Activation of Human Complement System by *Mucor polymorphosporus* Mycelia

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Abstract: Complement activation by *Mucor polymorphosporus* mycelia was studied using absorbed human serum in the presence or absence of chelators (EGTA or EDTA). We found that it presented low complement consumption when incubated with EGTA-Mg^{2+} or without chelators. Anionic sugars, such as sialic and glucuronic acids, binding to factor H may lead to C3b inactivation. Treatment with sialidase and/or glucuronidase slightly increased complement consumption. The presence of C3 fragments on the surface of the cells, observed by immunofluorescence, corroborates our findings.

Keywords: Complement system, fungi, *Mucor polymorphosporus*, sialidase treatment, glucuronidase treatment, mycelium.

Mucormycosis is an opportunistic infection caused by fungi of the *Zygomycetes* class, *Mucorales* order [1]. Several *Mucor* species have been associated with this disease including *M. ramosissiums* [2], *M. circinelloides* [3] and *M. indicus* [4]. The infection occurs after ingestion [5] or inhalation [6] of spores. The disease can manifest itself in different ways, such as, cutaneous [1] or sinusitis, which can evolve to pulmonary and rhinocerebral manifestations [7]. Several predisposing conditions, including diabetes, acidosis, severe neutropenia, leukemia and lymphoma have been associated with the incidence of mucormycosis [8, 9].

The complement system plays a crucial role in the humoral defense against microbial pathogens. It consists of more than 30 proteins that can be activated, leading to complement deposition onto the microbial surface prior to opsonization (C3b) and/or lysis (C5b-C9) of susceptible microorganisms [10]. These proteins can be activated by the classical pathway, usually involving antigen-antibody complex, or by the alternative pathway, where the initial activation step occurs by spontaneous hydrolysis of the C3 molecule. In addition, the lectin pathway involves activation through binding of the acute phase protein, mannose binding lectin (MBL) to mannose residues on the microbial surface [11].

In the present work, *M. polymorphosporus* mycelial form was used to study complement activation and the influence of surface sialic and glucuronic acids on the activation process.

MATERIALS AND METHODS

Fungal strain. *M. polymorphosporus* 1044 was obtained from Coleção de Culturas of Micoteca URM of Universidade Federal de Pernambuco (UFPE), Brazil. It was first isolated from an appendix biopsy of a patient in Pernambuco. For the experiments the fungus was cultured in Czapeck-Dox medium (CD) [12], pH 6.5 for 30 days with shaking at room temperature, to obtain sufficient fungal mass for our experiments.

Sialidase treatment. Mycelium (20 mg wet weight) was washed twice with phosphate buffered saline (PBS – 10mM NaH_2PO_4, 142mM NaCl) pH 6.0 and incubated for 2 hours at 37°C with 500µL of PBS pH 6.0 containing 0.2 U mL^{-1} of *Vibrio cholerae* sialidase (Sigma Chemical Co. USA). After removal of the supernatant, cells were washed twice with PBS pH 7.2 [13].

Glucuronidase treatment. Mycelium (20 mg wet weight), either pre-treated or not with sialidase, was washed twice with PBS pH 7.2 and incubated for 1 hour at 37°C with 1.0 mL of 0.1 M acetate buffer (pH 5.0) containing 1.0 U mL^{-1} of *Helix pomatia* β-glucuronidase (Sigma Chemical Co. USA). After removal of the supernatant, cells were washed twice with PBS pH 7.2.

Complement source. A pool of normal human serum (NHS) was obtained after 10ml of blood was drawn from 6 of our coworkers, with previous consent, to be utilized in the experiments. Two different sets were prepared. The first set was absorbed twice, each time with 100mg of mycelium of *M. polymorphosporus*, for 30 minutes at 4°C. After centrifugation at the same temperature, supernatant was collected and absorbed twice with a pellet of sheep erythrocytes (10^9 cells mL^{-1} final concentration) under the same conditions. The second set was absorbed only with sheep erythrocytes (10^9 cells mL^{-1} final concentration). The absorbed serum was aliquoted and kept at –80°C.

