collodion particles. Agglutination was only weak with known positive sera and often greater with heterologous sera. They also found a great variation in activity among antigen lots (68). Martin later reported that 65% of the sera from normal medical students would agglutinate erythrocytes sensitized with Blastomyces material if the test was made sensitive enough to react with positive cast sera (52).

Utilizing the lymphocyte transformation assay, Cox reported that 13 of 13 patients with positive histoplasmin reactions cross-reacted with blastomycin, although lower total counts were obtained. Slightly fewer cross-reactions occurred with sera from patients with coccidiodomycosis (11). This assay is probably the most sensitive of those discussed so far. This sensitivity may cause the test to react more consistently with the antigens shared among the crude filtrates of these fungi, and thereby exhibit a greater rate of cross-reactivity than the other tests described.

Whole Yeast Cells

Skin-test. Whole yeast cells have not been used

extensively in skin-testing. The history of that work is given in Table 4. The sensitivity can be seen to have varied widely among the studies, and testing for specificity has been minimal. The 1947 report of Howell is the same as that in Table 1 for blastomycin (34). He concluded that the yeast antigen was more sensitive, and more consistently so, than the culture filtrate, although the variations due to a particular lot, dilution, and animal sensitivity remained. The tabulated value for his work is for the 1:1000 dilution of his stock. At 1:100, sensitivity increased to 90% and the number of cross-reactions to 89%. At 1:2000, no cross-reactions were observed, but the sensitivity dropped to 63%.

Complement-fixation. In the first attempt at complement-fixation (CF) using whole yeast, Davis found them to be anti-complementary and so abandoned the test (15). This and further history is shown in Table 5.

In 1926, Mellon tested B. dermatitidis in various morphologic states. Infections with mycelial fragments, conidia, yeast, or in-between states gave mixed CF titers among the homologous and heterologous antigens (55). Salvin noted that the yeast gave a more sensitive test than culture filtrates but that adsorption studies indicated a very high cross-reactivity (66).

The breakage of cells as reported by Dulaney may have given excellent sensitivity by releasing cytoplasmic material (21). His use of this material in precipitin

TABLE 4

WHOLE YEAST CELLS: SKIN TEST

DATE-REFERENCE	SUBJECT	SENSITIVITY	CROSS-RXS	REMARKS
1911 Davis (15)	patients	50% (1/2)	-	
1926 Long (48)	g-pig	0%	-	
1936 Martin/Smith (54)	patients	67% (2/3)	-	heat-killed yeast
1940 Peck + (61)	patients	100% (2/2)	-	phenol-killed yeast
1947 Howell (34)	g-pig	84%	8%	best of a range, see text heat-killed yeast (1:1000)

TABLE 5

WHOLE YEAST CELLS: COMPLEMENT-FIXATION

DATE-REFERENCE	SUBJECT	SENSITIVITY	CROSS-RXS	REMARKS
1911 Davis (15)	g-pig	-	-	found anti-complementary
1925 Rebaudi (in: 50)	patient	+ (1/1)	-	
1926 Mellon (54)	rabbit	-	-	formalin-killed cells in differing morphologic states; mixed results
1930 Dulaney (21)	rabbit	100% (6/6)	-	mortar-ground yeast
1935 Martin (50)	patients	75% (3/4)	0%	killed or live yeast, 0.3% suspension
1936 Martin/Smith (54)	patients	67% (4/6)	-	mortar-ground yeast
1949 Salvin (66)	rabbit	74% (68/92)	74% (77/104)	formalin-killed yeast; 1:8 titer or higher = pos
1950 Martin (51)	patients	71% (5/7) 47% (8/17)	- -	"cutaneous" cases systemic cases
1964 Busey (55)	patients	50% (40/80)	-	

tests (sensitivity, 4 of 6) was probably also related to these components.

Overall, the information gained using whole yeast cells in skin and CF-testing has been more valuable in arguing for the superiority of the yeast phase versus the mycelial phase than it has been in diagnostic or epidemiologic work.

Yeast Fractions

The above review should clearly highlight the necessity of moving from crude to more purified antigen preparations if both sensitivity and specificity are to be improved. With the exception of the fractionation of blastomycin by Lancaster and Sprouse (45), most effort has been directed toward the yeast phase of B. dermatitidis. As noted above, comparisons between mycelial and yeast preparations generally identified the yeast as the more sensitive agent. In addition, since the yeast is the parasitic form, it has seemed more logical to look for useful antigens in this phase.

In attempting to prepare more defined antigens, four general sources have been investigated -- the filtrate or aqueous extracts of whole cells, the cell wall, the cytoplasm, and ethanol-precipitates of various preparations. These will be presented in the order of their historical appearance.

Ethanol-precipitates. The first antigen fraction to

be recorded as used in the serology of Blastomyces was an ethanol (EtOH)-precipitate prepared by H. T. Ricketts for precipitin tests (in: 15). The history of these fractions dates from then to the present as summarized in Table 6.

Using the same antigen, Davis reported precipitin titers ranging from 1:7 to 1:20 in 69% of his infected animals (15). The cross-reaction figure in the table is for normal guinea pigs which he found to have titers of 1:3 to 1:10 when positive. Neuber and Rohlich reported positive CF results with one patient's serum (in: 50), and Peck et al. latter found the 44-60% EtOH fraction to be the most sensitive for that purpose (61). They reported the presence of a mannose-containing polysaccharide in their precipitates, but this was not further characterized. Such a precipitate was also found to be more specific than cytoplasmic antigens in skin-testing (52).

Dyson and Evans introduced a series of studies notable for the numbers of animals and antigen lots involved, as well as for the striking sensitivities and specificities they recorded (22, 23). Several lots of precipitated antigen gave 100% sensitivity with 0% cross-reactions in skin tests. It is of interest to note that these preparations originated from yeast culture filtrates. This success was repeated by Knight and Marcus who also reported that the average skin-test induration on B. dermatitidis-infected guinea-pigs was much greater than on H. capsulatum-infected animals -- 6.0 vs 1.6 mm, respectively (44).

TABLE 6

YEAST FRACTIONS: ETHANOL-PRECIPITATES

DATE-REFERENCE	SUBJECT	PREPARATION[1]	TEST[2]	SENSITIVITY	CROSS-RXS	REMARKS
1905 Ricketts (in:15)	g-pig	cyto; 75%	PPT	-	-	distinct ppt seen
1911 Davis (15)	g-pig	cyto; 75%	PPT	69% (22/32)	9% (2/22)	x-rxs in normal pigs
			ST	0%	-	
1940 Peck + (61)	rabbit	exoAg/A; 60%	CF	-	-	44-60% fraction best
			ST	+ (2/2)	-	
1948 Keeney + (43)	rabbit	exoAg/B; 75%	PPT	+	(-)	
1954 Dyson + (22)	rabbit	exoAg/B; 50%	ST	100% (10/10)	0% (0/10)	2 antigen lots
		; 67%	ST	100% (13/13)	0% (0/15)	3 antigen lots
1955 Dyson + (23)	rabbit	exoAg/B; 67%	ST	97% (29/30)	0% (0/36)	6 antigen lots
		; 75%	ST	72% (18/25)	0% (0/30)	5 antigen lots
	g-pig	; 67%	ST	100% (9/9)	0% (0/7)	10 microgram level
1958 Knight + (44)	g-pig	exoAg/B; 67%	ST	100% (6/6)	0% (0/11)	
1960 Marcus + (49)	rabbit	exoAg/B; 67%	PPT	+ (1/1)	-	only homologous bands
			CF	+ (1/1)	+ (3/3)	
1961 Edwards + (24)	human	exoAg/B; 67%	ST	-	38% (8/13)	recruit survey
1977 Rippon + (63)	patients	exoAg/B; 84%	PPT	90% (18/19)	0% (0/40)	fresh sera only

1) Origin of antigen (cyto = cytoplasm; exoAg = acqueous extract, A, or broth filtrate, B); % ethanol used to precipitate the fraction tested.
2) CF = complement-fixation; ST = skin test; PPT = precipitin test; ELISA = enzyme-linked immunoadsorbant assay.

Edwards et al. surveyed 2131 recruits and found 21 to be skin test positive (24). Eight of thirteen histoplasmin-sensitive recruits also reacted to blastomycin and this is the figure entered in the table. These results are somewhat misleading since the disease state of these recruits was not known, and so the true sensitivity of specificity of the antigen remains in doubt.

The most extensive precipitin trial with patient sera was performed recently by Rippon et al. (62). Excellent sensitivity and specificity was reported when fresh sera were tested. Stored sera were virtually unusable. This group also found that the antigens derived from the "+" mating strains of the organism were uniformly more sensitive (100%) than those derived from the "-" strain. In addition, a protein from this preparation was eluted from polyacrylamide gels and found to have esterase activity and to form a precipitin band of identify with the whole ethanol-precipitate. Thus the presence of antibodies to an active enzyme was demonstrated.

With this last exception, the ethanol-precipitates have not been characterized for the specific components responsible for such excellent results. Work by Young (K. D. Young, Ph.D. Thesis, University of Oklahoma, Norman, 1980) has indicated that a component now known as the A antigen can be precipitated with the ethanol concentrations used by previous investigators. It is therefore very likely that the EtOH preparations that have been used could contain this

particular component, the importance of which will be discussed below.

Cytoplasmic. Cytoplasmic antigens have been prepared in several ways, the most common being grinding of cells in a mortar, sonification, and breakage via glass beads. Cell walls and debris were removed by centrifugation and the supernatant generally used without further purification. This fraction was the first to be precipitated with EtOH, and its use as a crude preparation begun soon afterward by Eggers (in: 14). Table 7 presents the history of these fractions.

After an early beginning, a long lag occurred before Martin began investigating cytoplasmic antigens in 1950 (51). He reported "good" results using a protein fraction for CF. A carbohydrate fraction fixed complement with rabbit, but not human, serum, while the protein fraction was effective with either. He later reported that the cytoplasm equalled or exceeded the sensitivity of whole yeast cells in CF (52). At the same time he reported good sensitivity of the fraction for skin-testing, but with an extremely high rate of cross-reactions. A much better showing was given by the extract of Dyson and Evans, but the small number of animals examined makes generalization questionable (22).

Campbell reported the testing of 50,000 sera from recruits (8). She noted that CF tests cross-reacted extensively with histoplasmin-positive sera, and that such

TABLE 7

YEAST FRACTIONS: CYTOPLASMIC

DATE-REFERENCE	SUBJECT	PREPARATION	TEST[1]	SENSITIVITY	CROSS-RXS	REMARKS
1906 Eggers (in:15)	rabbit	mortar	PPT	slight	-	1 pos of "several"
1914 Stober (70)	patients	ground	ST	50% (1/2)	-	
1953 Martin (52)	rabbit	sonified	CF	+ (1/1)	-	PPT positive in
	patients	"	CF	58% (40/69)	33% (5/15)	rabbit & patient sera
	"	"	AGGL	0% (0/34)	-	
	g-pig	"	ST	85% (11/13)	83% (10/12)	
1954 Dyson + (22)	rabbit	ground, H_2O	ST	100% (3/3)	20% (1/5)	best preparation
1960 Campbell (8)	recruits	ground, NaCl	CF	-	33% (/10,000)	survey, not patients
				39% (/1238)	69% (/1238)	
1966 Kaufman (40)	patients	mortar	CF	28% (8/21)	22% (2/9)	
1973 Kaufman + (41)	patients	broken	CF	41% (20/49)	77% (37/47)	
1974 Cox/Larsh (13)	g-pig	broken	ST	89% (24/27)	24% (4/17)	10-30K mol wt fraction best
1977 Rippon + (63)	patients	broken	PPT	90% (18/20)	0% (0/40)	fresh sera only
1979 Green + (30)	patients	broken	PPT	-	-	A antigen absent

1) As in Table 6; AGGL = agglutination.

cross reactions were worse with the precipitin and agglutination tests. Martin described the cytoplasm as "not very specific" (53) and Kaufman et al. recorded the very poor results shown in the table (40, 41). On the other hand, Odds et al. found this preparation more sensitive in CF tests than whole cells or cell walls (56). Kaufman et al. reported it to be the only fraction of sufficient sensitivity for CF (41).

The first real attempt at fractionating the cytoplasm seems to be that of Cox and Larsh (13). Four fractions were collected by ultrafiltration through membranes of decreasing pore size and tested for skin-test reactivity on guinea-pigs. The fraction between 10-30K molecular weight was shown to be the most active and least cross-reactive of the four. This preparation was more specific than blastomycin. It was also more sensitive than an alkali-soluble water-soluble (ASWS) antigen (88%-vs-78%) but gave more cross-reactions (23%-vs-10%). These investigators concluded that the cytoplasmic preparation might be very useful if further purified. Rippon et al. reported a 90% sensitivity in Ouchterlony precipitation with a crude preparation (63).

Green et al. reported that the A antigen of B. dermatitidis was absent in cytoplasmic preparations (30). However this study included only the one isolate, A373. Later work by Young (K. D. Young, Ph.D. Thesis, University of Oklahoma, Norman, 1980) revealed that the presence of

the A antigen in cytoplasmic preparations varied from isolate to isolate, and was associated with two distinct protein bands in polyacrylamide gel electrophoresis (PAGE).

Exoantigen. The term "exoantigen" was first coined by Standard and Kaufman to describe the antigenic material that could be extracted from whole yeast or mycelium by allowing it to stand in an acqueous solution for a period of days to weeks (69). These first applications were directed toward the identification of fungal cultures of H. capsulatum and C. immitis, but the antigens were soon used for serologic purposes as well.

The first mention of the exoantigens of B. dermatitidis was in 1978 (42). These extracts were subsequently used in serologic testing (30, 31). Several earlier investigations employed such extracts and so have been included in this section, the history of which is given in Table 8.

Peck et al. first reported the use of saline extracts of the yeast-phase of B. dermatitidis in 1940 (61). They gave no details in its reactivity, being more interested in their EtOH-precipitate, except to state that the two were probably equivalent antigens.

