Mannan-Specific Immunoglobulin G Antibodies in Normal Human Serum Mediate Classical Pathway Initiation of C3 Binding to *Candida albicans*

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Candida albicans activates both the classical and alternative complement pathways. Previous studies found that immunoglobulin G (IgG) in normal human serum (NHS) mediates classical pathway initiation. The goal of this study was to determine the role of candidal mannan-specific human IgG antibodies in complement activation. Mannan was purified from the yeast cells, and naturally occurring antimannan IgG was isolated from pooled NHS or plasma samples by immunoaffinity chromatography. Early activation and binding of C3, characteristics of classical pathway activity, were abolished in yeast- or mannan-absorbed serum but could be restored to absorbed serum with added purified antimannan IgG in a dose-dependent manner. Microscopically, the immunofluorescence pattern of initial C3 binding was diffuse over the entire cell surface for yeast cells incubated in NHS or in mannan-absorbed NHS supplemented with antimannan IgG but was asynchronous and focal for yeast cells incubated in EGTA-treated or mannan-absorbed NHS. The antimannan IgG level in serum samples from 21 donors varied from 17 to 570 $\mu\text{g/ml}$ of serum compared to 220 μg in pooled NHS samples. The rate of initial C3 binding to yeast cells correlated with the level of antimannan IgG in sera from different individuals ($r^2 = 0.94$) and could be accelerated in sera containing lower amounts of antimannan IgG with exogenous antimannan IgG. These observations identify antimannan IgG as the initiator of classical pathway C3 deposition on *C. albicans*. Given the variability in the levels of antimannan antibodies in sera from different individuals, the presence or absence of these antibodies may be an important determinant of host resistance to disseminated candidiasis.

The complement system, an innate defense mechanism, plays an important role in host resistance in animal models of candidiasis (2, 13, 14). In vitro studies have shown that both the classical and alternative pathways are involved in activation of the complement system and binding of C3 fragments to *Candida albicans* (9, 11). Deposition of C3 molecules following initiation of the classical pathway can be detected within 1 min after the yeast cells are introduced into normal human serum (NHS) (11). This binding of C3 is markedly delayed in NHS that had been absorbed by yeast cells to remove potential initiators of the classical pathway or in NHS that had been treated with EGTA to chelate Ca^{2+} required for classical pathway activity (11). However, classical pathway activity can be restored to yeast-absorbed NHS by the addition of immunoglobulin G (IgG) isolated from pooled NHS samples (11). The latter observation identifies a role for anti-*Candida* IgG as an initiator for the classical pathway. The specificity of the IgG which initiates the classical pathway has not been determined. Mannan is a major surface component of the candidal cell wall, and antimannan IgG is ubiquitous among healthy individuals without regard to age, race, or gender (12). These observations suggest the possibility that serum antimannan IgG mediates initiation of the classical pathway in NHS, which leads to binding of C3 fragments to candidal yeast cells.

The goals of our study were (i) to determine whether absorption of NHS with purified *C. albicans* yeast mannan abolishes the activity of the classical pathway in a manner similar to

that observed with whole yeast cells, (ii) to determine whether affinity-purified antimannan IgG restores classical pathway initiation kinetics to serum absorbed with either whole yeast cells or purified mannan, and (iii) to assess the correlation between the levels of antimannan IgG in sera from different donors and the ability of the sera to support classical pathway initiation. Our results identify antimannan IgG as the primary initiator of the classical complement pathway in sera from healthy adult donors.

MATERIALS AND METHODS

Yeast cells and isolation of mannan. Yeast cells of *C. albicans* CA-1, a serotype A strain provided by Jim E. Cutler, Montana State University, were used for all experiments and were maintained and grown as described previously (5, 11). Briefly, GYEP agar slants (2% glucose, 1% peptone, 0.3% yeast extract) were inoculated with yeast cells stored in 50% glycerol at -80°C and incubated for 24 to 48 h at 37°C . This slant culture was used to start 3-ml GYEP liquid cultures in 15-ml culture tubes shaken at 180 rpm at 37°C . The tube culture was passaged three times, each time for 24 h. The final tube culture was used to inoculate 250 ml of GYEP in a 1-liter flask that was shaken at 150 rpm at 37°C for 24 h. Yeast cells were killed with 1% formaldehyde at 4°C overnight, harvested by centrifugation, washed, resuspended in phosphate-buffered saline (1.9 mM NaH_2PO_4 , 8.1 mM Na_2HPO_4 , 154 mM NaCl; pH 7.2) (PBS) containing 0.02% azide, and stored at 4°C .

