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Aspergillus Conidia Activate the Complement by the Mannan-Binding Lectin C2 Bypass Mechanism

Chantal Dumestre-Pérard, Bertrand Lamy, Delphine Aldebert, Catherine Lemaire-Vieille, Renée Grillot, Jean-Paul Brion, Jean Gagnon, and Jean-Yves Cesbron

Innate immunity is the major host defense against invasive aspergillosis. To determine whether the collectin mannan-binding lectin (MBL) is involved in the initial protective immunity through complement activation against opportunistic fungal infections caused by *Aspergillus*, we performed in vitro studies on 29 different strains of *Aspergillus* conidia from five different species. Incubation of *Aspergillus* conidia in human normal serum leads to activation of the alternative pathway, whereas neither the classical nor the lectin pathways through C4 and C2 cleavage are activated. Complement response to conidia was investigated using a MBL-deficient serum and reconstitution experiments were conducted with MBL/MASPs complexes. We found that MBL can directly support C3 activation by a C2 bypass mechanism. Finally, a stronger activation of the alternative pathway was observed for the clinical strains isolated from patients with invasive aspergillosis, compared with the environmental strains. The Journal of Immunology, 2008, 181: 7100–7105.

Aspergillus is an ubiquitous fungus spread by small airborne conidia. Inhalation of conidia induces a wide range of pathology, from allergic bronchopulmonary aspergillosis to invasive pulmonary aspergillosis, depending on the host immune status. Owing to the increasing number of immunosuppressed patients, invasive aspergillosis has become an important cause of death in medical institutions (1, 2). Although *A. fumigatus* is the most frequent type of *Aspergillus* responsible for human infections (~90%), other species such as *A. niger, A. flavus, A. nidulans*, and *A. terreus* are capable of causing disease (1). Complement is one of the major actors in the first line of innate immune defense against pathogens. Recognition of microorganisms is mediated by pattern recognition molecules like C1q and mannan-binding lectin (MBL) (3). Both can bind specifically to pathogen-associated molecular patterns present on the surface of microorganisms, leading to effector mechanisms like complement activation and opsonophagocytosis of the microorganisms.

C1q and MBL share similar structure and functions, but with key differences. C1q engages a broad range of ligands, such as IgG or IgM Abs, envelope proteins of retroviruses, and lipopolysaccharides from Gram-negative bacteria (4). It is associated with C1r and C1s in the C1 complex and activates the classical pathway of complement with involvement of C2 and C4. By contrast, MBL binds to a wide range of microorganisms through carbohydrates recognition, like other collectins (5, 6). MBL forms a complex with MBL associated serine proteases (MASP-1, MASP-2, and MASP-3) that triggers activation of the lectin pathway and generation of C3 convertase via cleavage of C4 and C2 (7, 8). Besides the classical pathway (C1qC1rC1sC4C2, C4, C2, and C3) and the lectin pathway (MBL, ficolins, MASPs, C4, C2, and C3), complement activation can also be achieved by direct interaction with the alternative pathway (C3, factorB, factorD, and properdin) (for comprehensive reviews see Refs. 9, 10). Very recently, a MBL-dependent C2 bypass mechanism has been shown to activate directly C3 and the alternative pathway (11, 12). The integration of this fourth pathway is better understood through the schematic representation in Fig. 1, derived from Daha et al. (13).

Host defense against *Aspergillus* infection is primarily based on innate immunity mediated by macrophages and neutrophils (14). Members of the collectin family as MBL and lung surfactant proteins SP-A and SP-D have been identified as opsonin for *A. fumigatus* (15, 16). Although SP-A and SP-D have been shown to directly bind the pathogen onto the phagocyte, the involvement of MBL and complement in the course of aspergillosis are poorly documented. It has been shown that *A. fumigatus* conidia can activate the complement alternative pathway, with deposition of C3 fragments on fungal pathogens (17, 18). In contrast, a recent report has suggested that MBL-bound *A. fumigatus* may participate in the activation of lectin complement pathway (19). These studies only concern strains of *A. fumigatus* and do not take into account the pathogenicity of the strains.

In the present work, we have studied the complement activation process for 29 strains of *Aspergillus* from five different species. Although in invasive aspergillosis the hyphae might appear to be...
more relevant for physiopathology, we focalized our work on conidia, due to the extreme difficulty to obtain homogenous hyphae preparation at the same developmental level for all the strains studied. We found that none of the strains tested activate the classical or the lectin pathways of complement involving C4b2a. We show that these strains activate the alternative pathway and that MBL can directly support C3 activation by a C2 bypass mechanism. In addition, a significant difference in complement activation was observed according to the pathogenicity of the strains.