Martin tested saline extracts only superificially for CF activity, but reported good activity in agglutination studies and excellent sensitivity in skin-testing (52). Busey and Hinton, and Kaufman tested a form of this type antigen by using yeast broth filtrates (7, 41). These had

TABLE 8

YEAST FRACTIONS: EXOANTIGEN[1]

DATE-REFERENCE	SUBJECT	PREPARATION[2]	TEST[3]	SENSITIVITY	CROSS-RXS	REMARKS
1953 Martin (52)	rabbit	saline	CF	+ (1/1)	-	
	patients	"	"	- (0/1)	-	
	"	"	AGGL	75% (26/35)	-	
	g-pig	"	ST	92% (12/13)	17% (2/12)	
1967 Busey + (7)	patients	broth filt.	PPT	77% (64/80)	33% (16/49)	cross-rxs distinguish-able by band pattern
	"	"	CF	50% (33/65)	-	
1973 Kaufman + (41)	patients	broth filt.	PPT	59% (29/49)	34% (16/47)	distinguish by pattern; A and B bands named
1978 Kaufman + (42)	-	water, 50X	PPT	100% (56/56)	0% (0/99)	identification of cultures with anti-A serum
1980 Green + (31)	patients	(saline, DEAE-	CF	70% (10/16)	0% (0/5)	purified A antigen
	"	cellulose)	ELISA	92% (25/27)	20% (2/12)	used in all tests
	"	"	PPT	52% (13/25)	-	
	g-pig	"	ST	100% (13/13)	0% (0/15)	

1) Antigen prepared by saline- or water-extraction of whole cells; broth filtrates also included in table.
2) Extraction procedure.
3) As in Table 6.

lower sensitivity and higher cross-reactivity than the acqueous extracts.

After it was recongized that specific antigens could be extracted from the fungi with water, Kaufman and Standard used this procedure to show that cultures of B. dermatitidis could be identified with 100% accuracy (42). Green et al. identified the A antigen, and a new D antigen, in the water-soluble fraction (30). Using this preparation in immunoelectrophoresis, they removed specific precipitin arcs and by immunizing rabbits produced the first reference serum for the A antigen.

This reference serum was useful in monitoring further purification of saline extracts by anion exchange chromatography. With a purified A antigen fraction from this procedure, Green et al. reported a remarkably good combination of sensitivity and specificity in CF, the enzyme-linked immunosorbant assay (ELISA) procedure, and skin-testing (31). Although the ELISA test showed cross-reactions in 2 out of 12 patients with other mycoses, the titers were only 1:8. The only test to fall behind was the precipitin test which detected only half the known cases. However, the patient sera used for this study had been in storage, and the observation of Rippon et al. concerning the negative effects of serum storage may explain this much lower sensitivity (63).

The emphasis of these exoantigen preparations has been directed mainly toward the characterization of that component termed the A antigen. This antigen of B.

dermatitidis may be the best characterized and have the most diagnostic potential of all those studied to date. This antigen will be described in more detail separately.

The A. Antigen. The A antigen was first named by Kaufman et al. as that antigen forming a precipitin line located nearer the antigen well (41). A "B" antigen was also described which formed nearer the well containing patient serum.

Palmer et al. considered that precipitation with the A antigen was diagnostic for a current or recent case of blastomycosis, although a negative reaction was not conclusive proof of absence of the disease (57). That these preparations were not pure was first mentioned by Kaufman and Standard, who found that reference serum produced 2-3 precipitin bands in addition to the A band (42).

The first "A-only" reference serum was prepared by Green et al. as described earlier (30). Using this serum they monitored the purification and characterization of the A antigen. The antigen was found to be resistant to boiling for two minutes, and moderately resistant for periods up to 10 minutes. It was not degraded by trypsin, proteinases IV, V, or VII (Sigma), or nucleases. The protein-to-carbohydrate ratio of the preparation was 10:1. The active fraction could be eluted from anion exchange columns with 0.6 M NaCl (31).

That this particular preparation still contained extraneous components was shown by Young (K. D. Young, Ph.D. Thesis, University of Oklahoma, Norman, 1980). Five prominent glycoprotein bands could be visualized in PAGE analysis using Coomassie blue, and a minimum of 20 additional proteins were detected by a more sensitive staining technique. The components associated with the A antigenic activity were identified by a combination of comparison with other antigen preparations and affinity chromatography. Two proteins, one a glycoprotein, were reported to be the active components.

These proteins may correspond to the two very closely located precipitin lines, which can be mistaken for a single band, in the A-antigen/reference A-antiserum system. That the reference serum was not monospecific was also evidenced by its formation of at least two additional precipitin lines to other antigen preparations.

The effect of this characterization may be to stimulate production of purer preparations, thus hopefully avoiding possible cross-reactions with future heterologous sera. At least, such characterization will allow sensitive monitoring of antigen preparations for the presence and purity of the A antigen.

The location and function of this antigen are still in doubt. It was reported to be present in the yeast cell wall, but absent from the cytoplasm (30). This conclusion was reached from studies of only two isolates of B. dermatitidis. Later work showed that the cytoplasm can

contain substantial amounts of the A antigen, depending on the isolate (K.D. Young, Ph.D. Thesis).

The degree to which the A antigen has proven itself in diagnostic work and the extent of its characterization make it the most likely candidate for immediate serologic and immunologic use. However, further purification and analysis are required before even this single antigen of B. dermatitidis can be understood in terms of its relationship to the organism and to the disease.

ASWS. The alkali-soluble water-soluble (ASWS) fraction of the cell wall of B. dermatitidis was first referred to by Cox and Best (12). Comparison of this fraction from two isolates of different virulence revealed the compositions to be equivalent. The fractionation procedure for this antigen and its use in skin-testing was reported by Cox and Larsh (13). Overall, ASWS had somewhat lower sensitivity than cytoplasmic antigens but had a lower rate of cross-reactivity, also. Subsequent results using this preparation are summarized in Table 9.

The antigen was shown to be extremely sensitive in identifying infected guinea pigs by skin-test, macrophage migration inhibition (MIF) and lymphocyte transformation (LT) assays (17). The optimal dose was 100 micrograms, lower doses giving decreasing sensitivity. A similar study by Hall et al. repeated the excellent LT responses, and further established that the variation between two antigen lots was insignificant (32). Further animal studies

TABLE 9

YEAST FRACTIONS: ASWS[1]

DATE-REFERENCE	SUBJECT	TEST[2]	SENSITIVITY	CROSS-RXS	REMARKS
1974 Cox/Larsh (13)	g-pig	ST	56% (31/55)	0% (0/29)	48 hr: 10-30K mol wt fraction
	"	"	40% (22/55)	2% (1/51)	48 hr: whole ASWS
1976 Lancaster + (46)	g-pig	ST	100% (10/10)	0% (0/10)	slowest peak in preparative PAGE
1977 Deighton + (17)	g-pig	ST	88% (21/24)	7% (1/14)	100 µg
		MIF	83% (10/12)	0% (0/10)	"
		LT	92% (12/13)	0% (0/11)	"
1978 Hall + (32)	g-pig	LT	100% (28/28)	0% (0/8)	10 - 100 µg
1979 Cox (11)	human	LT	-	0% (0/11)	coccidiodin-reactive subjects
	"	"	-	100% (13/13)	histoplasmin-reactive subjects
1980 Hall + (33)	g-pig	ST	Var[3]	Var	depends on infecting strain
	"	LT	Var	Var	and strain used in antigen
	"	MIF	Var	Var	preparation

1) ASWS = alkli-soluble, water-soluble cell wall antigen; prepared as in Cox and Larsh (13).
2) As in Table 6; plus, MIF = macrophage migration inhibition factor; LT = lymphocyte transformation assay.
3) Variable.

revealed that the sensitivity and specificity of the antigen depended on the particular isolate used for animal sensitization (33). Of 3 isolates tested, skin-test sensitivity varied between 40-80%, and was correlated with the virulence of the infecting organism. The most virulent isolates elicited the greatest number of positive skin tests. The LT and MIF assays reacted well with antigens from all three isolates, but reflected the same type of variation among the three infected animal groups.

The success of these tests in animals has not been repeated in the one report with human subjects. Using the LT assay, Cox found that Blastomyces ASWS elicited reactions in 13 of 13 subjects with positive skin-tests to histoplasmin (11). She reported no cross-reactivity to 11 coccidiodin-sensitive subjects.

Only two attempts have been reported regarding the further purification of the ASWS antigen. Cox and Larsh found that the 10-30K molecular weight portion was slightly more sensitive in skin-tests than was the whole preparation (13). Lancaster and Sprouse isolated the slowest moving of four protein fractions in preparative PAGE and showed it to give optimum results in skin-testing guinea pigs (46). Neither of these fractions has been tested in patients.

The relationship of this antigen to other similar preparations from other pathogenic fungi has been investigated by immunoelectrophoresis (35). Five precipitin lines formed

between it and an anti-coccidiodin reference serum. These five components were also uniformly present in every antigen preparation from H. capsulatum and C. immitis that was tested. These results highlight the fact that the ASWS fraction is still only semi-pure.

The ASWS antigen is the only preparation of B. dermatitidis that can be accurately and reproducibly standardized on a weight basis (12, 13). It has shown exceptional usefulness in skin-testing guinea pigs but its usefulness in humans is still in question. Its sensitivity to variations in the infecting organism may tell more about the disease process and the organisms themselves than about the disease state of the individual.

The cross-reactivity in humans may be due to the multiple-component nature of the antigen, or it may reflect some important common link between infections caused by the pathogenic fungi. Either possibility will be more easily answered by utilizing the current methods of further purification mentioned above, and the devising of new procedures.

Monoclonal Antibody Production

The above discussions center around antigens purified by physical means and generally characterized by immunologic tests which contain multiple reactive components. An alternate approach is to purify specific antigens by immunological means, thereby partially combining

the isolation and characterization steps. A method has been developed in the past few years whereby pure antibodies may be produced which are specific for only one antigenic determinant. This development opens the way for purification of components by attaching such specific antibodies to affinity gels.

The application of this "hybridoma" technology to isolation of components of B. dermatitidis has been recently reported (K. D. Young, PhD Thesis, University of Oklahoma, Norman, 1980). In this instance, one protein of a cytoplasmic preparation of B. dermatitidis was isolated by affinity chromatography. Neither the immunologic or the diagnostic importance of this antigen has been studied. The establishment of a cell line secreting antibody specific for this protein does, however, make its isolation and characterization capable of being standardized, and underlines the usefulness of this method in future work with the pathogenic fungi.

CHEMICAL COMPOSITION

The study of the above antigens' biological activity and that of their chemical nature must eventually merge if they are to be truly understood. The chemical composition of B. dermatitidis' yeast cell walls has been most deeply studied and will be presented first.

Cell Wall

General composition. The overall sugar content of yeast cell walls has been measured by several workers. The major hexose is glucose (12, 19, 36, 38), and major amino sugar is glucosamine (12, 36, 38). Glucosamine is present in a 1.5X greater amount in yeast than in mycelial walls (64). Lipid has been reported (36, 37), and in many cases has been removed prior to cell wall analysis without significantly affecting the values of other components (19, 61). Chitin content has been reported to be greater in more virulent isolates (12), and may comprise between 24-43% of the cell wall (12, 19, 37). The yeast phase has 3 times the amount of chitin as the mycelial phase (37). Amino acids comprise 8-11% of the total composition (38).

Mannose constitutes only about 1% of the wall, but seems to be a major component in several extracts (19, 38, 61).

In one case, one-half of all the wall mannose appeared in an ethylenediamine-methanol-water soluble fraction (19). Mycelial walls generally contain more of this sugar (19). Galactose is also present in small amounts, often associated with mannose (37).

A recent study with fluorescent lectins has identified the following as present as terminal sugars: alpha-D-mannopyranose, beta-D-galactopyranose, beta-D-acetylgalactosamine, beta-D-N-acetylglucosamine, alpha-L-fucopyranose, and sialic and/or uronic acid (71).

In one instance, the similarity of the chemical compositions of the walls of B. dermatitidis and H. capsulatum chemotype II was advanced as a possible reason for the extensive serologic cross-reactivity between these two organisms (19).

Glucan type. From the solubilities of wall material treated with alkali, Kanetsuna et al. calculated that the yeast glucan present was 95% alpha glucan and 5% beta glucan, both probably 1-3 linked (38). Later use of beta-1, 3-glucanase was uniformly ineffective in degrading yeast cell walls, suggesting that beta-1, 3-glucan was either absent or present in undectable amounts (16, 19, 56). A slight cytoplasmic disruption, though, was seen in whole cells treated in this manner which may indicate a very low presence of the beta glucan (56).

Lipids. Lipids have been reported to comprise from 3-10% of the total yeast (20, 36, 59). Whole cells were 8-10%

lipid (59), and a breakdown between walls and cytoplasm yielded 3% and 12-18% compositions, respectively (20). Mycelium contained far less lipid in both fractions (20).

Peck and Hauser identified the lipid components isolated from whole cells (59). The phosphatide fraction comprised 24-34% of the total lipids and included the presence of carbohydrate, ethanolamine, and choline. Glycerophosphoric acid composed 3.1% of the total fraction, and fatty acids, 65%. The following fatty acids were present in these percentages of total lipids: oleic, 49.1%; palmitic, 8.2%; stearic, 4.2%; linoleic, 3.5%. An acetone-soluble fraction comprised 66-76% of total lipids and included 4% sterols (glycerol and ergosterol) and 88% fatty acids. These fatty acids were present: oleic, 55%; linoleic, 18.5%; palmitic, 9.7%; stearic, 4.8:.

Domer and Hamilton investigated the composition of the walls alone (20). The greatest single component was the triglyceride fraction. The most concentrated phospholipids were phosphatidyl choline, phosphatidylethanolamine and phosphatidylserine. Also present were diglycerides, sterols, sterol esters, and free fatty acids. In the yeast walls, oleic acid was predominate among the fatty acids, while mycelial walls had an equal or greater amount of linoleic acid. In the wall fraction, more oleic and less linoleic acid was present in all lipid classes than in cytoplasmic fractions.

All the work discussed above analysed the easily extractable lipids. Peck et al. examined the cells after the removal of these and found the cells to be composed of an additional 5.7% firmly bound lipid fraction (60). These consisted of much the same components as described previously, but their relative abundance not mentioned.

Protein. In 1940, Peck et al. reported that the protein fraction of whole cells was the skin-test active fraction (61). Taylor later showed that protein could be removed from the cell wall of whole organisms by trypsin treatment, thereby proving that some protein is located on the wall's exterior (72). Total protein in the walls may vary from 5-10% (12, 37, 38). Mycelial walls have 3-4 times more protein content than yeast walls (30%-vs-7%; in: 37). Cox and Best also reported that the walls of a more virulent isolate were composed of twice as much protein than a less virulent isolate (10.5%-vs-5%; in: 12).

ASWS. Before the explicit naming of this fraction, Kanetsuna and Carbonell reported on the composition of an alkali-soluble, non-acetic acid-precipitable preparation from yeast walls (38). The fraction was 32% hexose, 0% amino sugar, and 0% amino acids. The major constituent seemed to be a polysaccharide of glucose:galactose:mannose in a 1:5.2:6.8 ratio. This comprised most of the cell wall galactose and mannose, and was present in very small yield.