The mannan of CA-1 yeast was isolated by the method of Kobayashi et al. (7), which is based on previously described methods for isolation of yeast mannan (8, 16). *C. albicans* yeast mannan obtained through this method typically contains about 95% carbohydrate as mannose and about 2% protein (7). Briefly, yeast cells were grown for 24 h at 37°C in GYEP, harvested by centrifugation, washed with 0.9% NaCl, treated with acetone, and then resuspended in water. The suspension was autoclaved at 128°C for 5 h, and water-insoluble cellular debris was removed by centrifugation. The supernatant fluid was dialyzed against water at 4°C and lyophilized. The lyophilized material was dissolved in water, and mannan was precipitated with Fehling solution. The precipitate was dissolved in water containing Amberlite IR-120 (H^+) resin (Aldrich, Milwaukee, Wis.). After removal of the resin by centrifugation, the mannan solution was neutralized, dialyzed against water at 4°C , and lyophilized.

Serum samples and serum proteins. Serum samples were prepared from peripheral blood samples collected from 21 healthy donors after they had given

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their informed consent. An aliquot of 1.5 ml from each serum sample was removed, and the remainder was pooled. The serum preparations were stored at -80°C . Serum was heated for 30 min at 56°C for studies requiring heat-inactivated serum. C3 was isolated from frozen human plasma samples as previously described (10, 19) and stored at -80°C until use. C3 was labeled with ^{125}I as described previously (1) by use of IODOGEN reagent (Pierce, Rockford, Ill.).

Naturally occurring human antimannan IgG was isolated by affinity chromatography with immobilized mannan and then with protein A. The isolated candidal mannan described above was coupled to CNBr-Sepharose 4B at 1 to 5 mg of mannan to 1 ml of gel according to the manufacturer's directions (Pharmacia Biotech, Uppsala, Sweden). A column of mannan-Sepharose 4B was constructed and equilibrated with (PBS). The pooled serum samples described above or pooled human plasma samples from healthy donors (plasma samples purchased from a local blood bank) were applied to the column, which was washed with PBS at 4°C . Mannan-bound proteins were eluted with 4 M MgCl_2 containing 0.1 M HEPES, pH 7.2 (20). The eluate was dialyzed against PBS at 4°C , and IgG was isolated with immobilized protein A according to the manufacturer's directions (Pierce). The eluate from protein A was dialyzed against PBS at 4°C and concentrated in a stirred ultrafiltration cell (Amicon, Inc., Beverly, Mass.). Analysis of the concentrate by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis under reducing conditions followed by Coomassie blue staining showed only two bands at 55 and 22 kDa, characteristic of human IgG heavy and light chains. The concentration of the purified IgG was determined by measuring A_{280} , assuming 1.4 U of absorbance at 1 mg of protein/ml. Since the immunoaffinity purification procedure may inactivate a percentage of the purified antibodies (20), the proportion of antibody that retained binding activity after purification was estimated as follows. PBS alone or PBS containing 100 μg of purified IgG/ml (PBS-IgG) was absorbed with yeast cells twice, each time with 10^8 cells per ml. The difference in A_{280} between PBS and PBS-IgG before and after yeast absorption was used to estimate the percentage of active antibodies in the IgG concentrate. The percentage was found to be 37. Thus, the amount of purified IgG antibody used in experiments reported here reflects the proportion of the IgG that retained the ability to bind to yeast cells of *C. albicans*.