Hemolytic system. Sheep blood was drawn in Alsever's solution and then a sample was centrifuged (1400xg). The supernatant was removed and the pellet was washed twice with PBS pH 7.2. The pellet was then suspended in veronal buffered saline (VBS - 0.1% gelatin, 5mM sodium Veronal, 142 mM NaCl pH 7.35) containing Ca^{2+} (1.5×10^{-4}M) and

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Mg^{2+} (1 x 10^{-3}M) in order to contain 10^9 cells mL^{-1}. To this suspension, we added equal volume of rabbit anti-sheep erythrocytes antibodies (Sigma Chemical Co. USA) diluted in VBS (1/3200), as previously described by Lima & Silva [14]. The mixture was incubated 30 minutes at 37°C. The suspension was then calibrated to 5 x 10^8 cells mL^{-1} according to Mayer [15].

Complement activators. The following were used in complement activation experiments: *M. polymorphosporus* mycelia, treated or not with sialidase and/or glucuronidase; Zymosan A (Sigma Chemical Co. USA), which was employed as the activation control of the alternative pathway.

Complement activation. Samples of *M. polymorphosporus* mycelium (20 mg wet weight), either or not treated with sialidase and or glucuronidase, were incubated with human absorbed serum, treated or not with chelators (either 10mM EDTA or 10mM EGTA with 5mM $MgCl_2$) at 37°C for 60 minutes. The final dilution of the serum was 1/10 in VBS (with chelators) or VBS with Ca^{2+} and Mg^{2+} (as described above). Serum treated or not with chelators without activators was used as negative control and 5 mg of Zymosan was used as positive control of activation. After incubation, the samples were centrifuged (1400xg) at 4°C. The residual complement was quantified in the supernatant using the hemolytic system. The amount of hemoglobin released was determined in a spectrophotometer (Beckmann) at λ 540nm. Consumption percentages were determined according to positive and negative controls, after CH_{50} was calculated by von Krogh's equation [15].

Immunofluorescence assay. The pellets resultant from the activation were washed 3 times each with PBS pH 7.2 and resuspended in 1ml of PBS. 10µL samples of the fungus were distributed in immunofluorescence slides. After heat fixation, the slides were treated with 10µL of rabbit polyclonal fluorescein conjugated anti-human C3c (DAKO-immunoglobulins A/S. Denmark), placed at 37°C for 1 hour and then washed with PBS. In indirect assay the slides were treated with 10µL of rabbit monoclonal anti-human C3d (DiaMed-Latino America S.A. Brazil) or goat polyclonal anti-human MBL (Santa Cruz Biotech., Inc. USA) and incubated as previously described. 10µL of fluorescein conjugated goat anti-rabbit and rabbit anti-goat IgG were used, respectively, as secondary antibodies and then washed with PBS. Control systems consisting of fungal cells incubated only with secondary antibodies were used. Samples were counterstained with 1% Evan's blue dye, to suppress auto-fluorescence. The slides were microscopically observed on an Axioplan 2 epi-fluorescence microscope (Zeiss, Germany).

Statistical methods. Complement activation and immunofluorescence assays were repeated three times. Complement consumption mean values were taken into account for evaluation. Analysis of variance and two tailed two sample *t*-test were applied to compare results from each activation.

RESULTS

Complement activation

Mucor polymorphosporus mycelium was studied on its ability to activate the human complement system *in vitro*, after incubation with absorbed human serum, to remove any possible reacting antibodies against the fungus or sheep erythrocytes only, treated or not with chelators (EDTA or EGTA). Chelator addition to the serum had the purpose of checking the activation pathway utilized and possible cleavage of complement components by fungal ion independent enzymes [16]. The results obtained are shown in Table **1**. Serum incubated with EDTA showed no decrease in hemolytic complement activity (data not shown), while serum treated with EGTA-Mg^{++}, which triggers only the alternative pathway, demonstrated a low complement consumption when compared to the positive control (zymosan). In the absence of chelators we found a significant increase on complement consumption (3 folds higher $p<0.05$), but which was still low when compared to zymosan. The results regarding activation using serum absorbed only with sheep erythrocytes showed no statistical difference ($p>0.05$) and for that reason are not shown.

