Identification of Rabbit Antibodies Directed against *Candida albicans** (33019)

NANCY M. MATTHEWS[1] AND F. P. INMAN (Introduced by W. J. Payne)
Department of Microbiology, University of Georgia, Athens 30601

Candida albicans is a yeast-like fungus which is infective for man and other animals. At least two serological groups exist and these are referred to as A and B (1,2). Precipitating antibodies have been demonstrated in immune sera.

Stallybrass (2) has reported that rabbits respond immunologically to the cell wall polysaccharide and to a cytoplasmic protein, whereas humans respond only to the polysaccharide antigen. However, recently it was reported that immune serum from humans contained precipitating antibodies against two components of *Candida,* one of which evidently was mannan, and the other appeared to be a cytoplasmic constituent (3,4).

It has been reported (5,6) that the mannan component of the *Candida* cell wall is active serologically and is, in fact, the major antigen in the cell wall (1). The mannan isolated from *C. albicans* possesses a highly branched structure of alpha-1,2-linked mannose residues joined by alpha-1,6 linkages (7). The polysaccharide yielded positive precipitin reactions with sera from rabbits which had been immunized with the fungus.

Our purpose for doing this study was to identify the classes of rabbit antibody directed against *Candida* polysaccharide antigen. Since rabbit serum IgA is extraordinarily difficult to isolate in workable quantities, we restricted ourselves to investigation of IgG and IgM.

Materials and Methods. Candida albicans (McClung, K. U. 115) was maintained on Sabouraud's dextrose agar slants at 30°C. The yeast was transferred to fresh media about every 2 weeks.

The cells were grown in Sabouraud's dextrose broth at 30°C on a shaker, centrifuged, and washed 2 times with 0.16 *M* saline solution. The cells then were resuspended in the saline solution to give a 79% transmittance (10^6 to 10^7 cells/ml) on a Klett-Sommerson colorimeter equipped with a number 42 filter. The suspension was autoclaved for 15 min at 121°C. Stock suspensions were stored in a freezer at —20°C and the working suspension was kept refrigerated.

The cell wall antigen (mannan) was prepared by two methods. *A.* A soluble antigen was extracted by a modification of the procedure of Trevelyan and Harrison (8). A 10% (wet wt./vol) suspension of cells was autoclaved 15 min with 21% KOH. The insoluble mannan–copper complex formed on addition of Fehling's solution was washed by blending with 0.5 *M* NaOH, and then dissolved by addition of 6 *M* HCl and water. Mannan was precipitated with cold ethanol (50%), and after standing overnight, it was collected by centrifugation and washed with cold 50% ethanol. The mannan then was dissolved in 0.1% $BaSO_4$ in 0.02 *M* H_2SO_4, centrifuged to remove the insoluble salt, and finally dialyzed against 0.16 *M* saline solution buffered with 10^{-3} *M* sodium borate, pH 8. Merthiolate was added to inhibit bacterial growth, and the stock mannan was stored in the refrigerator.

B. Additional mannan was prepared essentially by the procedure of Sakaguchi *et al.* (9) except that the cells were autoclaved two times at 120°C in 0.05 *M* citrate buffer, pH 7. The purified mannan was lyophilized, redissolved in distilled water, and chromatographed on DEAE–cellulose. One fraction was eluted with water, and a second with 1 *M* NaCl solution. Polysaccharide in the eluates was detected through use of the Molisch test for carbohydrates. Only the first fraction was used in our work.

Four New Zealand white rabbits were immunized by repeated intravenous inoculation

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TABLE I. Agglutination of Killed *Candida albicans* by Rabbit Immune Serum before and after Absorption with Mannan Preparation A.

Rabbit no. (serum)	Serum dilution						
	10	20	40	80	160	320	640
1	+	+	+	+	+	±	—
2	+	+	+	+	+	±	—
3	+	+	+	+	+	—	—
4	+	+	+	+	+	—	—
A[a]	+	—	—	—	—	—	—
B[b]	+	+	+	+	—	—	—

[a] Sera (1–4) were pooled and absorbed with mannan.

[b] Control not absorbed with mannan.

with the stock killed suspension of *C. albicans* according to Campbell *et al.* (10). The serum titer was tested 6 days after the last injection. The animals were bled once each week for 1 month, and a booster injection of 1 ml was given 4 days prior to each bleeding.