Several experiments used NHS that had been absorbed with yeast cells or mannan-Sepharose 4B to remove potential initiators of the classical pathway. To produce once-absorbed serum, NHS was incubated for 60 min at 0°C with 10^8 yeast cells per ml of serum (mixed every 10 to 15 min) and separated from the cells by centrifugation. In the case of twice-absorbed serum, the absorption procedure was repeated. Unless otherwise specified, yeast-absorbed serum used for this study was produced by two yeast absorption procedures. To prepare mannan-absorbed serum, NHS was incubated with mannan-Sepharose 4B beads at a ratio of 6:4 (vol/vol) for 60 min at 0°C (mixed every 10 to 15 min) and separated from the beads by centrifugation. The absorbed serum was filtered through a $0.45\text{-}\mu\text{m}$ -pore-size filter and used fresh or frozen at -80°C for later use. Estimates of the final concentration in serum were corrected for the dilution that occurred during the absorption procedures. Serum complement activity following absorption with Sepharose 4B beads alone was evaluated, and no difference was found between untreated NHS and bead-absorbed NHS in the kinetics of C3 binding to yeast cells.

ELISAs for quantification of serum antimannan IgG. A modified method of Lehmann and Reiss (12) was used for the enzyme-linked immunosorbent assay (ELISA). Briefly, wells of Immulon 1 microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.) were coated overnight with 400 ng of the isolated mannan in 100 μl of coating buffer (15 mM Na_2CO_3 and 35 mM NaHCO_3 ; pH 9.5). The wells were washed and blocked with PBS containing 0.05% Tween 20 (PBS-T) and then incubated for 2 h with serial dilutions of serum samples or purified antimannan IgG of known concentration in 100 μl of PBS-T. After three washes, the wells were incubated for 90 min with 100 μl of PBS-T containing goat anti-human IgG antibodies conjugated with horseradish peroxidase (1/16,000; Southern Biotechnology Associates, Birmingham, Ala.). The wells were washed three times with PBS-T and incubated for 30 min with 100 μl of tetramethylbenzidine microwell peroxidase substrate solution (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). The reaction was stopped by the addition of 100 μl of 1 M H_3PO_4 . A_{450} was read within 30 min with a Ceres 900 ELISA workstation (Bio-Tek Instruments, Winooski, Vt.). The amount of antimannan IgG in various serum samples was expressed either in micrograms per milliliter, which was estimated from standard curves with purified antimannan IgG, or as the titer producing an optical density at 450 nm of 3, which was calculated by use of a four-parameter nonlinear regression function of SigmaPlot (Jandel Corporation, San Rafael, Calif.).

Quantitative analysis of C3 binding with ^{125}I -labeled C3. Binding of C3 to candidal yeast cells was analyzed by the procedure of Kozel et al. (11). Briefly, each complement binding medium contained (i) 40% absorbed or nonabsorbed serum, (ii) GVB-Mg-Ca (sodium Veronal [5 mM]-buffered saline [142 mM], pH 7.3, containing 0.1% gelatin, 1.5 mM CaCl_2 , and 1 mM MgCl_2), or GVB-Mg-EGTA (sodium Veronal [5 mM]-buffered saline [142 mM], pH 7.3, containing 0.1% gelatin, 5 mM EGTA, and 5 mM MgCl_2), and (iii) ^{125}I -labeled C3 sufficient to provide a specific activity of 50,000 cpm/ μg of C3 for the mixture of labeled and unlabeled C3 in serum (assuming that NHS contains 1,200 μg of C3/ml). The reaction medium was warmed to and kept at 37°C , and 2.0×10^6 yeast cells per ml of reaction medium were then added to initiate C3 binding. To study kinetics of C3 binding, 50- μl samples were withdrawn in duplicate at various time intervals and added to 200- μl portions of a stop solution (PBS, 0.1% SDS, 20 mM

EDTA) in Millipore MABV-N12 filter plates fitted with BV 1.2- μm -pore-size filter membranes (Millipore, Bedford, Mass.). The particles were washed five times with PBS containing 0.1% SDS. The membranes were removed, and the amount of radioactivity bound to the yeast cells collected on the membranes was determined with a Packard AUTO-GAMMA gamma counter. Specific binding was determined by subtracting the radioactivity of samples which used heat-inactivated serum from the total binding observed with NHS or other serum preparations. In some experiments, purified human antimannan IgG was added to the binding medium prior to the addition of yeast cells.