Table 1. Complement Consumption Percentage* of Treated and Non-Treated *Mucor polymorphosporus* After Activation with Absorbed Serum$^{\forall}$ Treated with or without Chelators (EDTA$^{\perp}$ or EGTA)

Samples	Serum + EGTA-Mg^{2+}	Serum + VBS
Mycelia[a]	11.05± 0.83%	37.23± 2.22%
Sialidase treated[c]	21.43± 0.93%	45.88± 1.63%
Glucuronidase treated[d]	11.60± 1.02%	37.86± 1.47%
Sialidase[c] + Glucuronidase treated[d]	24.23± 1.76%	43.20± 0.98%
Zymosan[b]	100%	100%

[a]20 mg, [b]5 mg, [c]0.2U mL^{-1} sialidase treatment at 37°C for 2 hours. pH 6.0, [d]1.0U mL^{-1} glucuronidase treatment at 37°C for 1 hour. pH 7.2.

*Percentages were calculated based on CH_{50} values of the positive and negative controls, obtained with von Krogh's equation [15]. $^{\forall}$ Serum absorbed only with sheep red blood cells (RBC) showed no significant difference from serum absorbed with sheep RBC and fungus ($p > 0.05$). $^{\perp}$ EDTA treatment did not demonstrate loss on the hemolytic activity and for that reason are not shown.

Sialidase and/or Glucuronidase Treatments

Treatment of *M. polymorphosporus* with sialidase and/or glucuronidase, allowed the removal of sialic and/or glucuronic acids from the cell surface, making it possible to study the influence of these acidic sugars on complement activation. The results are also displayed on Table **1**.

M. polymorphosporus treated with sialidase or both enzymes showed a slight but significant improvement on complement consumption when incubated with or without magnesium EGTA (20% higher $p<0.05$). However when compared to zymosan it still remained very low ($p<0.01$). On the other hand, glucuronidase treatment alone did not affect the complement consumption. Activation procedure using sheep erythrocytes only absorbed serum showed no statistical difference (data not shown).

Identification of C3 Fragments and MBL on Mycelial Surface

The binding of C3 fragments or MBL to the fungus was determined in the presence and absence of magnesium EGTA, which chelates Ca^{2+}, necessary for classical pathway.