Materials and Methods

Aspergillus strains and preparation of conidia

The following strains were tested: 18 A. fumigatus, 3 A. niger, 3 A. terreus, 3 A. flavus, and 2 A. nidulans. The A. fumigatus strains were divided in two groups: environmental strains were provided by air and surface samples and clinical strains were isolated from patients with proven or probable invasive aspergillosis, according to an international consensus (20). All other species were isolated from patients. Aspergillus were cultured on Sabouraud-chloramphenicol agar medium at 37°C for other species except for A. nidulans, which were grown on sterilized damp corn at 24°C. All studied isolates (clinical and environmental) have been cultured on Sabouraud-chloramphenicol agar medium at 37°C for A. fumigatus and at 27°C for other species except for A. flavus which were grown on sterilized damp corn at 24°C. All studied isolates (clinical and environmental) have been treated in the same way: Following primary culture and identification, the isolates were counted, filtered, aliquoted, and kept frozen until use. After thawing, conidia were washed twice with physiological saline solution (21).

Purified proteins

MBL was purified from pooled normal human plasma as described previously (22). In brief, MBL/MASPs complexes were isolated by chromatography on TSK mannose, the first step of MBL purification. These complexes were used for reconstitution experiments with MBL-deficient serum. Afterward, MBL was separated from the copurified MASPs by gel-filtration chromatography on a Superose 6 column and further purified by ion-exchange chromatography on a Mono-Q column. Purified MBL was used for binding experiments with Aspergillus conidia. Purification of human C1q from pooled normal human sera was performed as reported by Arland et al. (23).

Serum and serum reagents

All sera were stored in aliquots at −80°C. Normal human serum (NHS) was obtained from a healthy donor and contained all factors necessary for complement activation. The MBL concentration of this NHS was of 1400 ng/ml within the normal range (1500 ± 1150 ng/ml) (24). MBL-deficient serum also obtained from a healthy donor was characterized by a low concentration of MBL (<20 ng/ml), but otherwise by normal total complement activity and by normal levels of C1q, C4, and C3 (22). This serum was supplemented with 10 µg/ml purified MBL/MASPs complexes. Both NHS and MBL-deficient sera were negative for the presence of anti-Aspergillus Abs as tested by ELISA (25).

Binding of purified MBL to different strains of Aspergillus

Conidia at 10^7/ml were incubated with purified MBL at 0.5 µg/ml for 60 min, at 37°C. MBL protein was diluted in IBS buffer (5 mM Imidazol, 150 mM NaCl, 1 mM MgCl₂, 5 mM CaCl₂, (pH 7.3)) containing 0.1% Tween 20. After conidia were removed by centrifugation for 10 min at 14,000 × g at 4°C, MBL remaining in supernatants was measured using the ELISA described previously (24). As control (100%), purified MBL was incubated with IBS-Tween buffer. Binding of MBL to the conidia was calculated and expressed as percentage of the control.

Complement activation in serum incubated with conidia

Serum (252 µl) was incubated with conidia (36 µl) at 8 × 10^7/ml for 60 min at 37°C to allow for complement activation. The conidia/serum solution was centrifuged 10 min at 14,000 × g at 4°C. Serum was incubated without conidia as control. Total hemolytic assays and hemolytic C4 (see below), as well as MBL levels (24), were determined in the supernatants. Results are expressed as percentage of the control. Values in serum without conidia correspond to 100%.

Determination of complement hemolytic activity in sera incubated with conidia

Conidia of Aspergillus were incubated with NHS to allow complement activation as described above. The complement activity remaining in the supernatant was measured using a total hemolytic assay for classical pathway (TH50c) (time of hemolysis 50%) (26, 27). In brief, in TH50c assay, sheep erythrocytes (Ellitech) were sensitized with anti-erythrocyte Abs (Hémolysine, bioMérieux) at 1/4000 for 15 min at 37°C to form an active Ab/Ag complex. The conidia/NHS supernatant (25 µl) was then mixed at 37°C with 3 ml of sensitized erythrocytes at 4 × 10^7/ml diluted in DGBV⁻⁺ (2.5% glucose, 0.05% gelatin, 2.5 mM Veronal, 72.5 mM NaCl, 0.15 mM CaCl₂, 0.5 mM MgCl₂⁻). Lysis of erythrocytes was measured at O.D. 660 nm as a function of time on a spectrophotometer (Safas 190IDES) and the inflection point determined by the first derivative was defined as the time to reach 50% lysis. Complement activation by the conidia-decreased lysis of erythrocytes as the complement components required for the recognition of the Ab/Ag complex on the RBC were consumed during initial incubation. One hundred percent lysis was defined by the TH50c of the control serum without conidia. The remaining complement activity of the serum after incubation with conidia was expressed as the ratio (control TH50c sample TH50c × 100). As this test is performed with sheep erythrocytes in presence of Ca^2+ and Mg^2+ in highly diluted serum (1/120), it targets the classical complement pathway. The classical pathway activation was confirmed by the determination of hemolytic C4 as described in Ref. 28.