The polysaccharide composed of these three sugars was

also detected in this fraction by Cox and Best (12). But although present in the original ASWS fraction, further purification of the carbohydrates by Azuma et al revealed the absence of any galactomannan (3). Whether this is a general phenomenon or is isolate-dependent is not known. The hydrolysis of mannan-protein complexes is known to be one of the actions of alkali extractions (47), and so the appearance of this sugar in this fraction should be expected.

The absence of hexosamines in an ASWS preparation was later confirmed (64), but protein and amino acids were later detected (12, 14, 65). After treatment with alkali, yeast walls released twice as much protein as mycelial walls (65). The protein fraction most active in skin-testing was determined to be in the 10-30K molecular weight range (13). PAGE analyses of ASWS have performed and the results are given in Table 10. These will be discussed below in relation to such analyses for several protein preparations.

PAGE Analyses

Polyacrylamide gel electrophoresis data of various antigen preparations are organized in Table 10. Bands have been lettered according to the results presented by Cox and Larsh (14). Placement of an Rf value in one band or another does not imply an identity of these components since the data are from several reports which used varying procedures.

TABLE 10

PAGE ANALYSES OF ANTIGENS[1)]

BAND 2)	50K 3)	30K 3)	10K 3)	009BY 4)	ASWS 3)	ASWS 5)	COM-B 9)	KCB-B 9)	YF+ 6)	YF- 6)	MYC 7)	A-AG 8)
A	06*	04		04*				04*			05*	
B	09*	07		06*	07	11	08*	08*				
b		11		10							15	
C	24*	21		23	24			24*				18*20* 22*
D	26			26							27	26*
E	30	33	33	32			32			30 35	30* 31	29*
F	38	39		39*	43			42	40,44 46		40,43	
G	49*	50	50	53*		53		49			50	54
H	63*	66		62*					65		68*	
I	72	75	75			77	75	70	70	70 75	72	
J	80			81	85							
K	98	89	94	97*	99	99	99		95		97	

1) Protein bands; "*" = carbohydrate stainable also; values relative to dye front, dye front = 100.
2) Band designations as in (14); use as a comparative guide only.
3) Cytoplasmic mol. wt. fractions as in (14); ASWS as in (14); 7.5% gels.
4) Cytoplasmic fraction of Young (Thesis); 14% gels; also had 9 additional proteins with Rf = 17,20,35,41,45,55,64,78,89.
5) ASWS as in (46); 5% gels.
6) "+" or "-" strains of A. dermatitidis, as in (63); 8.5% gels.
7) As in (39); nucleoprotein at Rf = 31; 7.5% gels.
8) A antigen of Green et al. (31); data from Young (Thesis); 14% gels.
9) Commercial blastomycin (COM-B) or KCB-prepared blastomycin (KCB-B); as in (14); 7.5% gels.

The greatest number of proteins was detected by Young in a cytoplasmic preparation (K. D. Young, Ph.D. Thesis, University of Oklahoma, Norman, 1980). At least 20 proteins could be identified with Coomassie blue staining, and others by a more sensitive silver stain. Although the number of bands visualized in the 50K preparation (14) was less than in 009BY, the arrangement of the major peaks was the same. The prominent A and B doublet and the G, H and I triplet were both present, as well as equivalent bands between them. In most cases in the table, protein staining was accomplished with Amido Black, which, since less sensitive than Coomassie blue, may account for many of the omitted proteins.

Cox and Larsh found four bands (A, B, C and F) to be consistently present in their skin-test reactive 30K fraction (14). One of these four, they concluded, was probably responsible for the activity. In a study on ASWS, Lancaster and Sprouse determined that the slowest moving band in their preparation was the active skin-test component (46). This band falls in the range of the B band of Cox and Larsh. Using immunofixation, it has also been noted that immune rabbit serum fixes, to the extent measurable with this technique, only the B band (K. D. Young, unpublished observations). This band is also the only one common to 7 of the first 8 preparations in the table, which were skin-test reactive, though to varying degrees. No skin-test data was given for the remaining

three preparations. This B band has been determined to be a glycoprotein, where tested, and the evidence seems to indicate that further effort in purifying this component would be valuable.

The major glycoproteins of the A antigen are recorded in Table 10. Also recorded is one additional protein (Rf = 54) which only appeared in the A antigen after silver staining. The bands at Rf's 18 and 54 are those found to be active in Ouchterlony precipitin tests against a reference anti-A serum (K. D. Young, Ph.D. Thesis, University of Oklahoma, Norman, 1980). Neither of these can be unambiguously matched with proteins in the other preparations.

Although several antigens have been analyzed by PAGE, most of these have been preliminary characterizations of crude preparations. The two exceptions are the preparative work of Lancaster and Sprouse (46) and the use of PAGE to detect specific proteins removed by affinity chromatography (K. D. Young, PhD Thesis). The isolation and testing of the numerous components listed in Table 10 would be useful in identifying more precisely the roles played by the various antigens.

Miscellaneous

Extracellular enzymes. Since extracellular enzymes are potentially important antigens, the list of those associated with B. dermatitidis will be presented. The

following have been detected: acid and alkaline phosphatase (5, 63), leucine amino peptidase, alpha-esterase (63), beta-1,3-glucanase, N-acetyl-beta-D-glucosaminidase, and alpha-D-mannosidase (16). Those absent are: alpha- or beta- glucosidase and alpha- or beta-galactosidase (5, 16).

Two enzymes have definitely been shown to elicit antibodies in patient sera. A precipitin band showing catalase activity was reported by Andrieu et al. (2), and one with alpha-esterase activity was identified and purified by PAGE (63).

Mannose-containing antigens. In the discussions above, mannose-containing components have been reported isolated in several instances. These instances are summarized since the fractions in which these are found often give excellent sensitivity and specificity. The component is generally a galactomannan, although glucose also may be present. Such carbohydrate compounds have been identified in ethanol-precipitates (39, 59, 61), in whole cells (71), and in the ASWS antigen (12, 19, 38). However, in this last antigen, additional purification of the carbohydrates revealed the absence of galactomannan (3). Since these compounds occur so frequently in reactive preparations, it seems worthwhile to specifically isolate them for further testing.

Virulence correlations. Although not directly related to antigenicity, it is of interest to review the

factors which have been associated with the degree of virulence of B. dermatitidis. These are: increasing lipid content (18), including phospholipid (12); increasing protein and chitin content (12); cell wall material minus protein (72); and decreasing alkali-soluble material (12). This latter observation has obvious relevance to the isolation and use of the ASWS antigen.

SUMMARY

"Without these tools (skin-test and complement fixation antigens for epidemiological, diagnostic, and prognostic use) we are at least 50 years behind in our defining of the disease blastomycosis" (63). This statement by Rippon et al. emphasizes the need for a well defined antigen or group of antigens from Blastomyces dermatitidis, not only for the two tests named above, but for any serological or immunological test. Several different preparations have been reviewed which are beginning to approach the quality necessary for such a sensitive and specific antigenic tool. All of these require further characterization and, in most instances, purification.

One purified fraction isolated from blastomycin, the F fraction, has shown exceptional promise as a skin-test agent in guinea pigs but has not been further studied. A 10-30K molecular weight fraction of the yeast cytoplasm has shown good reactivity, and contains a protein shown by PAGE to be common to several skin-reactive preparations. This fraction has not been further purified.

An ASWS preparation has been partially characterized and shown to be especially sensitive and specific in animals, though not yet in humans. Purification of this fraction by

PAGE has uncovered a highly reactive protein which may be related to one isolated from the cytoplasm. Investigations using this purified component have not been attempted in humans.

The A antigen has been well characterized regarding its applicability to human diagnosis. It has been partially purified and two of its components associated with its reactivity. It also has probably the best possibility as an immediate serologic tool. Even here, though, current preparations contain much extraneous material which could conceivably create cross-reactivity problems in the future.

The ethanol-precipitate antigens which have shown such superior results in the past have not been employed recently, nor have they been extensively characterized. This fraction may contain the reactive A antigen or other antigens deserving of study. Hopefully, the use of this technique can be encouraged as one starting point for isolation procedures.

The importance and isolation of enzymes and mannose containing antigens have probably not received adequate attention. An almost uniform identification of mannose in reactive preparations argues for purification procedures based on its presence.

Finally, use of hybridoma technology to produce antibodies specific for antigens of B. dermatitidis promises to improve our understanding of the organism and to help isolate purer antigenic fractions.

The search for antigens of importance in the immunology of B. dermatitidis should not be confined to any one or all of the antigens discussed above. A variety of components will likely be necessary for complete understanding of the disease and for diagnostic use. It has been observed that "information of clinical value can be obtained from immunological reactions to several different antigens" (35), thereby encouraging continuous efforts to obtain as many as possible.

LITERATURE CITED

1. Abernathy, R. S., and D. C. Heiner, 1961. Precipitation reactions in agar in North American blastomycosis. J. Lab. Clin. Med. 57:604-611

2. Andrieu, S., J. Biguet, L. Dujardin, and T. Vaucelle. 1969. Etude antigenique des agents des mycoses profondes par l'analyse comparee des milieux de culture. I. Histoplasma capsulatum et H. duboisii. Relations avec H. farciminosum, Gymnoascus demonbreunii, Blastomyces dermatitidis et Paracoccidioides brasiliensis. Mycopath. Mycol. Appl. 39:97-108.

3. Azuma, I., F. Kanetsuna, Y. Tanaka, Y. Yamamura, and L. M. Carbonell. 1974. Chemical and immunological properties of galactomannans obtained from Histoplasma duboisii, Histoplasma capsulatum, Paracoccidioides brasiliensis and Blastomyces dermatitidis. Mycopath. Mycolog. Appl. 54:111-125.

4. Balows, A. 1963. Comparison of Blastomyces dermatitidis vaccine and blastomycin as skin testing agents for the diagnosis of blastomycosis. Mycopath. Mycolog. Appl. 19:151.

5. Beneke, E. S., R. W. Wilson, and A. L. Rogers. 1969. Extracellular enzymes of Blastomyces dermatitidis. Mycopath. Mycolog. Appl. 39:325-328.

6. Busey, J. F. 1964. North American blastomycosis. Gen. Prac. 30:88-95.

7. Busey, J. F., and P. F. Hinton. 1967. Precipitins in blastomycosis. Amer. Rev. Resp. Dis. 95: 112-113.

8. Campbell, C. C. 1960. The accuracy of serologic methods in diagnosis. Ann N. Y. Acad. Sci. 89:163-177.

9. Campbell, C. C., and G. E. Binkley, 1953. Serologic diagnosis with respect to histoplasmosis, coccidioidomycosis, and blastomycosis and the problem of cross-reactions. J. Lab. Clin. Med. 42:896-906.

10. Chick, E. W. 1970. North American blastomycosis. p. 465-506. In: E. Uehlinger (Ed.), The Pathologic Anatomy of Mycoses. Human Infection with Fungi, Actinomycetes, and Algae. Springer-Verlag, Berlin.

11. Cox, R. A. 1979. Cross-reactivity between antigens of Coccidioides immitis, Histoplasma capsulatum, and Blastomyces dermatitidis in lymphocyte transformation assays. Infect. Immun. 25:932-938.

12. Cox, R. A. and G. K. Best. 1972. Cell wall composition of two strains of Blastomyces dermatitidis exhibiting differences in virulence for mice. Infect. Immun. 5:449-453.

13. Cox, R. A., and H. W. Larsh. 1974. Isolation of skin test-active preparations from yeast-phase cells of Blastomyces dermatitidis. Infect. Immun. 10:42-27.

14. Cox, R. A., and H. W. Larsh. 1974. Yeast- and mycelial-phase antigens of Blastomyces dermatitidis: comparison using disc gel electrophoresis. Infect. Immun. 10:48-53.

15. Davis, B. F. 1911. The immunological reactions of oidiomycosis (blastomycosis) in the guinea pig. J. Inf. Dis. 8:190-240.

16. Davis, T. E., Jr., J. E. Domer, and Y-T. Li. 1977. Cell wall studies of Histoplasma capsulatum and Blastomyces dermatitidis using autologous and heterologous enzymes. Infect. Immun. 15:978-987.

17. Deighton, F., R. A. Cox, N. K. Hall, and H. W. Larsh. 1977. In vivo and in vitro cell-mediated immune responses to a cell wall antigen of Blastomyces dermatitidis. Infect. Immun. 15:429-435.

18. DiSalvo, A. F., and J. F. Denton. 1963. Lipid content of four strains of Blastomyces dermatititis of different mouse virulence. J. Bacteriol. 85:927-931.

19. Domer, J. E. 1971. Monosaccharide and chitin content of cell walls of Histoplasma capsulatum and Blastomyces dermatitidis. J. Bacteriol. 107:870-877.

20. Domer, J. E., and J. G. Hamilton. 1971. The readily extracted lipids of Histoplasma capsulatum and Blastomyces dermatitidis. Biochim. Biophys. Acta. 231:465-478.

21. Dulaney, A. D. 1930. Immunologic studies in blastomycosis. J. Immunol. 19:357-370.

22. Dyson, J. E., Jr., and E. E. Evans 1954. Skin test antigens from yeast phase cultures of Blastomyces dermatitidis and Histoplasma capsulatum. Univ. Mich. Med. Bull. 20:53-61.

23. Dyson, J. E., Jr., and E. E. Evans 1955. Delayed hypersensitivity in experimental fungus infections. The skin reactivity of antigens from the yeast phase of Blastomyces dermatitidis. J. Invest. Dermatol. 24:447-452.

24. Edwards, P. Q., R. A. Knight, and S. Marcus. 1961. Skin sensitivity of human beings to Histoplasma capsulatum and Blastomyces dermatitidis polysaccharide antigens. Amer. Rev. Resp. Dis. 83:528-534.

25. Emmons, C. W., C. B. Binford, J. P. Utz, and K. J. Kwong-Chung. 1977. Blastomycosis. p. 342-364. In: Medical Mycology, 3rd ed., Lea & Febiger, Philadelphia.

26. Emmons, C. W., B. J. Olson, and W. W. Eldridge. 1945. Studies of the role of fungi in pulmonary disease. I. Cross reactions of histoplasmin. Public Health Rep. 60:1383-1394.

27. Gilchrist, T. C. 1894. Protozoan dermatitis. J. Cutan. Gen. Dis. 12:496-499.

28. Gilchrist, T. C. 1896. A case of blastomycotic dermatidis in man. Johns Hopkins Hosp. Rep. 1:269-83.

29. Gilchrist, T. C., and W. R. Stokes, 1898. The presence of an oidium in the tissues of a case of pseudo-lupus vulgaris. Johns Hopkins Hosp. Rep. 7:129-133.

30. Green, J. H., W. K. Harrell, J. E. Johnson, and R. Benson 1979. Preparation of reference antisera for laboratory diagnosis of blastomycosis. J. Clin. Microbiol. 10:1-7.