Immunofluorescence analysis of binding of C3 or antimannan IgG to yeast cells. The pattern of C3 binding to the yeast cell surface was analyzed with Oregon green 514-conjugated anti-human C3 antibodies. Briefly, rabbit anti-human C3 antiserum was purified by immunoaffinity on a column of C3-Sepharose 4B, and the IgG fraction was isolated by use of immobilized protein A (Pierce). Conjugation of the rabbit anti-C3 IgG to Oregon green 514, a fluorescein derivative, was achieved by following the manufacturer's recommendations (Molecular Probes, Eugene, Oreg.). The ratio of dye to IgG in the conjugate was estimated to be 6. Yeast cells were opsonized in the same manner as described above for the quantitative analysis, except that the reaction mixtures did not contain ^{125}I -labeled C3. At time intervals of 4, 8, and 16 min, 200- μl samples were transferred to 900 μl of ice-cold PBS containing 10 mM EDTA, and unbound C3 was removed by three washes with PBS. The bound C3 was detected by incubation of the cells for 1 h at 4°C with 250 μl of the Oregon green conjugate diluted 1:25 in PBS containing 1% bovine serum albumin (Sigma, St. Louis, Mo.). The cells were washed twice in PBS, pelleted, suspended in one drop of Vectashield (Vector Laboratories, Inc., Burlingame, Calif.), and applied to microscope slides that had been coated with poly-L-lysine (Sigma).

Similarly, the pattern of antimannan IgG binding to the yeast cells incubated in the C3 binding medium containing either NHS or the purified antimannan IgG antibodies was detected by fluorescein-conjugated goat anti-human IgG diluted 1:100 (Southern Biotechnology Associates). Yeast cells incubated in the C3 binding medium alone served as a control for nonspecific binding of the indicator antibodies.

The fluorescence images of C3 or antimannan IgG binding patterns displayed on the yeast surface were acquired at 1- μm intervals through individual cells by an epifluorescence microscope equipped with a Photonic Science Colour Cool-View image acquisition system (East Sussex, United Kingdom) and were processed with the aid of image analysis software including Image-Pro Plus (Media Cybernetics, Silver Spring, Md.) and MicroTome (VayTek, Fairfield, Iowa). Each stack of acquired images from a single cell was digitally deconvolved and projected onto a single plane.

RESULTS

Absorption of NHS to mannan abolishes the classical pathway initiation of C3 binding. Previous studies found that classical pathway initiation of C3 binding to yeast cells of *C. albicans* is blocked in serum that had been preabsorbed with the homologous yeast cells but can be restored to the yeast-absorbed serum by the addition of IgG isolated from pooled NHS samples (11). Because the surfaces of *C. albicans* yeast cells are rich in mannan, initial experiments were conducted to determine whether mannan-reactive serum component(s) were involved in initiation of the classical pathway. Mannan was isolated from the yeast cells and coupled to Sepharose 4B beads. The immobilized mannan was used to remove mannan-reactive serum component(s) from pooled NHS samples. Similarly, NHS was absorbed either once or twice with yeast cells. The titer of antimannan IgG in the mannan- or yeast-absorbed human serum was measured by an ELISA in which antimannan IgG was detected with anti-human IgG antibodies. Mannan absorption reduced the amount of antimannan IgG to 0.2% of that of untreated NHS; one yeast absorption procedure reduced it to 1.2%, and two yeast absorption procedures reduced it to 0.8%. This result indicates that yeast absorption removed significant quantities of antimannan IgG.

Subsequently, we examined the classical pathway activity of mannan-absorbed serum. Candidal yeast cells were incubated for 1 to 36 min in a complement binding medium containing 40% NHS, 40% NHS containing Mg-EGTA, or 40% yeast- or mannan-absorbed NHS. Figure 1 presents the results of one representative experiment from five independent assays. The results show that accumulation of C3 fragments on yeast cells incubated in untreated NHS was rapid and detectable within 1 min. How-

FIG. 1. Kinetics of C3 binding to yeast cells in mannan- or yeast-absorbed serum. Yeast cells were incubated in a C3 binding medium containing 40% NHS (●), 40% NHS preabsorbed with mannan-Sepharose beads (▼), 40% NHS preabsorbed with the homologous yeast cells (■), or 40% NHS supplemented with Mg-EGTA (◆).

ever, there was a 6-min delay before detectable amounts of C3 were bound to the yeast cells incubated in NHS containing Mg-EGTA or in NHS preabsorbed with yeast, a result that is similar to that of an early study conducted under similar conditions (11). The blockade of early deposition of C3 molecules in mannan-absorbed serum was much longer, approximately 12 min (Fig. 1).