Antibody-containing globulins were prepared by precipitation of serum with 18% Na_2SO_4 and subsequent chromatography on DEAE–cellulose and Sephadex G-200 (11).

Immunoelectrophoresis was done with 0.85% ionagar in 0.05 *M* sodium barbital buffer, pH 8.2. The agar was poured onto a glass plate and electrophoresed at 2 mA per linear cm for 90 min. A similar system was used for analyses by double diffusion in gel. The plates were allowed to develop about 18 hours at room temperature.

Whole cell agglutination tests were performed according to Campbell *et al.* (10). Serial dilutions of sera or immunoglobulin preparations were prepared, and an equal volume of the stock suspension of *Candida* was added. The presence or absence of agglutinated cells was determined after 18 hours incubation at 37°C.

Agglutination tests of absorbed preparations were performed essentially as above, except that mannan solutions were added to the diluted sera or immunoglobulin preparations. For the experiment reported in Table I, 1 ml of mannan preparation A was added to each tube, and for the results shown in Table II, 2 drops of the more concentrated preparation B were added. The mixtures first were incubated 5 hours at 37°C, and then overnight at 4°C. One ml of stock *Candida* suspension was added to the absorbed antibody-containing supernatant solutions subsequent to centrifugation. Control tubes contained the same initial amount of antiserum or immunoglobulin preparation as the experimental tubes, and were diluted with a volume of 0.16 *M* saline solution equal to the volume of mannan added to the experimental tubes.

The IgM-containing fraction was reduced at room temperature in 0.05 *M* Tris–0.5 *M* NaCl buffer with 0.1 *M* 2-mercaptoethanol for 1 hour. The mixture then was alkylated with 0.2 *M* iodoacetamide. After 30 min the reduced and alkylated protein was dialyzed against two changes of 0.16 *M* NaCl buffered with 10^{-3} *M* sodium borate, pH 8.

Experimental Results. A soluble cell wall antigen was extracted from *C. albicans*. The two procedures were designed to isolate an antigen rich in mannan. Previous investigators studied the chemical nature of similarly prepared antigens and found them composed of mannan (12), glucose (1), glucosamine (13), and protein (14). Our preparation B mannan was not precipitable by 5 or 95% trichloroacetic acid, but an amino acid analysis of the hydrolyzed mannan preparation indicated a small amount of protein was present. The hydrolyzate was devoid of glycine and methionine. Kessler and Nickerson (15) also found the protein component of their mannan preparation to contain no methionine. Although we did not thoroughly analyze our antigen preparations, we do have a soluble cell wall antigen that seems to resemble closely mannan preparations reported in the literature.

Table I shows the results of whole cell agglutination tests with sera from immunized rabbits. Two rabbits' sera had possible titers of 1:320, and all sera had a definite titer of 1:160. When whole cell agglutination tests were conducted on the absorbed and unabsorbed sera, the serum absorbed with mannan preparation A exhibited no agglutination when diluted 1:10; the unabsorbed serum agglutinated cells up to a dilution of 1:80.

IgG and an IgM-containing fraction were

TABLE II. Whole Cell Agglutination Tests with Purified Immunoglobulins before and after Absorption with Mannan Preparation B.

Immunoglobulin	Dilution of immunoglobulin							
	10	20	40	80	160	320	640	1280
IgG[a]	+	+	+	±	—	—	—	—
IgG (absorbed)[a]	±	—	—	—	—	—	—	—
IgM[b]	ND[d]	+	+	+	+	+	±	±
IgM (absorbed)[b]	ND	+	—	—	—	—	—	—
IgM (reduced)[c]	ND	±	—	—	—	—	—	—

[a] Concentration before dilution was 14 mg/ml.
[b] Concentration before dilution was 8 mg/ml.
[c] Reduced with 0.1 *M* 2-mercaptoethanol and alkylated with iodoacetamide.
[d] Not done.

isolated from the pooled immune serum and analyzed immunoelectrophoretically (Fig. 1). When the electrophoresed antigens were tested with goat antirabbit *immunoglobulins,* only one line appeared. When antirabbit *serum* was used to develop the plate one line appeared against IgG, but at least two were evident near the IgM well. These results indicated the IgG was free of contaminating proteins, but this was not true of the IgM fraction. Evidently IgM was the only immunoglobulin present in that fraction, although there was at least one other serum component.