31. Green, J. H., W. K. Harrell, J. E. Johnson, and R. Benson 1980. Isolation of an antigen from Blastomyces dermatitidis that is specific for the diagnosis of blastomycosis. Current Microbiol. (Dec. issue).

32. Hall, N. K., F. Deighton, and H. W. Larsh. 1978. Use of an alkali-soluble water-soluble extract of Blastomyces dermatitidis yeast-phase cell walls and isoelectrically focused components in peripheral lymphocyte transformations. Infect. Immun. 19:411-415.

33. Hall, N. K., F. Deighton, and H. W. Larsh. 1980. An alkali soluble water extract of yeast phase cells walls of Blastomyces dermatitidis. p. 209-217. In: H.-J. Preusser (ed.). Medical Mycology Proceedings. Mycological Symposium of the Twelfth International Congress of Microbiology. Gustav-Fisher-Verlag, New York.

34. Howell, A., Jr. 1947. Studies of fungus antigens. I. Quantitative studies of cross-reactions between histoplasmin and blastomycin in guinea pigs. Public Health. Rep. 62:631-651.

35. Huppert, M., J. P. Adler, E. H. Rice, and S. H. Sun 1979. Common antigens among systemic disease fungi analyzed by two-dimensional immunoelectrophoresis. Infect. Immun. 23:479-485.

36. Kanetsuna, F. 1967. Estudio bioquimico del Paracoccidiodes brasiliensis. Acta Cient Venezolana Suppl. 3:308-317.

37. Kanetsuna, F., L. M. Carbonell, R. E. Moreno, and J. Rodriguez. 1969. Cell wall composition of the yeast and mycelial forms of Paracoccidioides brasiliensis. J. Bacteriol. 97:1036-1041.

38. Kanetsuna, F., and L. M. Carbonell. 1971. Cell wall composition of the yeastlike and mycelial forms of Blasstomyces dermatitidis. J. Bacteriol 106:946-948.

39. Kaskhin, J. P., A. I. Drosdov, and V. A. Ponomarenko. 1978. Comparative immunochemical studies of different antigens of deep mycosis pathogens. Ann. Immunol. (Inst. Pasteur) 129:287-300.

40. Kaufman, L. 1966. Serology of systemic fungus diseases. Public Health Rep. 81:177-185.

41. Kaufman, L., D. W. McLaughlin, M. J. Clark, and S. Blumer 1973. Specific immunodiffusion test for blastomycosis. Appl. Microbiol. 26:244-247.

42. Kaufman, L., and P. Standard. 1978. Immuno-identification of cultures of fungi pathogenic to man. Curr. Microbiol. 1:135-140.

43. Keeney, E. L., and N. Eriksen. 1948. The chemical isolation and biologic assay of extracellular antigenic fractions from pathogenic fungi. J. Allergy 20:172-184.

44. Knight, R. A., and S. Marcus. 1958. Polysaccharide skin test antigens derived from Histoplasma capsulatum and Blastomyces dermatitidis. Amer. Rev. Tubercul, Pul. Dis. 77:983-989.

45. Lancaster, M. V., and R. F. Sprouse, 1976. Preparative isotachophoretic separation of skin test antigens from blastomycin purified derivative. Infect. Immun. 13:758-762.

46. Lancaster, M. V., and R. F. Sprouse. 1976. Isolation of a purified skin test antigen from Blastomyces dermatitidis yeast-phase cell wall. Infect. Immun. 14:623-625.

47. Lee, C.H., S. K. Tsang, R. Urakabe, and C. K. Rha. 1979. Disintegration of dried yeast cells and its effect on protein extractability, sedimentation property, and viscosity of the cell suspension. Biotechnol. Bioeng. 21:1-17.

48. Long, E. R. 1926. Specific necrosis in the infectious granulomas. Demonstration in the testis in experimental tuberculosis, bacillus abortus infection and blastomycosis. J. Amer. Med. Assoc. 87:1441-1445.

49. Marcus, S., G. A. Hill, and R. A. Knight. 1960. Antigens of Blastomyces. Ann. N. Y. Acad. Sci. 89:193-201.

50. Martin, D. S. 1935. Complement-fixation in blastomycosis. J. Infect. Dis. 57:291-295.

51. Martin, D. S. 1950. Practical applications of immunologic principles in the diagnosis and treatment of fungus infections. Ann. N. Y. Acad. Sci. 50:1376-1379.

52. Martin, D. S. 1953. Serologic studies on North American blastomycosis. Studies with soluble antigens from untreated and sonic-treated yeast phase cells of Blastomyces dermatitidis. J. Immunol. 71:192-201.

53. Martin, D. S. 1957. Evaluation of skin tests and serologic methods in fungus infections. J. Chron. Dis. 5:580-591.

54. Martin, D. S., and D. T. Smith. 1939. Blastomycosis (American blastomycosis, Gilchrist's disease). I. A. review of the literature. Amer. Rev. Tuberculosis 39:275-304.

55. Mellon R. R. 1926. Studies in microbic heredity. VII. Observations on the genetic origin of the several types of fungi found in the lesions of blastomycosis hominis. J. Bacteriol. 11:419-431.

56. Odds, F. C., L. Kaufman, D. McLaughlin, C. Callaway, and S. O. Blumer. 1971. Effect of chitinase complex on the antigenicity and chemistry of yeast-form cell walls and other fractions of Histoplasma capsulatum and Blastomyces dermatitidis. Sabouraudia 12:138-149.

57. Palmer, D. F., L. Kaufman, W. Kaplan, and J. J. Cavallaro. 1977. Serodiagnosis of Mycotic Diseases. Charles C. Thomas Pub., Springfield, Illinois, 191 pp.

58. Pates, A. L. 1948. Precipitin reactions in experimental histoplasmosis and blastomycosis. Science 108:383-385.

59. Peck, R. L., and C. R.Hauser. 1938. Chemical studies of certain pathogenic fungi. I. The lipids of Blastomyces dermatitidis. J. Amer.Chem. Soc. 60:2599-2603.

60. Peck, R. L., and C. R. Hauser. 1940. Chemical studies of certain pathogenic fungi. III. Further studies on the lipids of Blastomyces dermatitidis and Monilia albicans. J. Biol. Chem. 134:403-412.

61. Peck, R. L., D. S. Martin, and C. R. Hauser. 1940. Polysaccharides of Blastomyces dermatitidis. J. Immunol. 38:449-455.

62. Ricketts, H. T. 1901. Oidiomycosis (blastomycosis) of the skin and its fungi. J. Med. Res. 6:374-546.

63. Rippon, J. W., D. N. Anderson, S. Jacobsohn, M. S. Hoo, and E. D. Garber. 1977. Blastomycosis: specificity of antigens reflecting the mating types of Ajellomyces dermatitidis. Mycopath. 60:65-72.

64. Roy, I., and J. W. Landau. 1972. Composition of the alkali resistant cell wall material of dimorphic Blastomyces dermatitidis. Sabouraudia 10:107-112.

65. Roy, I., and J. W. Landau. 1972. Protein constituents of cell walls of the dimorphic phases of Blastomyces dermatitidis. Can. J. Microbiol. 18:473-478.

66. Salvin, S. B. 1949. The serologic relationship of fungus antigens. J. Lab. Clin. Med. 34:1096-11-4.

67. Sarosi, G. A., and S. F. Davies. 1979. Blastomycosis. State of the art. Amer. Rev. Resp. Dis. 120:911-938.

68. Saslaw, S., and C. C. Campbell. 1949. A comparison between histoplasmin and blastomycin by the collodion agglutination technique. Public Health Rep. 64:290-294.

69. Standard, P. G., and L. Kaufman. 1976. Specific immunological test for the rapid identification of members of the genus Histoplasma. J. Clin, Microbiol. 3:191-199.

70. Stober, A. M. 1914. Systemic blastomycosis. A report of its pathological, bacteriological and clinical features. Arch. Intern. Med. 13:509-556.

71. Stoddart, R. W., and B. M. Herbertson. 1978. The use of fluorescein-labelled lectins in the detection and identification of fungi pathogenic for man: a preliminary study. J. Med. Microbiol. 11:315-324.

72. Taylor, J. J. 1964. Enhanced toxicity in chemically altered Blastomyces dermatitidis. J. Bacteriol. 87:748-749.

73. Tenenberg, D. J., and A. Howell, Jr. 1948. A complement-fixation test for histoplasmosis. I. Technic and preliminary results on animal sera. Public Health. Rep. 63:163-168.

74. Veterans Administration Cooperative Study: Blastomycosis. I. A review of 198 collected cases in Veterans Administration Hospitals. 1964. Amer. Rev. Resp. Dis. 89:659-672.

CHAPTER 2

IDENTIFICATION OF THE ACTIVE PRECIPITIN COMPONENTS IN A PURIFIED PREPARATION OF THE A ANTIGEN OF *BLASTOMYCES DERMATITIDIS*.

ABSTRACT

A purified A antigen preparation of *Blastomyces dermatitidis* was determined to be composed of five major glycoprotein bands, visible with Coomassie blue and periodic acid-Schiff staining of polyacrylamide gels. A minimum of 20 additional protein bands were detected using a silver stain which is 100 times more sensitive than the Coomassie method. Two components of this mixture were determined to be associated with the A antigenic activity of *B. dermatitidis*. Of several antigen preparations examined in Ouchterlony precipitation tests, those reactive with a reference anti-A antiserum contained the slowest moving of the "Coomassie blue" bands. The antigen preparations without precipitin reactivity lacked this protein band. Two protein bands were shown to disappear from an antigen preparation after incubation with an affinity gel linked to the reference anti-A serum. One of the bands was the slowest "Coomassie blue" band and the other was a fast-migrating protein detectable only with the

silver stain. The characterization of the components responsible for the A antigenic activity has important applications in the production and standardization of serologic reagents for the diagnosis of blastomycosis.

INTRODUCTION

The A antigen of Blastomyces dermatitidis was first described in 1973 by Kaufman et al. (11) who found the activity in yeast broth filtrates. The antigen was subsequently used to identify B dermatitidis with 100% accuracy while giving no cross-reactions to various other fungi (12). A reference antiserum developed in 1979 (8) aided Green et al. In using anion-exchange chromatography to purify an antigen fraction showing only A banding in Ouchterlony tests (9). This "purified" A antigen was found to be resistant to several proteases, nucleases, and to short periods of boiling. In this form, the antigen also showed promise as a serological tool in identifying active cases of blastomycosis by complement-fixation, skin-testing, ELISA procedures and Ouchterlony methods (9).

The continuing bane of fungal antigens as serological tools has been a combination of low sensitivity and high cross-reactivity. Although no cross-reactions with other fungi have been reported when the "A" antigen is used in Ouchterlony tests, Green et al. reported that such testing is only positive in 52% of known human cases. The same problem arose using complement-fixation where

only 70% of the cases gave positive results. And although the ELISA procedure detected 92% of known cases, it had a high (20%) rate of cross-reacting with various other fungal disease sera (9).

Crude extracts and impure antigen preparations have long been used in fungal serologic and epidemiologic studies. Such preparations have been shown to contain high numbers of common antigenic components which complicate the interpretation of immunologic tests (1,3,10). It is possibel that the usefulness of the "A" antigen of B. dermatitidis is still suboptimal because of contaminants in even the most recent pure preparations. It is, therefore, the purpose of this paper to more exactly identify those components associated with the "A" antigenic activity. Gel electrophoresis, affinity chromatography, and comparisons of various antigenic preparations are presented to show that two specific proteins or glycoproteins can be identified as the active components of the "A" antigen. The presence of these proteins in polyacrylamide gels can thus be used as a marker to identify antigen preparations which contain the "A" antigen, and as a standard against which the purity of various preparations may be measured.

MATERIALS AND METHODS

Antigen preparation. Cell cytoplasmic and cell-wall supernatant antigens were prepared by inoculating one liter amounts of BHI broth with a log phase culture of the respective isolate and incubating 3 days on a rotary shaker. The cells were killed by adding either formaldehyde (0.2% final concentration) or merthiolate (0.05% final concentration) and refrigerating for 48 hours. Whole cells were collected by centrifugation at 5000 g for 10 min and were washed 3 times with distilled water. A 50% suspension of cells was made in distilled water, added to an equal volume of 0.45-0.50 mm diameter glass beads, and broken for 90 sec in a CO_2-cooled Braun homogenizer. Cell debris was removed by centrifugation at 20,000 g for 30 min and the supernatant designated the "cytoplasmic" antigen. The cell wall debris was washed 3 times with distilled water, suspended 1:1 in distilled water, and stored at 4 C for at least 4 weeks. The water-soluble portion of this preparation was designated the "cell-wall supernatant" antigen. The cytoplasmic antigen from one isolate (SCB-2) was precipitated by adding either one or two volumes of 95% ethanol (EtOH) to give a 50% and 67% EtOH-precipitate, respectively.

The various antigens are listed in Table 1. The "OU" series was prepared in this laboratory. All other antigens were prepared and supplied by Sharon Harris and Stan Bauman of Immunomycologics, Inc., Norman, OK. The "Marshfield" isolates are clinical isolates from Marshfield, Wisconsin. The SCB and Davis isolates are also clinical isolates. All but two of the antigens, OU-S/CW and OU-D/CW, are cytoplasmic preparations. Purified "A"-antigen and reference "A"-antiserum were supplied by Dr. W. K. Harrell of CDC. The preparation and purification of this antigen is described by Green et al. (9).

Ouchterlony immunodiffusion. Immunodiffusion was performed in prepared slides from Immunomycologics, Inc., Norman, OK. Samples were delivered in 10 microliter amounts. Antisera were allowed to diffuse into the gel for 30 min before antigens were added to the test slides. Slides were read at 24 and 48 hr. Each was then developed for 5 min with phosphotungstic acid, according to the supplier's directions, in order to enhance visualization of faint bands. Rabbit anti-mouse IgG, anti-mouse IgM, and pure mouse IgC and IgM from myeloma lines MOPC-21 and MOPC-104E were obtained from Bionetics Laboratory Products.