Antimannan IgG antibodies from NHS restore classical pathway initiation kinetics to absorbed serum. Since serum IgG reactive with candidal mannan is ubiquitous in the general population (12) and mannan absorption introduced a marked reduction in the level of antimannan IgG in serum, we sought to determine the effect of antimannan IgG from NHS on the kinetics of activation and binding of C3. Mannan-specific IgG antibodies were isolated from pooled serum or plasma samples by immunoaffinity chromatography. With affinity-purified IgG as a standard, pooled serum samples from 21 donors were found to contain 220 μ g of antimannan IgG per ml. Kinetics of C3 binding to yeast cells incubated for 1 to 36 min in 40% yeast- or mannan-absorbed serum supplemented with the purified antimannan IgG to the level found in the pooled serum samples was compared to that in untreated NHS or absorbed NHS. The results (Fig. 2) showed that the addition of the mannan-specific IgG restored the characteristic early classical pathway initiation kinetics to either mannan- or yeast-absorbed serum.

Next, we determined the dose-dependent effects of antimannan IgG on restoration of the classical pathway activity to absorbed serum. Because mannan or yeast absorption introduced a 6- to 12-min delay in the initiation of C3 binding to the yeast cells (Fig. 1), we viewed the accumulation of C3 molecules on the yeast cell for up to 6 min as an immediate consequence of classical pathway initiation. Thus, we used the 6-min accumulation of C3 molecules per cell as an overall measure of the classical pathway activity. Yeast cells were incubated for 6 min in a complement binding reaction mixture containing 40% yeast- or mannan-absorbed pooled sera supplemented with 0 to 280 μ g of antimannan IgG per ml of reaction medium. Figure 3 shows that the number of C3 molecules bound to candidal cells increased in response to increasing amounts of antimannan IgG and reached a plateau at about 100 μ g of IgG per ml of reaction mixture. This corresponds to approximately 250 μ g of antibody per ml of undiluted serum. A reduction in C3 binding occurred when the amount of IgG exceeded 100 μ g

FIG. 2. Restoration of classical pathway initiation kinetics to mannan- or yeast-absorbed serum with purified human antimannan IgG antibodies. Yeast cells were incubated in a C3 binding medium containing 40% NHS (●), 40% absorbed NHS (○), or 40% absorbed NHS that was supplemented with purified human antimannan IgG antibodies (▼). The amount of supplemented antimannan IgG antibodies was 88 μ g per ml of binding medium, which corresponds to 220 μ g of antimannan IgG found per ml of pooled sera used in this study.

of IgG per ml of reaction mixture. Taken together, these observations indicate that mannan-specific IgG initiates the classical pathway in a dose-dependent manner.

The serum antimannan IgG level correlates with the amount of early C3 binding to yeast cells. The antimannan IgG levels in serum samples from 21 donors were determined with purified antimannan IgG as a standard and were found to vary from 17 to 570 μ g per ml of serum. This wide range of naturally occurring antimannan IgG in serum prompted us to examine the classical pathway initiation kinetics of sera from different individuals. Sera from 11 donors were selected by virtue of their titers which were low, medium, and high relative to the titer of pooled sera from the 21 donors. To minimize potential variation of sera from individual donors in the amplification activity following the initial deposition of C3 molecules, yeast cells were incubated only for 1 and 2 min in 40% sera from donors. The number of C3 molecules bound per yeast cell per minute over this early time interval was used as a measure of the initial C3 binding. The results showed that the level of antimannan IgG in sera from different individuals highly correlated with the rate of initial C3 binding ($r^2 = 0.94$ [Fig. 4]).