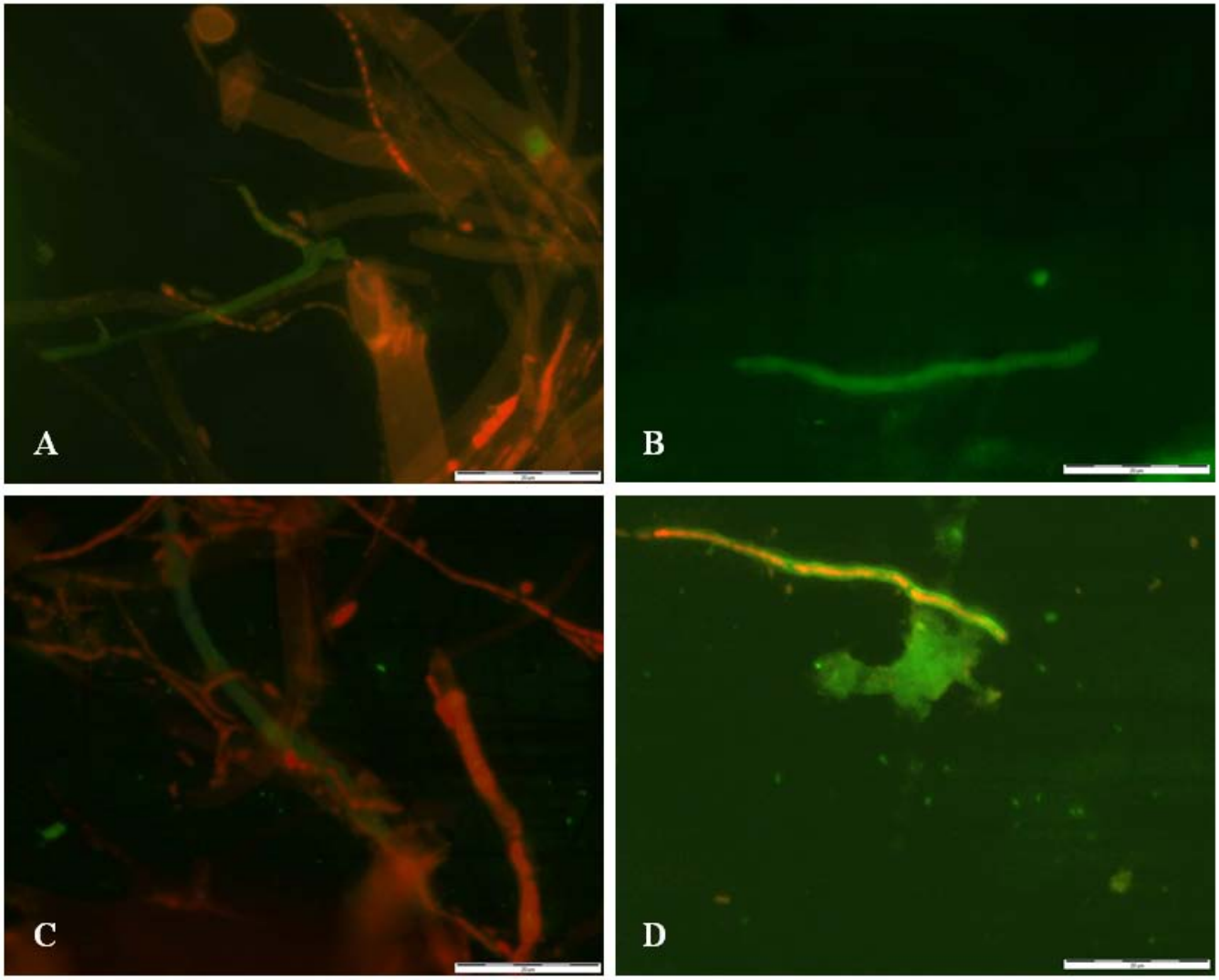

Fig. (1). Direct immunofluroescence of C3 fragments using rabbit anti human C3c labeled with fluorescein. **1A** - Mycelia incubated with serum + EGTA-Mg^{2+}. **1B** - Mycelia incubated with serum without chelators. **1C** - Mycelia treated with sialidase incubated with serum + EGTA-Mg^{2+}. **1D** - Mycelia treated with sialidase incubated with serum without chelators. Red fluorescence indicates counterstain with Evan's blue dye to suppress auto-fluorescence and unspecific antibody binding. (White bar indicates 20μm).

Direct fluorescence technique was used to detect C3 fragments deposited on sialidase treated or not mycelial forms, incubated in serum with EGTA-Mg^{2+} (Fig. **1A** and **1C**) or without chelators (Fig. **1B** and **1D**). All conditions cited above show that some, but not all, hyphae have C3 fragment deposition onto the surface. Mycelia treated or not with sialidase showed similar fluorescent aspects.

Indirect immunofluorescence was used to detect C3d deposited, as a result of cleaved C3b during the activation process, on sialidase treated (Fig. **2A** and **2B**) or not (Fig. **2C** and **2D**) mycelia. The cells treated with sialidase still presented C3d attached to its surface. The same technique was used to detect MBL, but no fluorescence was observed on any sample (data not shown). This was done with both serum absorbed with fungus and sheep red blood cells or red blood cells alone.

DISCUSSION

Since 1961, it is known that *Rhizopus oryzae*, a member of the *Mucorales* order, has low resistance against human serum [17]. Although that is true, it was also noted that this resistance was not due to complement activation, since the inhibition remained after heating the serum to 56°C for 2 hours. It has been demonstrated [18] that while complement activation was not directly involved in eliminating the fungus, it produced a chemotactic factor that attracted neutrophils and monocytes. This factor was directly related to complement system since heating it to 56°C or EDTA addition inhibited the activity. This information is critical to determine that even though complement activation is not critical to eliminate the fungus itself, it still has to occur in order to attract neutrophils, so that they can clear the infection. It is not yet clear how this activation occurs.