Polyacrylamide gel electrophoresis. PAGE was performed in an apparatus constructed as per the instructions of Ogita and Markert (15). This apparatus allowed the

TABLE 1

ANTIGENS

ANTIGEN	ISOLATE	PREPARATION[1]	PROTEIN[2] (mg/ml)
OU-S	SCB-2	formalin	1.0
OU-D	Davis	formalin	1.4
008BY	CDC-A373	merthiolate	2.0
009BY	CDC-A373	merthiolate	1.0
205B3	Marshfield #1	formalin	0.7
008B3	Marshfield #1	formalin	0.6
007B3	Marshfield #1	merthiolate	0.4
009B3	Marshfield #2	formalin	1.1
00AB3	Marshfield #2	merthiolate	0.5
OU-S/CW	SCB-2	formalin, cell-wall	ND[4]
OU-D/CW[3]	Davis	formalin, cell-wall	ND
OU-S/50[3]	SCB-2	formalin, EtOH-ppt	ND
OU-S/67[3]	SCB-2	formalin, EtOH-ppt	ND

1) Method of killing. Unless otherwise stated, all are cytoplasmic preparations.
2) Protein was measured by the method of Lowry as modified by Garvey et al. (7), or by the Coomassie blue dye-binding method of Bradford (2) as modified by Spector (18).
3) Designates the antigen fractions precipitated from the OU-S antigen by either 50% or 67% EtOH treatments.
4) ND = not done.

formation of gels measuring 63 x 72 x 0.8 mm. The exact procedures and formulations of all solutions used in this study are tabulated in the same paper (15). Only two alterations were made -- first, glycerol was omitted from the acrylamide-bis solutions; and second, 1 ml of 1% bromphenol blue was added directly to the upper reservoir instead of to each individual sample dilution. Running gels of 8%, 14%, or 20% acrylamide-bis were overlaid with a 4% stacking gel. Gels were electrophoresed until the dye front was between 3-5 mm from the bottom edge of the gel.

Proteins were visualized in the gels by staining for 1 hr in 0.1% Coomassie Blue R-250 in 50% TCA. Gels were then destained in 10% acetic acid for 1-2 hr at 60 C or for 24-48 hr at 25 C. In selected cases, proteins were stained by the modified silver stain of Oakley et al. (14), which is 100 times more sensitive than the Coomassie blue method.

Gels were stained for carbohydrates by the periodic acid-Schiff (PAS) method of Zacharius et al. (19).

Affinity chromatography. Antibodies were linked to Affi-Gel 10 (Bio-Rad) according to the manufacturer's directions. After pre-washing, 30-40 mg of antibody were added to 4-5 ml of gel and allowed to react overnight at 4^{o} C with mild agitation. The antibody-linked gel was poured into a 1 cm diameter column, unabsorbed antigen washed out with Tris-HCl buffer (0.1 M, pH 8.0, 0.15-0.5 M

NaCl), and allowed to stand in the same buffer for 1 hr to saturate any remaining sites. The gel was washed with glycine-HCl elution buffer (0.05 M, pH 2.4) and rewashed with Tris buffer. Antigen (1.3-1.5 mg in 2.5 ml Tris) was added and incubated for 1 hr at room temperature. Unadsorbed antigen was washed out with Tris buffer, and adsorbed antigen removed by the glycine-HCl buffer. The gel was immediately rewashed with Tris buffer and stored at 4 C.

RESULTS

Ouchterlony Precipitation with Anti-A Reference Serum.

Precipitin lines of identify were formed between the purified A antigen and the following antigen preparations: OU-S, OU-S/CW (slight), OU-D, OU-D/CW (very slight), 205B3 (slight), OU-S/50, and OU-S/67. Lines were absent in the following antigen preparations: 008BY, 009BY, 00AB3, 009B3, 007B3, and 008B3 (fig. 1). Close inspection of the line formed midway between the purified A antigen and the anti-A reference serum revealed it to be composed of two very close lines which are fused. This can be most clearly seen in the photograph of the reactions involving the two ethanol precipitate antigens, although the same phenomenon seemed to occur with all reactive preparations. (fig. 1).

PAGE of Purified A Antigen

Purified A antigen was electrophoresed in 14% and 20% polyacrylamide gels and stained for protein by the Coomassie blue and silver methods. The results are shown in Figures 2 and 3. Only five protein bands appeared when stained with Coomassie blue, with Rf values between 0.19 and 0.29 in the 14% gel and between 0.10 and 0.17

Figure 1. Precipitin bands between anti-A reference serum and various antigen preparations. The center wells of the A and B templates, and the #1 well of the C template contained anti-A serum. Template A tested these antigens: well #1, purified A antigen; #2, 205B3; #3, 00AB3; #4 009B3; #5, 007B3; #6, 008B3. Template B tested these antigens: well #1, purified A antigen; #2, OU-S; #3, OU-S/CW; #4, 009BY; #5, OU-D/CW; #6, OU-D. Template C tested: well #2, OU-S/50; #5, OU-S/67.

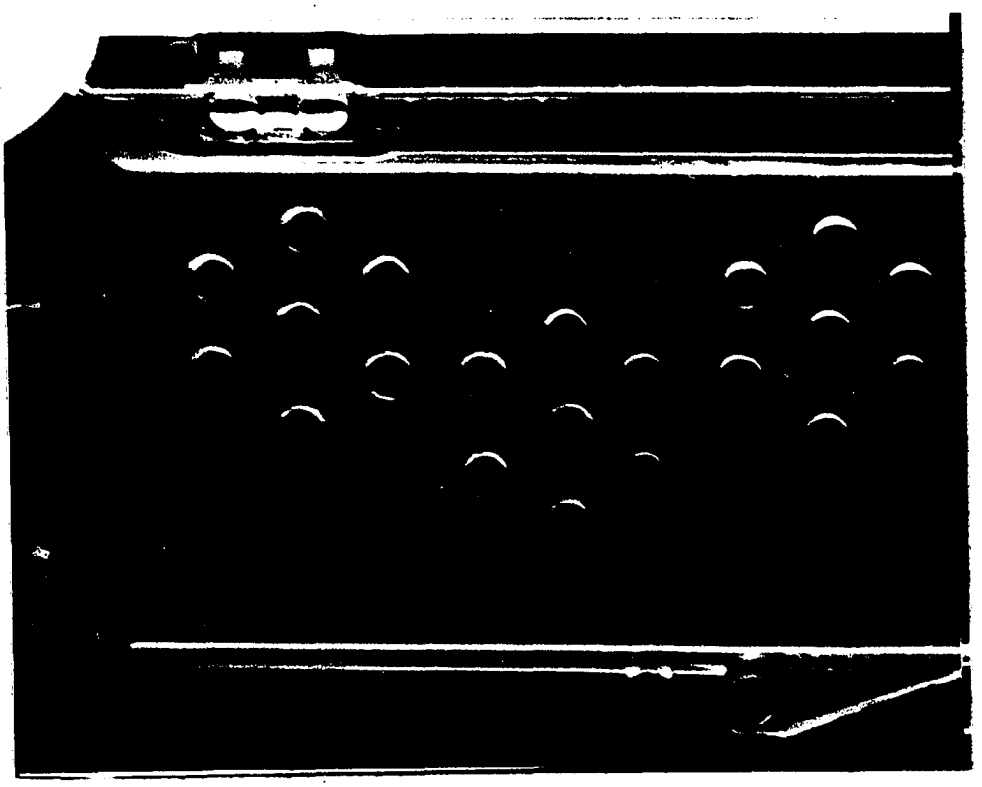
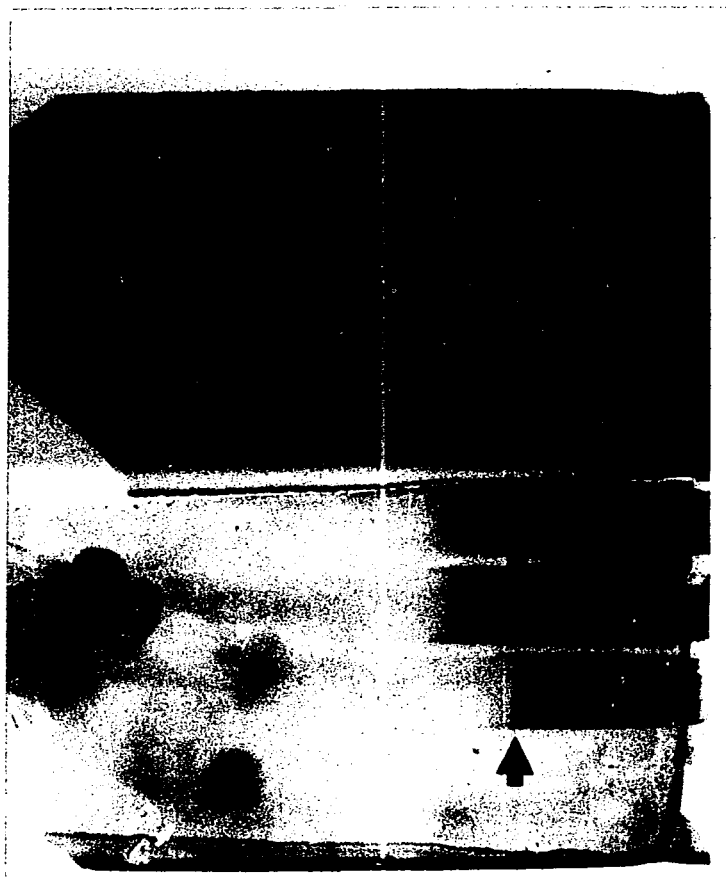
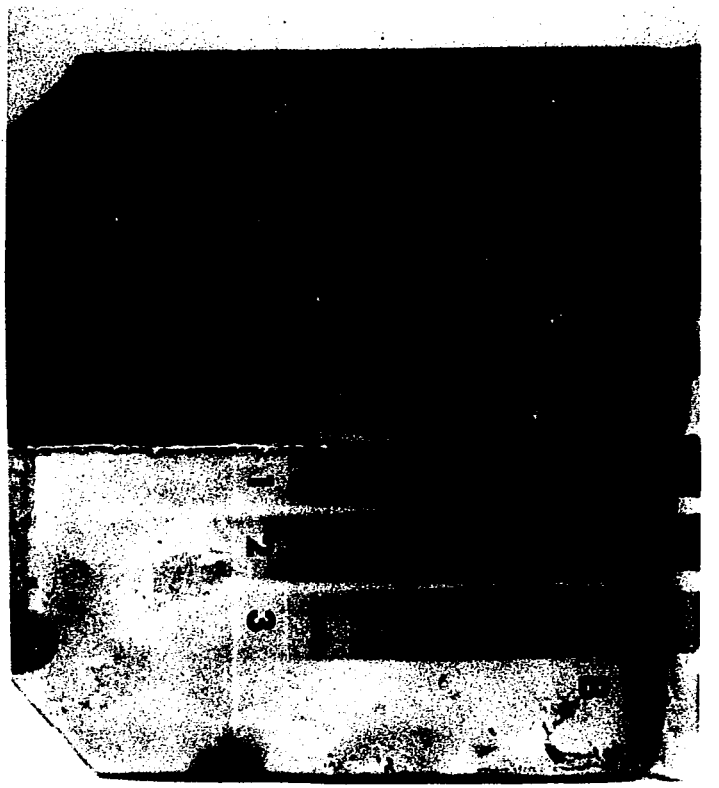


Figure 2. Protein stain of 14% gels. Track #1, OU-S; #2, OU-S after passage through affinity column containing anti-A antigen reference serum; #3, purified A antigen. Gel A, Coomassie blue stain; gel B, silver stain. Arrow locates common protein band which is partially removed by affinity chromatography from the OU-S preparation in track #2, gel A.

Figure 3. Protein stain of 20% gels. Arrangement as in Figure 2. Arrow locates common protein band removed by affinity chromatography from the OU-S preparation in track #2, gel B.





in the 20% gel. The silver stain revealed a minimum of 25 well-defined protein bands with the most rapidly moving band having an Rf of 0.54 and 0.27 in the 14% and 20% gels, respectively. PAS staining revealed 5 carbohydrate bands corresponding to the 5 seen with Coomassie blue. The silver stain also revealed numerous a-ditional bands in the OU-S preparation which reproduced photographically as an almost solid black track.

Comparison of Purified A Antigen With Other Preparations.

Gel scans of polyacrylamide gels are shown in Figures 4 and 5. These gels compare the purified A antigen with the OU-S preparation, which reacted with anti-A serum in Ouchterlony tests, and with the 008BY preparation, which was non-reactive. Both the 14% and 20% gels revealed only one common band between the reactive OU-S preparation and the purified A antigen. This particular band appeared only faintly, if at all, in the non-reactive 008BY preparation, and then only in the 20% gel. The correspondence between the slowest-moving protein of the purified A antigen (in the Coomassie-stained gels) and the largest band in OU-S can be seen in Figure 2.

Several other antigens were electrophoresed to compare the presence or absence of this particular band with the reactivity of the preparations in Ouchterlony precipitation tests. These gels are shown in Figures 6 - 8. In all cases, presence or absence of the marked band

Figure 4. Gel scan of 14% gels stained with Coomassie blue. Antigen preparations are: A = OU-S; B = purified A antigen; C = 008BY. Arrows designate bands held in common.

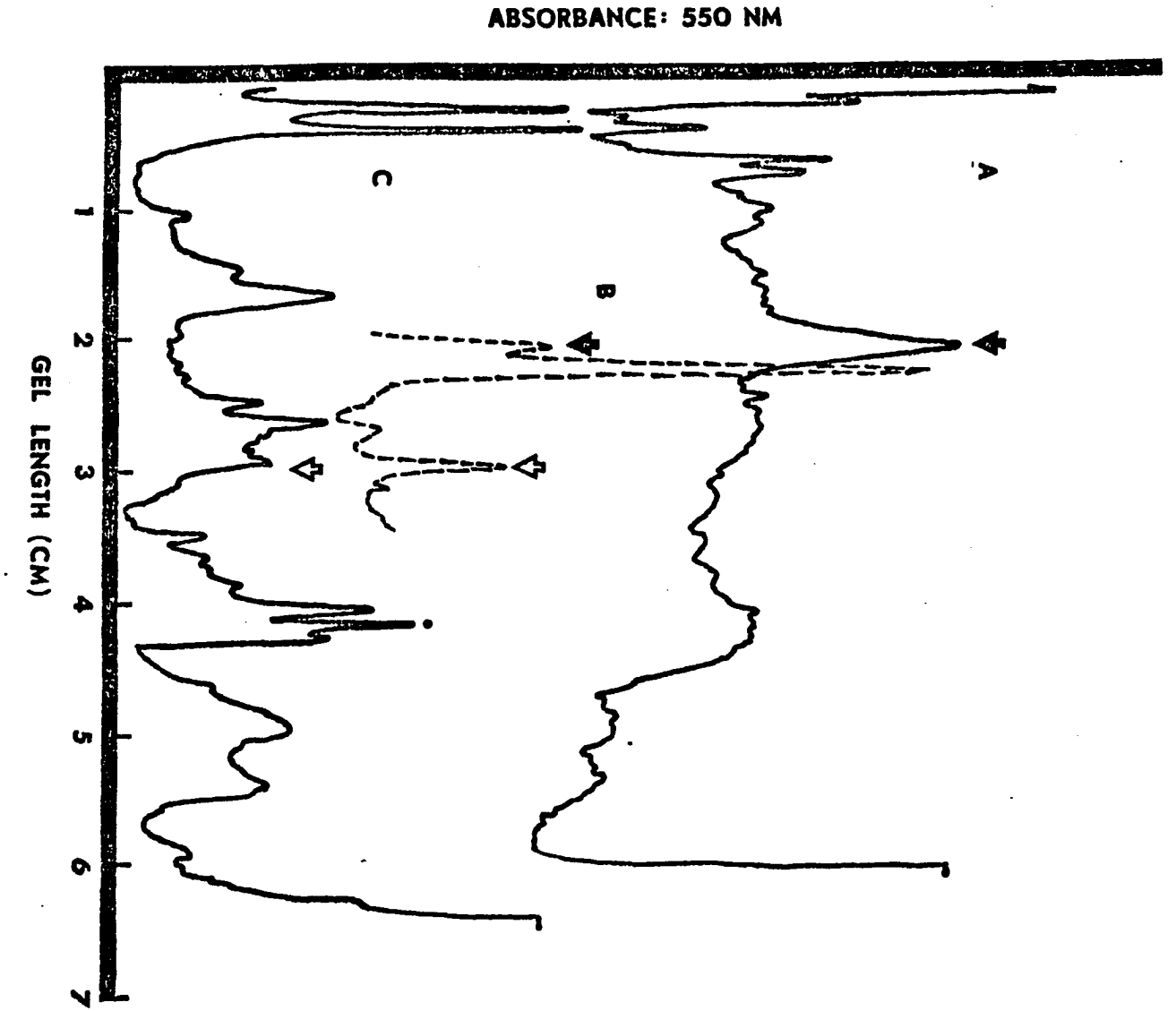

ABSORBANCE: 550 NM
GEL LENGTH (CM)
1
2
3
4
5
6
7
A
B
C

Figure 5. Gel scan of 20% gels stained with Coomassie blue. Legend as in Figure 4.