Confirmation of the latter was obtained when the IgM-enriched fraction was examined in the analytical ultracentrifuge (Fig. 2). It was found to contain primarily IgM, as

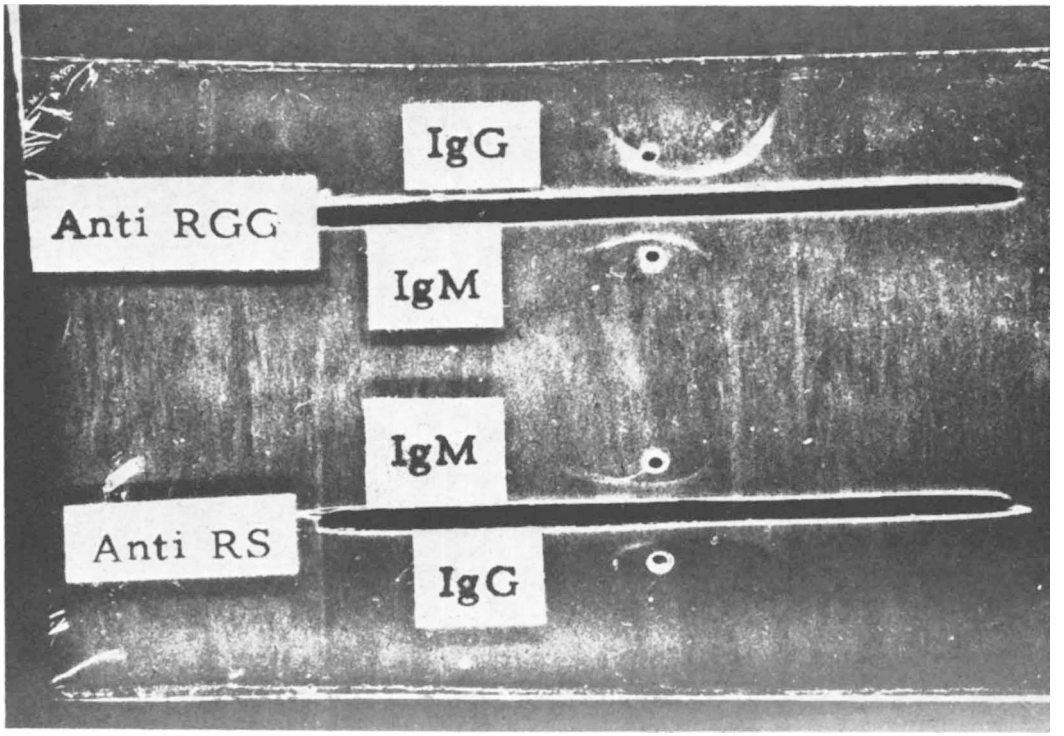

FIG. 1. Immunoelectrophoresis of IgG and IgM-enriched fractions. Abbreviations used: Anti-RGG, antirabbit immunoglobulins; Anti-RS, antirabbit whole serum.

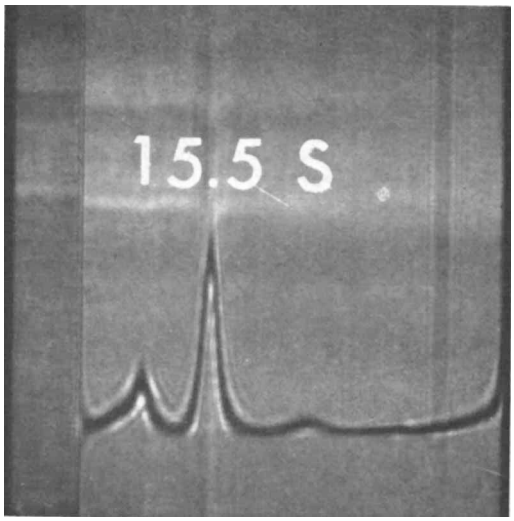

FIG. 2. Schlieren pattern of IgM-enriched fraction. The protein was dialyzed against 0.32 *M* NaCl solution containing 10^{-3} *M* sodium borate, pH 8. Protein concentration was 6 mg/ml, and photograph was made 12 min after reaching 60,000 rpm. Sedimentation is left to right.

well as two or three other proteins present in small amounts. The sedimentation value, $S_{20,w}$, of the IgM was 15.5.