Donor sera with antimannan titers lower than that of the pooled NHS were chosen to assess the effect of exogenous antimannan IgG on classical pathway initiation kinetics. In one experiment, sera from donors 234 and 240 were used. The

FIG. 3. Dose-response effect of exogenous antimannan IgG on early activation and binding of C3 to *C. albicans*. Yeast cells were incubated for 6 min in a C3 binding medium containing either 40% mannan-absorbed serum (○) or 40% yeast-absorbed serum (●) and containing various amounts of purified human antimannan IgG per milliliter of binding medium.

antimannan IgG levels in sera from donors 234 and 240 were 20 and 13%, respectively, of that of the pooled sera. Yeast cells incubated in serum from either donor exhibited a delay of about 4 min in the initiation of C3 deposition compared to the value for yeast cells incubated in pooled sera (Fig. 5). The delays were largely abrogated when the level of antimannan IgG in sera from these two individuals was raised to the level found in pooled NHS by the addition of exogenous antimannan IgG (Fig. 5). However, the added IgG did not seem to alter the maximum binding of C3 to yeast cells incubated in serum from either donor alone (Fig. 5). Similar observations were found with sera from three other donors whose antimannan IgG levels were 8, 24, and 47% of the pooled NHS (data not shown). These observations indicate that the level of naturally occurring antimannan IgG in human sera directly influences the rate of early accumulation of C3 on the yeast cells.

Antimannan IgG restores a synchronous pattern of C3 binding to yeast cells in mannan-absorbed serum. The above observations describe quantitatively the effect of antimannan IgG

FIG. 4. Correlation between serum antimannan IgG titer and the ability of sera from different individuals to support early binding of C3 to *C. albicans* cells. Yeast cells were incubated in 40% sera from different donors (●) and sampled after 1 and 2 min of incubation. The number of C3 molecules bound to individual yeast cells per minute was determined. The rate of initial C3 binding to yeast cells incubated in pooled NHS (○) is shown as a reference point but was not included in the correlation analysis.

FIG. 5. Effect of exogenous antimannan IgG on the kinetics of C3 binding to yeast cells in sera from two donors with low levels of naturally occurring antimannan IgG. Yeast cells were incubated in a C3 binding medium containing 40% pooled NHS (●), 40% donor serum (▽), or 40% donor serum supplemented with antimannan IgG (▼). Donor 234 had 43 μ g of antimannan IgG per ml of serum, and donor 240 had 28 μ g/ml. Purified human antimannan IgG was added to the level found in pooled NHS (88 μ g per ml of binding medium, which corresponds to 220 μ g per ml of serum).

on the accumulation of C3 on *C. albicans*. Previous observations found that the microscopic pattern of initial C3 binding to the cell surface of *C. albicans* through the classical pathway is distinct from the pattern observed only if the alternative pathway is operative (11). The initial binding of C3 molecules activated through the alternative pathway is limited to focal sites on the cell surface, whereas the initial binding of C3 molecules activated via the classical pathway is diffuse over the entire surface (11). Consequently, we sought to determine the pattern of C3 activation and binding in mannan-absorbed serum with or without the addition of exogenous anti-mannan IgG antibodies. The C3 binding patterns for yeast cells incubated for 4, 8, or 16 min in mannan-absorbed NHS supplemented with antimannan IgG antibodies were compared to the patterns observed in either NHS, NHS-Mg-EGTA, or mannan-absorbed NHS. The binding patterns of C3 molecules were detected with fluorophore-tagged anti-C3 IgG antibodies (Fig. 6). An asynchronous, focal initiation pattern was observed on yeast cells after a 4-min incubation in NHS treated with Mg-EGTA or after an 8-min incubation in NHS absorbed with mannan. In contrast, a uniform and apparently synchronous pattern of C3 binding was observed after a 4-min incubation on yeast cells incubated in NHS or mannan-absorbed serum that was supplemented with antimannan IgG antibodies. In parallel with these immunofluorescence assays for C3

FIG. 6. Immunofluorescence patterns of C3 binding to the yeast cell surface. Yeast cells were incubated in 40% NHS, 40% NHS containing Mg-EGTA, 40% mannan-absorbed NHS, or 40% mannan-absorbed NHS supplemented with exogenous antimannan IgG at 88 μ g per ml of binding medium and stained with affinity-purified rabbit anti-human C3 IgG antibodies conjugated with Oregon green 514. The patterns of C3 binding were visualized with fluorescence microscopy.

binding patterns, we examined the distribution of epitopes recognized by the isolated antimannan IgG antibodies. Yeast cells incubated for 1 min either in NHS or in PBS containing antimannan IgG and stained with fluorophore-tagged goat antibodies against human IgG exhibited a bright and uniformly distributed fluorescence staining (data not shown). No staining was observed on yeast cells incubated in PBS containing the goat antiserum alone. These microscopic observations provide further experimental evidence that polyclonal antimannan antibodies in NHS are required for the early and synchronous initiation of the complement cascade, which leads to deposition of C3 fragments onto the cell surface.