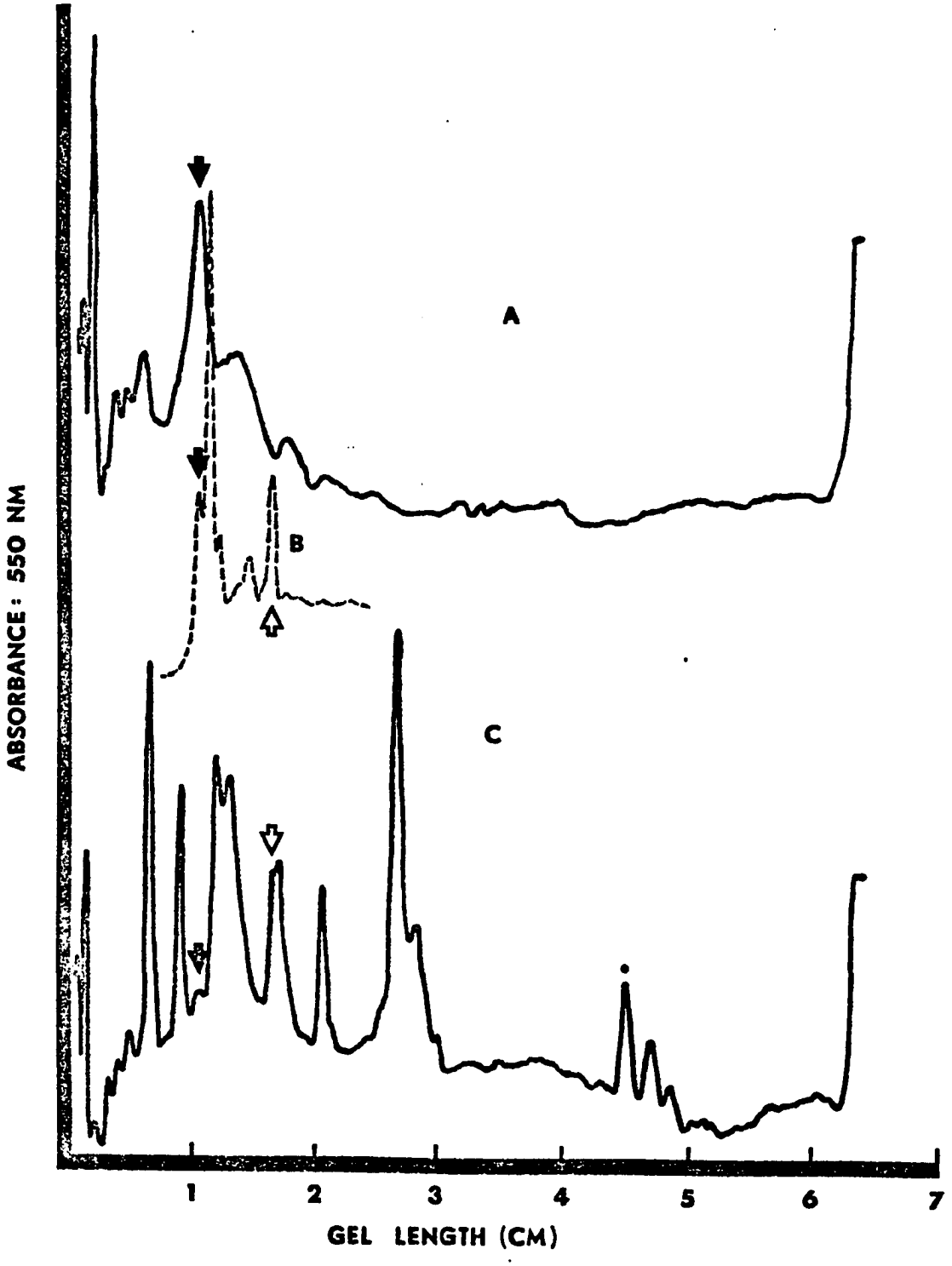
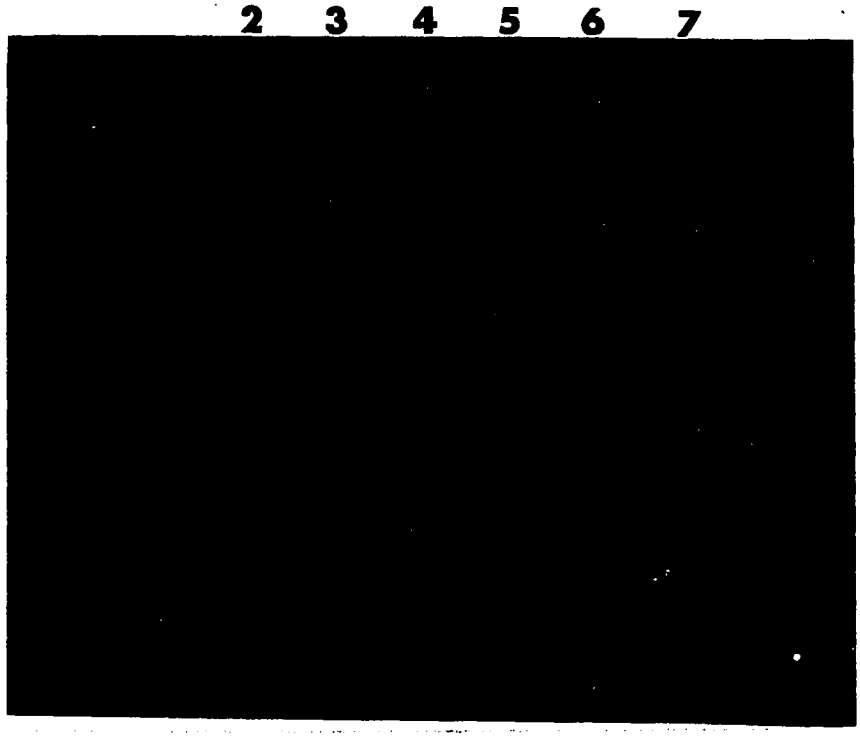

A
B
C
ABSORBANCE : 550 NM
1
2
3
4
5
6
7
GEL LENGTH (CM)

Figure 6. Coomassie blue stain of 20% gel. Track #2, OU-S; #3, OU-D; #4, 205B3; #5, 008BY; #6, OU-S/CW; #7, OU-D/CW. Arrow locates position of the "OU-S/A-antigen" common protein band.

Figure 7. Coomassie blue stain of 20% gel. Track #1, 008BY; #2, 009BY; #3, 00AB3; #4, 009B3; #5, 007B3; #6, 008B3. Arrow locates the position at which the "OU-S/A-antigen" common band should appear.


2 3 4 5 6 7

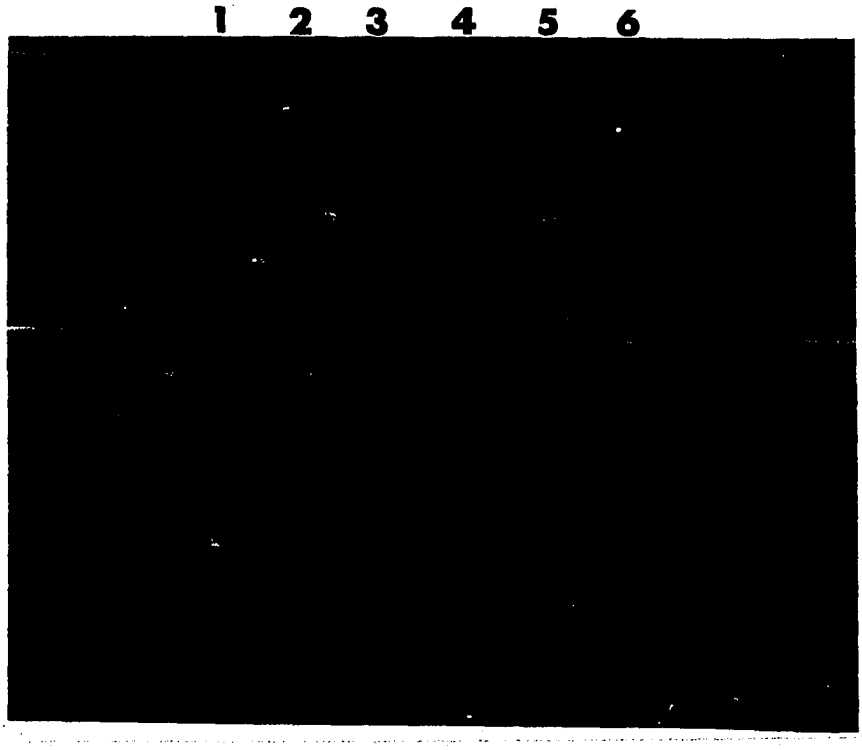

1 2 3 4 5 6

Figure 8. Coomassie blue stain of 20% gel. Track #1, OU-S; #2, OU-S/50; #3, OU-S/67; #4, purified A antigen. Arrow locates the "OU-S/A-antigen" common protein band.

1 2 3 4

paralleled the presence or absence of precipitin reactivity to the anti-A reference serum. The corresponding bands in OU-D and 205B3 in Figure 6 were much lighter than in OU-S and were not clearly reproduced photographically. The A antigenic activity was shown to be precipitated with ethanol treatment (Fig. 8).

In addition, these results demonstrate that lot-to-lot variation of antigen preparations may be absent (008By-vs-009BY, Fig 7), or slight (007B3-vs-00AB3, Fig. 7). Strain-to-strain variations may occur in relatively few protein bands (007B3-vs-00AB3 and 008B3-vs-009B3, Fig. 7, and OU-S-vs-OU-D, Fig. 6), or in many bands (OU-S or OU-D vs any antigen in Fig. 7). Preparation by killing with formalin versus merthiolate yielded only minor differences (007B30vs-008B3 and 00AB3-vs-009B3, Fig. 7).

Affinity Chromatography

The removal of protein bands from OU-S by affinity chromatography through an anti-A-linked gel is shown in Figures 2 and 3. In both figures, the non-adsorbed OU-S had been adjusted to the same protein concentration as the applied OU-S. Figure 2 most clearly shows the reduction of the slowest-moving band of the A antigen from OU-S (Coomassie blue-stained gel). This band is the one previously shown to be common to both preparations (Figs. 4 and 5). An additional, rapidly-migrating protein band,

which appeared only after silver staining, was completely absent from the antigen exposed to the affinity gel but was present in both the OU-S antigen before application to the Gel and in the purified A antigen (fig. 3).

DISCUSSION

Although the A antigen of Blastomyces dermatitidis has been shown to be of use in the serologic diagnosis of blastomycosis (9, 11, 12, 16), the antigen itself has been poorly characterized. The purification by ion exchange chromatography of an antigen fraction containing reactivity to a reference anti-A serum (8, 9) has allowed us to more exactly characterize the components responsible for the A antigenic activity.

The purified preparation of Green et al. (9) consists of at least 25 proteins and glycoproteins, as determined by a combination of Coomassie blue, PAS, and silver staining of polyacrylamide gels. Such a mixture may be the reason that cross-reactions with other mycotic disease sera continue to occur in the highly sensitive ELISA procedure (9). The use of a mixture may be appropriate in less sensitive techniques, such as Ouchterlony precipitation, but an unacceptable increase in false negative reactions also occurs (9). As long as extraneous material is included in antigen preparations, the absence of cross-reactions cannot be guaranteed.

Although previous reports suggest that precipitation

tests yield a single band between the A antigen and anti-A serum, we have noted that the reaction is more likely one giving two fused bands. This correlates well with the findings that two specific components, identified by comparison with other antigen preparations and by affinity chromatography, are involved in the A antigenic activity.

The identification of these two components opens the way to their further purification, using polyacrylamide gel electrophoresis as a sensitive indicator of purity. In the past, Ouchterlony tests have been the primary method used to assay various antigen fractions for the presence of the A antigen. Although this may be useful as a preliminary test, a more sensitive assay is needed if very pure preparations are to be produced. Huppert et al. (10) have suggested the use of two-dimensional electrophoresis as a tool for both standardizing and assessing the purity of antigen preparations. The major drawbacks to this use of the method include the amount of time and material required to perform the test, its relative insensitivity, and its reliance on an antigen-antibody reaction. This latter is restricting since a large quantity of antibody must be used, and since antigens of possible significance may go undetected if specific antibody is unavailable. The staining of proteins in polyacrylamide gels is a rapid and very sensitive procedure, especially if the new silver stain is employed,

and many samples may be compared in detail. For comparative purposes, a standard is now available using the purified A antigen with knowledge of its specific reactive components. Hopefully, much purer preparations will soon become available.