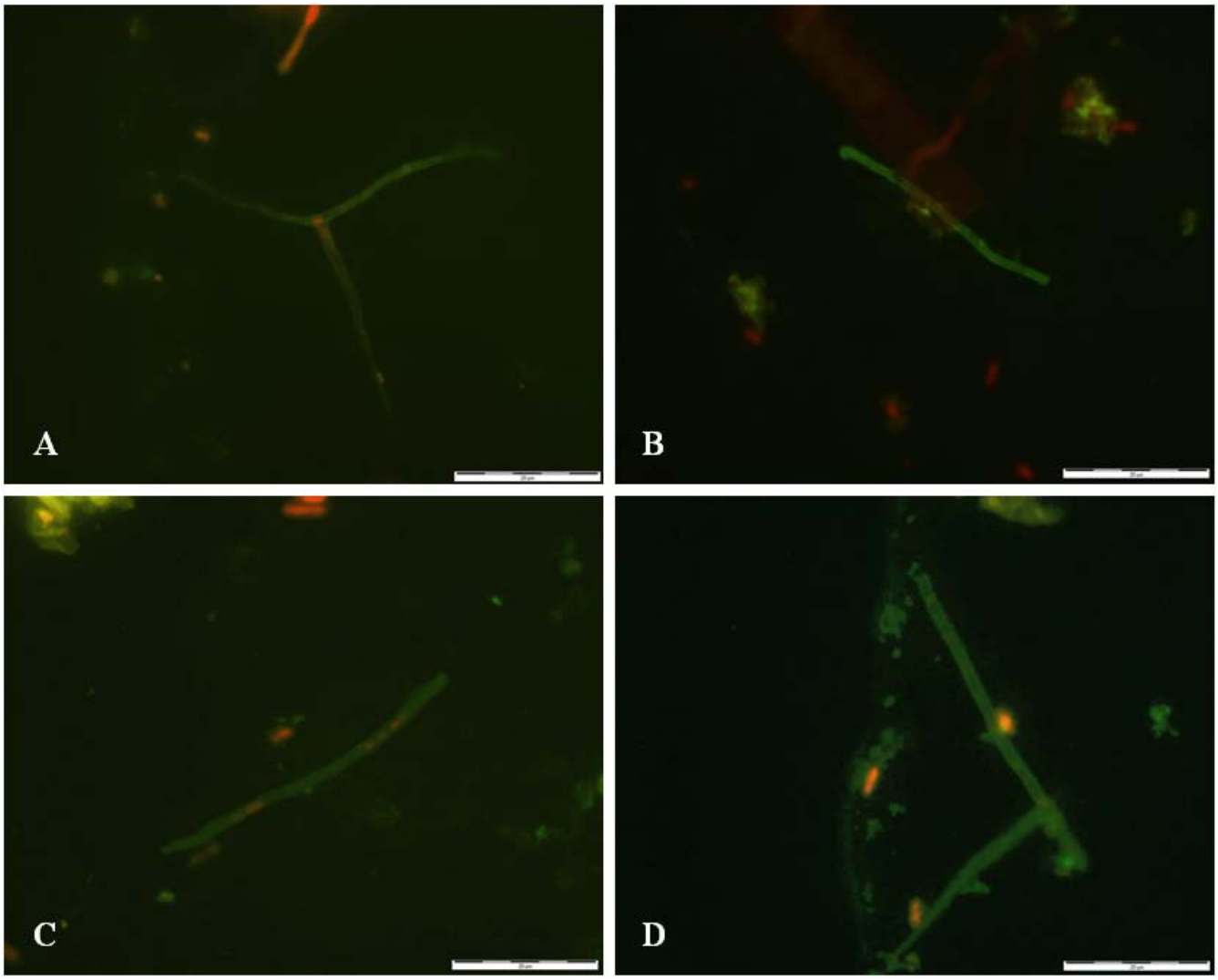

Fig. (2). Indirect immunofluroescence of C3d fragments using rabbit anti human C3d and goat anti rabbit IgG labeled with fluorescein. **2A** - Mycelia treated with sialidase incubated with serum + EGTA-Mg^{2+}. **2B** - Mycelia treated with sialidase incubated with serum without chelators. **2C** - Mycelia incubated with serum + EGTA-Mg^{2+}. **2D** - Mycelia incubated with serum without chelators. Red fluorescence indicates counterstain with Evan's blue dye to suppress auto-fluorescence and unspecific antibody binding. (White bar indicates 20μm).