The activity of IgG and IgM against mannan was tested by double diffusion in gel (Fig. 3). Doubling dilutions of the mannan preparation A were placed in the outer wells. Antibody-containing IgG or IgM, respectively, was placed in the center well. A single precipitin band was observed for each. This clearly demonstrates antibody activity in each immunoglobulin preparation. When the IgM-containing preparation was reduced and alkylated, the precipitin band no longer appeared.

The evidence to this point indicated both IgG- and IgM- type antibodies were produced in response to *Candida* cells. We wished to confirm this with mannan prepared under less harsh conditions.

Whole cell agglutinin tests were performed using IgG and the IgM-containing preparations (Table II). The IgG agglutinated cells up to a dilution of about 1:80. When the IgG was absorbed with mannan preparation B, much of its antibody activity was removed.

The IgM-enriched preparation agglutinated cells when it was diluted as high as 1:1280. Much of its antibody activity was lost after absorption with mannan preparation B. A portion of the IgM preparation was reduced and alkylated. As expected, most of the antibodies' ability to agglutinate cells was de-

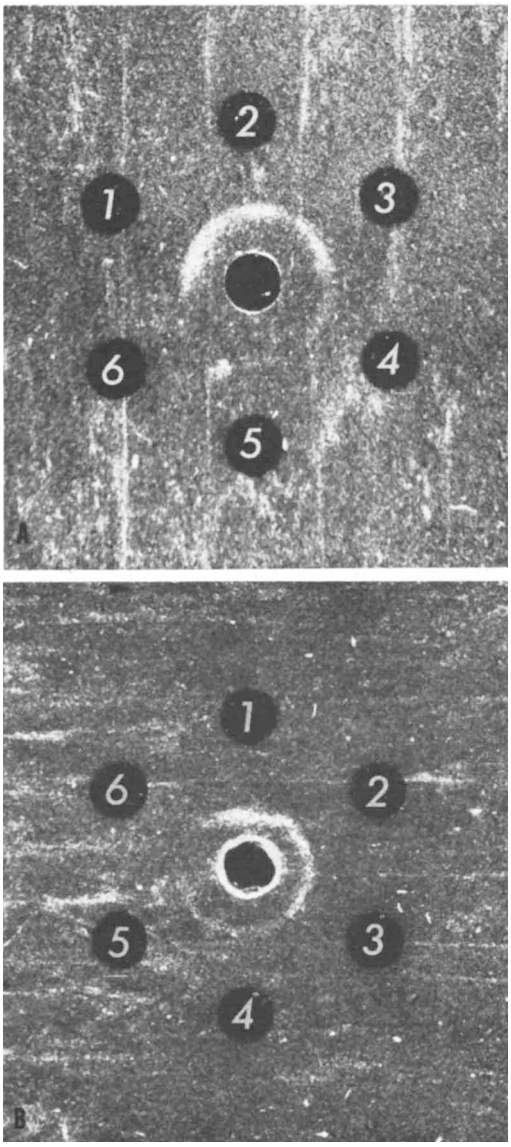

FIG. 3. Demonstration of antimannan activity in immunoglobulin preparations by double diffusion in agar. Doubling dilutions of mannan preparation A were placed in outer wells with undiluted mannan in well 1. A. Center well contained IgG (6 mg/ml). B. Center well contained the IgM-enriched fraction (6 mg/ml).

stroyed. Clearly, then, antibody activity is attributable to both IgG and IgM.

Discussion. Serum collected from immunized rabbits periodically was titered to determine when sufficient antibody against the cells was present. A large volume of pooled serum from each rabbit then was collected. When whole cell agglutination tests were performed, each serum pool was found to contain agglutinins in sufficient quantity. That a large proportion of the antibody was directed against cell wall mannan was demonstrated by the significant loss of agglutinin activity when the serum was absorbed with mannan.

Since our main concern was the identification of the classes of antibody directed against the cell wall antigen, it was necessary to separate the antibody-containing globulins. The IgG contained no contaminating serum proteins, and although the IgM-containing fraction comprised two or three proteins in addition to IgM, they were not of the immunoglobulin classes (Figs. 1 and 2).