DISCUSSION

A previous study from our laboratory showed that absorption of pooled NHS samples by yeast cells of *C. albicans* removes serum factors that are required for the activation of the classical pathway (11). In this study, we demonstrate that these serum factors primarily consist of antimannan IgG antibodies. This conclusion is based on five lines of evidence. First, absorption of NHS with immobilized candidal yeast mannan reduces the serum antimannan IgG titer and abolishes the early deposition of C3 that is characteristic of classical pathway initiation (Fig. 1). Second, the addition of purified antimannan IgG restores the early classical pathway kinetics to serum that had been absorbed either by immobilized mannan or by homologous yeast cells (Fig. 2) in a dose-dependent manner (Fig. 3). Third, the level of antimannan IgG in sera from different donors quantitatively correlates with the rate of initial C3 binding to the yeast cells (Fig. 4). Fourth, the classical pathway initiation of complement binding in sera from different donors with lower amounts of antimannan IgG is slower than that in pooled NHS but can be accelerated by the addition of exogenous antimannan IgG antibodies (Fig. 5). Last, the addition of exogenous antimannan IgG antibodies restores the rapid and synchronous C3 binding pattern to mannan-absorbed serum (Fig. 6); moreover, this uniform binding is consistent with the distribution of mannan epitopes recognized by the antimannan antibodies alone (data not shown).

The requirement for antimannan antibody in classical pathway initiation further demonstrates the importance of surface-

displayed *Candida* mannan in host-yeast interactions. *Candida* mannan is structurally similar to that of *Saccharomyces cerevisiae* and is a complex glycoprotein composed of mannose polymers and oligomers attached in different ways to a peptide (for a review, see reference 15). It is conceivable that antimannan antibodies used in this study may contain antibodies that are reactive with either carbohydrate or protein components or both. Although determination of the fine specificity of the antimannan antibodies was beyond the scope of this study, our recent observations from separate studies suggest that putative antibodies reactive with mannan protein components have little or no role in activation of the classical pathway by *C. albicans* yeast. First, treatment of yeast cells with periodate completely abolishes the abilities of the cells to bind antimannan antibodies and to initiate the classical pathway (22). Second, treatment of yeast cells with proteolytic enzymes such as proteinase K, pronase, or trypsin has no discernible effect on the ability of the cells to bind antimannan antibodies or to support classical pathway initiation of complement deposition to the cells (21). These observations are consistent with the contention that components of *C. albicans* mannan carbohydrate are recognized by the antimannan antibody in NHS.

Absorption of NHS with either the purified candidal mannan or homologous yeast cells abolished early deposition of C3 in a manner similar to the treatment of serum with EGTA (Fig. 1). However, detection of C3 molecules deposited onto yeast cells in the mannan-absorbed serum was further delayed (about 12 min) compared with the yeast-absorbed or EGTA-treated serum (about 6 min). There may be more than one factor that contributed to this difference. One possibility for the discrepancy between the activities of yeast- and mannan-absorbed sera may be related to residual amounts of antimannan IgG after absorption. For example, the antimannan titer in mannan-absorbed serum was 0.2% of that in untreated NHS, 0.8% in twice-absorbed serum, and 1.2% in once-absorbed serum. It has been reported that absorption of serum once with 10^8 of the same yeast cells as used in this study produced a delay of 4 min in the detection of C3 binding to yeast cells, whereas absorption twice, each with 10^8 yeast cells, introduced a delay of 6 min (11). Our observation that restoration of classical pathway initiation to the mannan-absorbed NHS with antimannan IgG was dose dependent (Fig. 3) provides further evidence in support of this explanation. With regard to the difference between EGTA-treated NHS and mannan-absorbed NHS in the delay of C3 binding initiation (6 versus 12 min [Fig. 1]), there may be at least two mechanisms. One is that mannan absorption altered the serum in some manner that diminished alternative pathway activity, which may have resulted in a slower initiation of complement binding in mannan-absorbed serum (12 min) than in yeast-absorbed or EGTA-treated serum (6 min). The observation that the addition of exogenous antimannan IgG restored classical pathway initiation kinetics to the mannan-absorbed serum (Fig. 2) suggests that such a putative alteration in alternative pathway activity may not be apparent when a second mechanism for the initiation of the complement system is available, i.e., the classical pathway. An alternative explanation is that the naturally occurring antimannan antibody in NHS may facilitate initiation of the alternative pathway. Such an antibody would be unavailable in mannan-absorbed serum. This antibody-dependent activation of the alternative pathway has been described for zymosan by Schenkein and Ruddy (18). Experiments currently in progress in our laboratory suggest that antimannan antibody does, indeed, play a role in activation of the alternative pathway by *C. albicans* yeast. In preliminary experiments, we have found that the kinetics for binding of C3 to *C. albicans* yeast in mannan-