This study helps elucidate the interaction between *Mucor polymorphosporus* and the human complement system. Although opportunistic, this fungus is capable of infecting human beings, since our sample was obtained from an appendix biopsy.

The results presented on Table **1** indicate that *M. polymorphosporus* is not a good activator of the complement system, when using serum absorbed with both the fungus and sheep erythrocytes and also when using serum absorbed with sheep erythrocyte alone. This indicates that any specific antibody against the fungus or acute phase proteins, which could bind to the fungus, did not affect human complement activation. Ram *et al.* [19] and Blodin *et al.* [20] state that sialic and glucuronic acids have anticomplementary activity. In order to verify if these substances could be responsible for the low complement consumption, the fungus was treated with sialidase and/or glucuronidase. The results showed a slight but significant improvement ($p<0.05$) regarding complement consumption. Sialic acid present on pathogens favors the binding of factor H to C3b [21, 22]. This interaction leads to C3b cleavage, by factor I, into iC3b and C3d, thus blocking the alternative pathway [23]. The presence of C3d on our samples, but not on zymosan, suggests that this pathway reaches C3b formation, which is degraded to C3d. C3d is not able to bind the additional C3 convertase components, thus impossibilitating this pathway from continuing. This points out that this pathway was negatively regulated.

After complement activation, C3b and iC3b deposited on microorganisms act as opsonins being recognized by phagocyte receptors CR1 [24] and CR3 [25], respectively. Therefore, C3b evidence is important to verify if there was an effective complement response with opsonizing fragments, which could lead to phagocytosis of the microorganism. Several studies have shown that C3b and iC3b are present on fungal surface after complement activation, such as *Candida albicans* [26], *Aspergillus fumigatus* [27], *Cryptococcus neoformans* [28] and *Blastomyces dermatitidis* [29], and thus, could facilitate binding to macrophages.

Immunofluorescence was used to detect C3 fragments on fungal surface indicating that, in spite of low complement

consumption, the activation was capable of generating opsonizing fragments (C3b; iC3b) which form ester or amide bonds with the activating particle [30].

The differences perceived when serum was incubated with or without EGTA-Mg^{2+} evidenciate that there was participation of more than one pathway. MBL absence on the fungus excludes the lectin pathway. This could have happened since polyanionic structures present on the fungal surface could be blocking MBL binding to the surface [31]. Seeing as absorbed serum was used, antigen-antibody complex activation can also be ruled out. Likewise, the use of serum absorbed only with sheep red blood cells (data not shown), rendered the same consumption results, which excludes the possibility that any acute phase protein binding to the fungus, during the absorption, interferes with the activation process. If neither antigen-antibody complex nor MBL are influencing complement activation, other acute phase proteins like C-reactive protein [32] or direct binding of C1 [33] could still trigger the activation.

Other surface structures, such as terpenes or proteins, could be acting as potential anticomplementary agents and would be influencing the activation. Triterpenes isolated from the fungus *Ganoderma lucidum* such as ganoderiol F, ganodermanondiol and ganodermanontriol have shown strong anticomplement activity against the classical pathway [34]. Furthermore, it has been shown that *Candida albicans* is able to bind factor H [35] and C4b binding protein – C4BP [36]. Therefore, various other structures are capable of interfering in the activation process, resulting in low complement consumption.

M. polymorphosporus is not a good activator of the complement system, although there is some activation, which is confirmed by the deposition of C3 fragments. The deposition of C3d on the fungal surface suggests a negative regulation of the alternative pathway. Furthermore, the presence of sialic acids interferes negatively on the complement activation by *M. polymorphosporus*, since that removal of sialic acid slightly improves the consumption.

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