The location of the A antigen has also been addressed. Kaufman et al. (11) first located it in yeast culture filtrates. It has since been most commonly prepared by extracting whole yeast cells with water or saline over a several-week period (8, 9, 12). Reference anti-A serum has been reported to be adsorbed by yeast cell walls but not by cytoplasmic preparations (9). These previous investigations have been performed with only two isolates of B. dermatitidis, A295 and A373. We have verified that the A antigen is absent, or present in insignificant amounts, in the cytoplasm of A373 (antigens 008BY and 009BY). However, other isolates have shown significant amounts of A antigen in cytoplasmic preparations (OU-S and OU-D). This has two ramifications. First, preparation of large quantities of the A antigen can be made more quickly by breaking whole cells, although contamination with extraneous material may be greater than in aqueous extracts. Second, the precise location and function of the A antigen remains in doubt, and several isolates must be included in future studies examining these questions.

The finding that the A antigen can be precipitated by ethanol may have some historical, and perhaps practical,

implications. As early as 1911, Davis (4) isolated a fraction from yeast cytoplasm by ethanol precipitation which yielded a fairly sensitive precipitin antigen for infected guinea pig serum. Many investigators have since employed ethanol precipitation to isolate surprisingly sensitive antigens (K. D. Young, Ph.D. thesis, University of Oklahoma, Norman, 1980). Antigens from yeast culture filtrates have been reported to detect 100% of infected guinea pigs or rabbits by skin test while having 0% cross-reactions with animals having other mycoses (5, 6, 13). In a recent study, Rippon et al. reported successfully detecting 90% (18 of 19) known human cases by Ouchterlony precipitation, with 0% (0 of 40) cross-reactions with sera from patients having other systemic mycoses (17). None of these preparations was characterized in depth, and the possibility that they contained what is now termed the "A antigen" seems quite high. Incorporation of an ethanol precipitation step may therefore be useful in future purification schemes.

Rippon et al. have noted that without the tools of skin-test or complement-fixation antigens for epidemiological, diagnostic, and prognostic use, "we are at least 50 years behind in our defining the disease blastomycosis (17)." Much of the problem has been due to impure antigen preparations and an imprecise understanding of the reactive agents. Hopefully, the identification of particular reacting components will help alleviate some of the problems involved in closing such a large gap.

REFERENCES

1. Andrieu, S., J. Biguet, L. Dujardin, and T. Vaucelle. 1969. Etude antigenique des agents des mycoses profondes par l'analyse comparee des milieux de culture. I. Histoplasma capsulatum et H. duboisii. Relations avec H. farciminosum, Gymnoascus demonbreunii, Blastomyces dermatitidis et Paracoccidioides brasiliensis. Mycopath. Mycol. Appl. 39:97-108.

2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

3. Cox, R. A. 1979. Cross-reactivity between antigens of Coccidioides immitis, Histoplasma capsulatum, and Blastomyces dermatitidis in lymphocyte transformation assays. Infect. Immun. 25:932-938.

4. Davis, B. F. 1911. The immunological reactions of oidiomycosis (blastomycosis) in the guinea pig. J. Inf. Dis. 8:190-240.

5. Dyson, J. E., Jr. and E. E. Evans 1954. Skin test antigens from yeast phase cultures of Blastomyces dermatitidis and Histoplasma capsulatum. Univ. Mich. Med. Bull. 20:53-61.

6. Dyson, J. E., Jr. and E. E. Evans 1955. Delayed hypersensitivity in experimental fungus infections. The skin reactivity of antigens from the yeast phase of Blastomyces dermatitidis. J. Invest. Dermatol. 24:447-452.

7. Garvey, J. S., N. E. Cremer, and D. H. Sussdorf 1977. Methods in Immunology, 3rd ed., W. A. Benjamin Inc., Reading, Mass., pp 87-89.

8. Green, J. H., W. K. Harrell, J. E. Johnson, and R. Benson 1979. Preparation of reference antisera for laboratory diagnosis of blastomycosis. J. Clin. Microbiol. 10:1-7.

9. Green, J. H., W. K. Harrell, J. E. Johnson, and R. Benson 1980. Isolation of an antigen from Blastomyces dermatitidis that is specific for the diagnosis of blastomycosis. Current Microbiol. (Dec. issue).

10. Huppert, M., J. P. Adler, E. H. Rice, and S. H. Sun 1979. Common antigens among systemic disease fungi analyzed by two-dimensional immunoelectrophoresis. Infect. Immun. 23:479-485.

11. Kaufman, L., D. W. McLaughlin, M. J. Clark, and S. Blumer 1973. Specific immunodiffusion test for blastomycosis. Appl. Microbiol. 26:244-247.

12. Kaufman, L., and P. Standard. 1978. Immuno-identification of cultures of fungi pathogenic to man. Curr. Murr. Microbiol. 1:135-140.

13. Knight, R. A., and S. Marcus. 1958. Polysaccharide skin test antigens derived from Histoplasma capsulatum and Blastomyces dermatitidis. Amer. Rev. Tubercul. Pul. Dis. 77:983-989.

14. Oakley, B. R., D. R. Kirsch, and N. R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. 105:361-363.

15. Ogita, A.-I., and C. L. Markert. 1979. A miniaturized system for electrophoresis on polyacrylamide gels. Anal. Biochem. 99:233-241.

16. Palmer, D. F., L. Kaufman, W. Kaplan, and J. J. Cavallaro. 1977. Serodiagnosis of Mycotic Diseases. Charles C. Thomas Pub., Springfield, Illinois, 191 pp.

17. Rippon, J. W., D. N. Anderson, S. Jacobsohn, M. S. Hoo, and E. D. Garber. 1977. Blastomycosis: specificity of antigens reflecting the mating types of Ajellomyces dermatitidis. Mycopath. 60:65-72.

18. Spector, T. 1978. Refinement of the Coomassie blue method of protein quantitation. A simple and linear spectrophotometric assay for 5.0 to 50 micrograms of protein. Anal. Biochem. 86:142-146.

19. Zacharius, R. M., T. E. Zell, J. H. Morrison, and J. J. Woodlock. 1969. Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. 30:148-152.

CHAPTER 3

PRODUCTION AND CHARACTERIZATION OF HYBRIDOMA-DERIVED ANTIBODIES TO *BLASTOMYCES DERMATITIDIS*.

ABSTRACT

A hybridoma cell line was isolated which produced monoclonal antibody to one protein component of a yeast-phase cytoplasmic antigen of *Blastomyces dermatitidis*. The IgM antibody product was characterized by immunodiffusion, autoradiography of polyacrylamide gels, and cellulose acetate electrophoresis. By attaching the antibody to an affinity gel, one major protein band was identified as the antigen for which the antibody was specific. The usefulness of this approach to serologic analysis of fungal pathogens is discussed.

INTRODUCTION

In the past few years a technique has been developed which allows the production of large quantities of monoclonal antibodies (8, 10, 12). Such antibodies are extremely pure and are specific for only one antigenic determinant. They can, therefore, be used as extremely sensitive probes or isolation tools for their respective antigen.

The production of these hybridoma-derived antibodies for pathogenic fungi was first reported for Blastomyces dermatitidis (K. D. Young and H. W. Larsh, Abstr. Ann. Meet Am. Soc. Microbiol., 1980, F1, p. 319) and for Cryptococcus neoformans (N. K. Hall and R. Blackstock, Abstr. Ann. Meet. Am. Soc. Microbiol., 1980, F2, p. 319). The former report outlined the optimum conditions for production of hybridomas when using B. dermatitidis. The latter reported isolation of 9 hybridomas producing antibodies having differing specificities to cells of C. neoformans, Histoplasma capsulatum, and Coccidiodes immitis. Neither of these previous reports included a characterization of the antigens for which the antibodies were specific.

We report the isolation of a hybridoma line secreting antibody to a cytoplasmic preparation of B.

dermatitidis. By linking this antibody to an affinity gel, the protein for which the antibody is specific was purified and characterized by polyacrylamide gel electrophoresis. The implications of this technique for the study of the pathogenic fungi is discussed.

MATERIALS AND METHODS

Antigen preparation. A cytoplasmic fraction from B. dermatitidis cells broken by glass beads in a Braun homogenizer was supplied by Sharon Harris and Stan Bauman of Immunomycologics, Inc., Norman, Ok. This "009BY" preparation was derived from merthiolate-killed yeast cells of the CDC isolate, A373, and contained 1.0 mg/ml protein.

Production of hybridomas. C57BL/6J mice (Jackson Labs) were injected with approximately 5 x 10^5 formalin-killed B. dermatitidis yeast cells at 2-week intervals for 8 weeks. The first three injections were I.P., and the last, I. V. in the tail vein. Mice were sacrificed 3 days after the final injection and their spleens removed.

The following hybridization procedure is a composite drawn from Kennett (7), Shulman (13), and Gefter (5). Spleens were placed in sterile Eagle's Minimal Essential Medium (EMEM) without calcium and magnesium and minced through wire mesh screens into tissue-culture dishes. The cell suspension was gently drawn into a syringe, dispenses through 20G, 22G, and 26G needles, and centrifuged at 400-500G for 10 min. The cell pellet was suspended in 5 ml of 4 C 0.17 M NH4C1 for 10 min, diluted with 10 ml Dulbecco's

Modified Eagle's Medium (DMEM), pelletted, washed twice more with DMEM, and resuspended in 5 ml of EMEM-fusion medium.

NS-1 myeloma cells (Hybritech, Inc.) were maintained in DMEM plus 8-azaguanine (10^{-4} M) and cells in mid-log were used for fusions. NS-1 and spleen cells were combined in approximately a 1:10 ratio, pelletted, washed twice with EMEM-fusion, and repelletted. All supernatant was removed and the pellet loosened by tapping. A 30% PEG solution in EMEM-fusion had been previously adjusted to pH 7.4-7.6 and maintained at 37 C. The pelletted cells were covered with 0.2 ml of this 30% PEG solution for 8 min, during which the pellet was centrifuged at 400-500G for 3-6 min. The pellet was then gently suspended in 5 ml of EMEM-fusion medium, mixed in an additional 5 ml of EMEM-fusion, centrifuged, and washed once in the same medium. The pellet was suspended in 15-16 ml of DMEM plus hypoxanthine (10^{-4} M) and thymidine (1.6×10^{-4} M),and 50 microliters were distributed to each of the 60 interior wells of 5 microtiter plates (Linbro). After incubating 24 hr in a 5% CO_2 atmosphere, 50 microliters of "HAT" selective medium was added to each well, and 100 microliters of DMEM plus hypoxanthine and thymidine added after incubating 7 days. Colonies appeared 10-20 days after the last addition of medium and were transferred to new medium in microtiter dishes, then to larger culture dishes if found to be producing antibody.

Ascites fluid containing hybridoma antibodies was

produced by injecting at least 10^6 hybridoma cells I.P. into Pristane-primed BALB/C mice. Fluid was drawn after 10-14 days. Hybridoma cells were frozen by suspending to 5-10 x 10^6 cells/ml in cold 5% dimethylsulfoxide in fetal calf serum (FCS) and cooling first to -20 C for 1 hr. then transferring to a -70 C freezer. Antibodies from selected hybridoma culture supernates were partially purified by the 50% ammonium sulfate precipitation method of Jonak (14). The precipitate from 500 ml of supernate was resuspended in 7 ml of PBS, pH 7.2, and dialyzed against the same.

Maintenance medium was composed of DMEM containing NaHCO3 and glutamine, 10% FCS, 100 units/ml Penicillin, and 100 micrograms/ml streptomycin. EMEM-fusion medium was EMEM without calcium and magnesium, pH 7.4-7.6. All tissue culture media was obtained from GIBCO. Polyethylene glycol was obtained from Koch-Light, Ltd. All other chemicals were from Sigma.

The Blastomyces-specific hybridoma line has been placed with Dr. N. K. Hall of the University of Oklahoma Health Sciences Center, Oklahoma City.

ELISA procedure. Hybridoma antibodies specific for B. dermatitidis were detected by enzyme-linked immunosorbant assay (ELISA). Antigen was suspended in carbonate-bicarbonate buffer, pH (Dynatech Labs, Inc.) overnight at 4° C. Plates were rinsed 3 times in 3 rinses in PBS. Supernatants (0.1 ml) from cultures having hybridoma growth were added, incubated 1 hr at room

temperature, and washed 3 times in PBS. Peroxidase-linked goat anti-mouse IgG (heavy and light chain-specific, Bionetics) was diluted 1:100, 0.1 ml added to each well, nncubated for 1 hr at room temperature, and washed 3 times in PBS. The reagent solution of 0.15% H_2O_2 and 0.1% O-phenylenediamine was added in 0.1 ml amounts to each well, incubated 1 hr at room temperature, and read spectrophotometrically at 492 nm. Controls consisted of wells containing no antigen or antigen plus uninoculated medium, medium from NS-1 cells alone, or normal mouse serum.

Autoradiography of polyacrylamide gels. Localization of immunoglobulins in polyacrylamide gels was accomplished by the method of Bonner and Laskey (1). Hybridoma or myeloma cells were suspended to a final concentration of 0.1-1.0 x 10^6 cells/ml in 1.0 ml DMEM plus hypoxanthine and thymidine. 3H-leucine was added to give 1.0-4.0 micro-Ci/ml. The suspension was incubated 24 hr at 37^o in 5% CO_2. The cells were removed by centrifugation and the supernatant stored at -20^o C.

Portions of the supernatants were separated on SDS polyacrylamide gels, and the gels fixed in 50% TCA for 30 min. The gels were then soaked in two successive 20X volumes of dimethyl sulfoxide (DMSO) for 30 min each. The gels were next placed in a 4X volume of 22.2% (w/v) 2,5-diphenyloxazole in DMSO for 3 hr, after which they were soaked in a 20X volume of distilled water for 1 hr. The gels were dried on Watman #4 paper in a Bio-Rad gel dryer for 1-2 hr

and exposed to Kodak X-OMAT R film at -70° C for 3-7 days. The X-ray film is more sensitive if pretreated with light before exposing the gels (11). Pretreatment consisted of a 20-sec exposure to a yellow 20 watt "bug-light" placed approximately 20 feet from the film. The bulb was shielded with cardboard containing a 1" slit through which the light could pass. The film was developed according to the manufacturer's directions (9).

Cellulose acetate electrophoresis. Electrophoresis was performed in a Beckman Microzone apparatus, using Millipore membranes. One microliter samples were applied and electrophoresed at 250V for 20 min in 0.05 barbital buffer, pH 8.6. Proteins were stained in 0.4% amido black in 7% acetic acid, destained with 5% acetic acid, and cleared with 30% cyclohexanone in 95% ethanol.

Other procedures. Polyacrylamide gel electrophoresis, Ouchterlony immunodiffusion, and affinity chromatography have been described previously (K. D. Young, Ph.D. Thesis, Chapter 2).