absorbed serum which was treated with EGTA and supplemented with purified antimannan IgG were identical to the kinetics observed in EGTA-treated unabsorbed serum (21).

Classical pathway complement activation was restored to yeast- or mannan-absorbed serum with affinity-purified antimannan antibodies in a dose-dependent manner (Fig. 3). However, when the supplemented exogenous IgG exceeded 100 µg per ml of binding medium containing 40% absorbed serum, a reduction in C3 binding was observed (Fig. 3). The suppressive effect of the polyclonal antimannan IgG antibodies on the binding of C3 was unexpected, and to our knowledge, a similar phenomenon has not been reported in the literature. The mechanism for this phenomenon is unknown. Excess numbers of antimannan IgG antibodies bound to the cell surface may exclude the binding of C3b generated through alternative pathway amplification and/or the binding of complement factors required for amplification of C3 binding. Antimannan IgG antibodies with particular epitope specificities may be suppressive in that their binding alters the microenvironment of the cell surface. Such alteration may influence the binding of newly generated metastable C3b and/or regulators of the amplification cascade. The modulation of binding of C3b amplification cascade regulators by cell surface architecture has been noted in different organisms. For example, C3b molecules bound to nonencapsulated or encapsulated *Cryptococcus neoformans* yeast cells follow distinctly different kinetics for conversion to iC3b when the yeast cells are incubated with factors H and I (17). In another example, membrane-associated sialic acid on sheep erythrocytes favors the binding of factor H, relative to factor B, to cell-bound C3b, a mechanism that is responsible for the inability of sheep erythrocytes to activate human alternative complement pathway (6). While in group A streptococci, cell surface M protein promotes the dissociation of C3b-Bb in the presence of factor H (4).

The rate of classical pathway-mediated C3 binding to *C. albicans* was highly correlated with the level of naturally occurring antimannan IgG (Fig. 4); moreover, the rate of early C3 binding in sera from different donors with lower levels of antimannan IgG could be accelerated by the addition of exogenous antimannan antibodies (Fig. 5). The ability of this naturally occurring anti-*Candida* IgG to rapidly activate the complement system for deposition of potentially opsonic C3 fragments on the yeast may be important in initial defense against the yeast. However, the significance of rapid classical pathway initiation of the C3b amplification cascade has not been determined with certainty. Gelfand et al. (2) examined the role of the classical complement pathway in host defense against experimental disseminated candidiasis. No difference was found in either mortality or tissue distribution of the yeast between normal and C4-deficient guinea pigs that had been infected with *C. albicans* (2). The study noted, however, that these animals had no detectable levels of agglutinating antibody to the yeast (2); thus, an absence of a role for the classical pathway in host resistance is not surprising. Han and Cutler (3) found that an IgM monoclonal antibody specific for a mannan epitope (Mab 6.1) protects mice against disseminated candidiasis (as measured by survival and kidney infection), whereas another IgM directed against a different surface determinant (Mab 6) did not protect the mice. Since IgM antibodies are known to initiate the classical complement pathway, the observation of IgM-mediated protection implies an important role for the early and rapid initiation of complement opsonization via the classical pathway. It is attractive to speculate that the presence of

antimannan antibodies in human serum contributes to host resistance and that the absence of such antibodies is an indicator of increased susceptibility to disseminated candidiasis.

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