Both the IgG and IgM-containing preparations had antibody activity against mannan prepared by either of the two procedures. When tested by double diffusion in agar, a single precipitin line appeared when either immunoglobulin preparation was used as a source of antibody.

The IgM comprises five similar subunits joined together by inter-subunit disulfide bonds (16). Each subunit, freed from IgM by reduction with 2-mercaptoethanol (2-ME) and alkylation to prevent repolymerization, apparently is capable of binding to only one antigenic site (17). Consequently, the visible antibody activity of IgM such as precipitation or agglutination, is lost subsequent to reduction. Since the antibody activity of IgG persists when similarly reduced and alkylated, mild reduction serves as a simple test for antibody activity attributable to IgM. We found that precipitin activity in agar gel of our IgM-containing preparation was lost following reduction. Therefore, we conclude that the antibody activity in this fraction is due to IgM.

Additional evidence that anti-*Candida* antibodies in immune rabbit serum comprise both IgG and IgM was obtained by whole cell agglutination tests using the purified immunoglobulins. When IgG was absorbed with mannan, much of the agglutinin activity was lost. The IgM agglutinated cells at dilutions considerably higher than those of IgG. This may reflect the fact that IgM is more active on a molar basis than IgG (17). After absorption of the IgM with mannan, the antibody preparation's ability to agglutinate cells was diminished greatly. Extensive loss of visible antibody reaction was noted also following reduction with 2-ME. This is consistent with the previous observations that antibody activity in this preparation of immunoglobulin is associated with IgM.

Summary. The nature of antibody globulins produced by rabbits immunized with heat-killed *Candida albicans* was investigated. Most of the antibodies were directed against a cell wall antigen rich in mannan. The agglutinin activity of immune serum, purified IgG, and a preparation highly enriched in IgM could be removed by absorption with mannan. In addition, when the IgM-containing preparation was reduced with 2-ME and alkylated, antibody activity was lost. We concluded that the rabbit makes antibodies of the IgG and IgM classes in response to intravenous injections of killed *Candida albicans*.

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Transfer of Antibiotic Resistance (R factor) in the Mouse Intestine* (33020)

THEODORE C. SALZMAN AND LYDIA KLEMM
(Introduced by Edward J. Hehre)

Department of Microbiology and Immunology and Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461

In *E. coli* and other species of the family *Enterobacteriacae* the genes responsible for antibiotic resistance are generally part of extrachromosomal elements (episomes) known as R factors (1–5). During conjugation R factors are transmitted to susceptible organisms with a frequency varying from about 10^{-2} to less than 10^{-7}/hour per donor cell (6). However, bacteria recently acquiring an R factor can transmit with a frequency greater than 1/hour per donor cell (6). In a given instance, the frequency of transfer depends upon the specific R factor, the specific mating pair, the growth medium, and the conditions of growth. However, R factors replicate faster than the bacterial chromosome and, *in vitro,* it is not unusual for a high proportion of a population of susceptible bacterial cells to acquire drug resistance when grown under suitable conditions with an appropriate donor.

The widespread prevalence of antibiotic resistant *Enterobacteriacae* in man and domestic animals results, primarily, from the selective pressure of antibiotic therapy in these species. However, the infectivity of R factors, their capacity for autonomous replication, and the lack of any known adverse effect upon bacterial cells, suggest that they can survive and spread among bacteria in nature without the selective pressure of antibiotic therapy.

The normal habitat of most species of *Enterobacteriacae* is the mammalian intestinal tract and several strains can be found in the same gut. Although there is epidemiologic (6) and experimental (7,8) evidence that R factor transfer can occur in the intestine, little is known of the rate of spread of R factors among populations of susceptible bacteria.

For conjugation to occur in the intestinal tract efficiently, the animal must necessarily be colonized by large numbers of donor and recipient bacteria. It has proven difficult to experimentally colonize animals with most nonenteropathic strains of *Enterobacteriacae* unless the animals' indigenous flora has first been suppressed by oral antibiotics. Moreover, heavy colonization is usually not long lasting; presumably the normal inhabitants of the intestinal flora grow back and again repress the implanted strains. The antibiotics may also favor the growth of resistant organisms and therefore the proportion of resistant bacteria in the flora may not be a measure of the efficiency of R factor transfer alone.

This study presents results derived from the purposeful colonization of germ-free mice. Obviously, bacterial antagonism from indi-

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