RESULTS

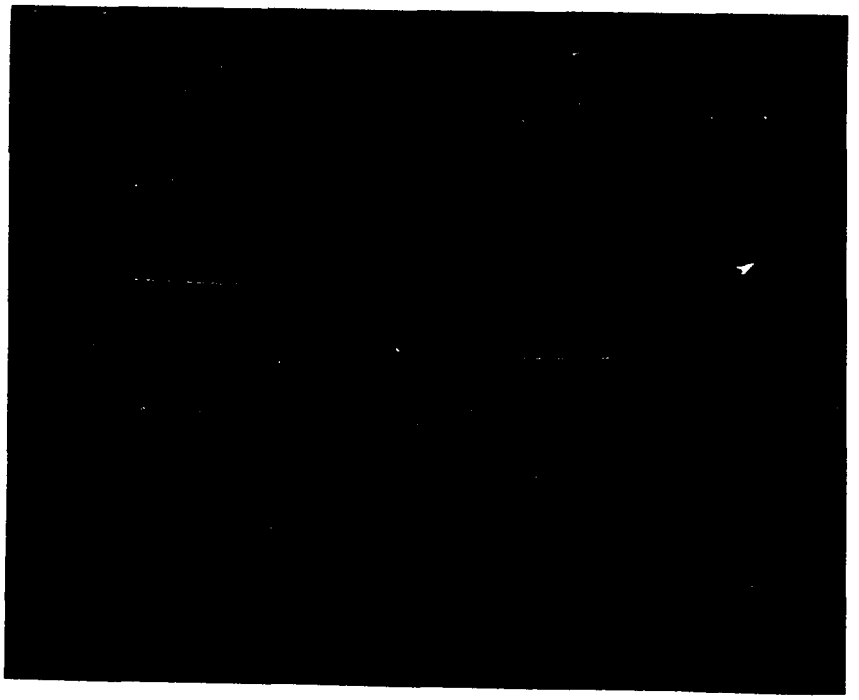
Of the hybridomas isolated, one (OU31/B6) produced absorbance reading 6 times that of control values in the ELISA procedure, and was selected for further characterization. Analysis of the culture supernatant and ammonioum sulfate-precipitated fractions by Ouchterlony immunodiffusion, cellulose acetate electrophoresis, and autoradiography indicated that OU32/B6 produced IgM.

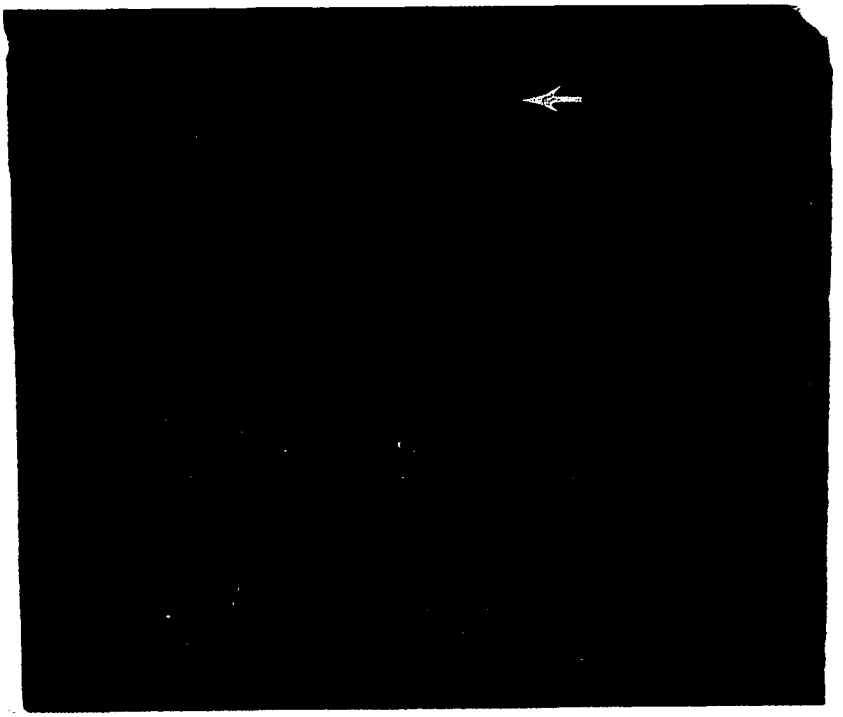
The autoradiography results in Figure 1 show that OU32/B6 produced only IgM. Heavy IgG chains are absent from the supernatant. In addition, the hybridoma cells secreted antibody in much greater amounts than may have been present in the original myeloma line. This is clear from tracks #2 and #4 in the gel (Fig. 1). Use of ^{3}H-leucine in amounts greater than 1 uCi/10)6) cells reduced the amount of antibody secreted (tracks #3 and #5, Fig. 1), probably because of lethal effects.

Ascites fluid contained IgM and IgG as determined by Ouchterlony tests. A precipitin band formed between this preparation and the 009BY antigen (data not shown). This band was not present when the ammonium sulfate-precipitated antibody was tested and so may be an artifact due to other

Figure 1. Autoradiography of hybridoma antibodies in polyacrylamide gels. Antibodies were separated in a 14% SDS polyacrylamide gel. Myeloma (NS-1) and hybridoma (OU32/B6 and OU32/C1) cells were incubated in various concentrations of ^{3}H-leucine as follows: lane #1, NS-1 + 1.0 uCi; #2, OU32/C1 + 1.0 uCi; #3, OU32/C1 + 2.0 uCi; #4, OU32/B6 + 1.0 uCi: #5, OU32/B6 + 4.0 uCi. Film was exposed to the gel for 165 hours before developing. The top, middle, and lower arrows locate the position of IgM heavy chains, IgG heavy chains, and light chains, respectively, as determined in a separate gel stained for protein.

Figure 2. PAGE analysis of protein eluted from hybridoma antibody-linked affinity column. Proteins were electrophoresed through a 14% polyacrylamide gel and stained for protein by the silver method. Lane #1 contains 009BY antigen (100 ug/ml). Lane #2 contains the material eluted from the affinity column. The eluate was concentrated 100X prior to electrophoresis. The arrow locates the position of the eluted band.

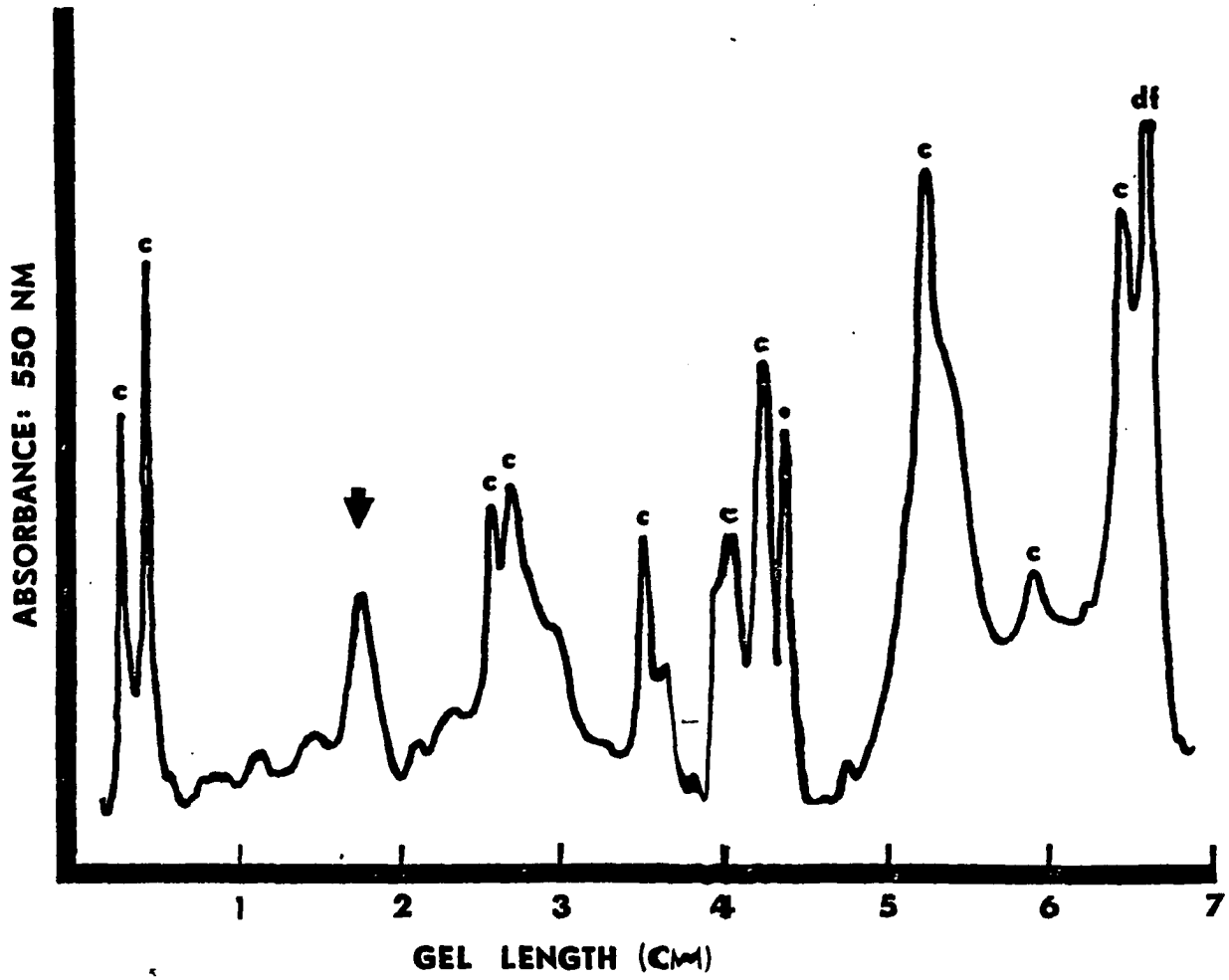




components in the ascites fluid. Therefore, the precipitated antibody was used in subsequent procedures.

Affinity chromatography using the ammonium sulfate-precipitated antibody was successful in purifying one component from the 009BY antigen preparation as shown in Figure 2. The location of this band relative to the other protein components of the antigen preparation is shown in Figure 3. The scan of Fig. 3 and the silver stain of Fig. 2 were different gels, processed separately, and so a number of bands in Fig. 3 do not appear in the photograph of Fig. 2.

Figure 3. Gel scan of 009BY antigen preparations separated on a 14% native polyacrylamide gel, stained with Coomassie blue, and destained in 10% acetic acid. A small "c" indicates bands also staining for carbohydrate with PAS. The small dot indicates a non-specific, opaque "junk" band which appears in unstained gels. The arrow designates the protein which corresponds to the band eluted from an OU32/B6 antibody-linked affinity column.


ABSORBANCE: 550 NM
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GEL LENGTH (CM)

DISCUSSION

The progression of blastomycosis in man and experimental animals has been difficult to assess, primarily because suitable immunologic monitoring techiques have been unavailable. Although the refinement of the A and ASWS antigens (4, 6) offers a great improvement in the diagnosis of blastomycosis, they are not necessarily the only antigens of use in diagnosis or in the study of the disease's development.

Studies of cross-reactions among patients with diseases caused by these three organisms indicate that different antigens of B. dermatitidis may be involved in the elicitation and detection of different immunologic properties, in this case between skin tests and lymphocyte transformation (3). It is possible that protective or diagnostic components may be absent in some preparations or overshadowed by other antigens (2). In either case, such relevant antigens could escape notice, especially since all current antigen and antibody preparations contain such a multiplicity of individual components.

The development of a technique to produce large quantities of monospecific antibodies by fusing a tumor

cell line and spleen cells from an immunized animal is already revolutionizing serological analyses (8, 10, 12). In the present report we have applied this technology to isolate a hybridoma cell line which produces IgM antibody against a cytoplasmic preparation of Blastomyces dermatitidis.

The antigen for which this antibody is specific has been isolated by affinity chromatography. Gel electrophoresis has revealed this preparation to contain one protein band. The development of such a cell line allows the isolation and characterization of this antigen to be standardized. This makes available for the first time a monospecific antibody to, and a single component antigen of B. dermatitidis.

Charlotte Campbell observed that "accurate serologic analysis...can not be achieved except by the isolation and characterization of each antigenic component an organism produces, related as well as unrelated, and the further correlation of these components' activities with antibodies produced throughout all stages of an infection (3)." Not until 20 years later has this ideal finally become achievable. Hopefully, a "library" of hybridomal lines can be developed, each producing an antibody to a different component of the pathogenic fungi. Such a library will prove invaluable in increasing not only our diagnostic capabilities, but also our ability to study the disease process in greater detail.

REFERENCES

1. Bonner, W. M., and R. A. Laskey, 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83-88.

2. Campbell, C. C. 1960. The accuracy of serologic methods in diagnosis. Ann N. Y. Acad. Sci. 89:163-177.

3. Cox, R. A. 1979. Cross-reactivity between antigens of Coccidioides immitis, Histoplasma capsulatum, and Blastomyces dermatitidis in lymphocyte transformatioin assays. Infect. Immun. 25:932-938.

4. Cox, R. A. and H. W. Larsh. 1974. Isolation of skin test-active preparations from yeast-phase cells of Blastomyces dermatitidis.

5. Gefter, M. L., D. H. Margulies, and M. D. Scharff. 1977. A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. Somatic Cell Genet. 3:231-236.

6. Green, J. H., W. K. Harrell, J. E. Johnson, and R. Benson 1980. Isolation of an antigen from Blastomyces dermatitidis that is specific for the diagnosis of blastomycosis. Current Microbiol. (Dec. issue).

7. Kennett, R. H., K. A. Denis, A. S. Tung, and N. R. Klinman, 1978. Hybrid plasmacytoma production: fusions with adult spleen cells, monoclonal spleen fragments, neonatal spleen cells and human spleen cells. Curr. Topics Microbiol. Immunol. 81:77-91.

8. Kennet, R. H., T. J. McKearn, and K. B. Bechtol. 1980. Monoclonal Antibodies. Hybridomas: A new Dimension in Biological Analyses. Plenum Press, New York, 423 pp.

9. Kodak instruction sheet, revised 2-1980. Detection of labeled compounds on chromatograms by autoradiographic procedures. Eastman Kodak Co., Rochester, N. Y. 14650.

10. Kohler, G. and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495-497.

11. Laskey, R. A. and A. D. Mills. 1975. Quantitative film detection of 3H and 14C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56:335-341.

12. Melchers, F., B. M. Potter, and B. N. Warner (Ed.). 1978. Lymphocyte hybridomas. Cur. Topics Microbiol. Immunol. 81:1-246.

13. Shulman, M., C. D. Wilde, and G. Kohler 1978. A better cell line for making hybridomas secreting specific antibodies. Nature 276:269-270.