The complement activity remaining in the supernatant was also measured using a total hemolytic assay for alternative pathway (TH50a) (time of hemolysis 50%), derived from the method described by Riches and Stanworth (29). In brief, rabbit erythrocytes (BioMérieux) were washed three times with DGBV-Mg-EGTA (2.5% dextrose, 0.05% gelatin, 2.5 mM Veronal, 72.5 mM NaCl, 5 mM MgCl₂⁻, 3 mM EGTA). The conidia/NHS supernatant (50 µl) was then mixed at 37°C with 1 ml of rabbit erythrocytes diluted at 0.05% in DGBV-Mg-EGTA. The remaining complement activity of the serum after incubation with conidia was calculated as described previously for TH50c. This test favors the alternative complement pathway because rabbit erythrocytes were used and the reaction occurred in the presence of Mg^2+ and EGTA in serum diluted at 1/20.

Statistics

Statistical analysis was performed with the R software from the R Foundation for Statistical Computing (http://www.r-project.org/foundation). R is available as Free Software under the terms of the Free Software Foundation’s GNU General Public License. For all tests developed in this study, results were considered significant at p < 0.05. Correlations were analyzed using a Spearman rank test. Differences between data sets were computed with the nonparametric Mann-Whitney U test.

Results

Conidia of Aspergillus incubated in vitro with NHS led to C3 cleavage without classical convertase C4b2a involvement

To test the activation of the classical pathway by conidia of Aspergillus, NHS was incubated with or without 10^5 conidia/ml. The complement activity remaining in the supernatant was measured using a total hemolytic assay for classical pathway (TH50c). Consumption of complement factors caused by complement activation during initial incubation led to a diminution of remaining complement activity expressed by a percentage of TH50c lower than 82%, which is the minimum value of the normal range. Complement activation by the classical pathway was found with all the isolates tested excepted with four isolates of A. fumigatus and one isolate of A. terreus (Fig. 2).
Aspergillus ACTIVATES THE COMPLEMENT VIA MANNAN-BINDING LECTIN

In a similar manner, the activation of the alternative pathway by conidia of Aspergillus was investigated using a total hemolytic assay for alternative pathway (TH50a). All the strains of Aspergillus activate significantly the alternative pathway (Fig. 2). A. niger showed the strongest complement activation of alternative pathway (TH50a of 57%). A. flavus, A. terreus, A. nidulans, and A. fumigatus showed the same profile of complement activation (TH50a between 49–62%).

To gain fuller insights into complement activation by conidia, key complement components were assayed. First, the classical pathway activation was investigated using a hemolytic C4 assay, measuring the remaining C4 activity in NHS first incubated with conidia. The hemolytic C4 test was conducted with the ten strains that showed a marked reduced TH50c. Unexpectedly, normal hemolytic C4 were found in all tested strains (Fig. 3), attesting an absence of C4 activation. This demonstrates both that reduction in TH50c is not dependent on formation of the classical convertase C4bC2a, and that neither the classical nor the lectin pathways are involved. Reduction of TH50c results from consumption of complement protein factors during incubation of conides in normal human serum. We have shown that there is no C4 consumed. Therefore the factors consumed are located downstream from C4, i.e., C3 and terminal complement pathway.

To confirm the activation of the alternative pathway, C3 cleavage was analyzed for ten strains of Aspergillus that induced the most significant activation. C3 cleavage was observed with all strains tested (data not shown). Taken together, these results show that conidia of Aspergillus incubated in vitro with NHS led to C3 cleavage without classical convertase C4bC2a involvement.

FIGURE 2. Total complement hemolytic assays. The remaining activities of the classical pathway TH50c (dark symbols on the left) and the alternative pathway TH50a (gray symbols on the right) were measured in sera after incubation with conidia from different strains of Aspergillus (n = 29). Results were expressed as the ratio: (control TH50 without conidia)/(sample TH50) × 100. Bars correspond to the mean values. The normal values have been determined as the mean ± 2 SDs. The minimum values of their range are represented by solid and dotted lines for TH50c and TH50a, respectively.

FIGURE 3. Hemolytic assays for classical pathway (TH50c) and C4. The remaining complement activity in the serum incubated with conidia from different strains of Aspergillus was measured in TH50c and hemolytic C4 assays. Hemolytic C4 tests were conducted only with strains that showed a marked reduced TH50c. Hatched bars represent TH50c and gray bars represent hemolytic C4. Data are represented as a mean ± SD of three different experiments. The normal values have been determined as the mean ± 2 SDs. The minimum values of their range are represented by solid and dotted lines for TH50c and hemolytic C4, respectively.

FIGURE 4. Binding of purified MBL to conidia from different strains of Aspergillus. Percentage of MBL binding was determined as described in Materials and Methods. Horizontal bars correspond to the mean values. The initial MBL level added corresponds to 100%.

To confirm this possibility, we investigated the role of MBL in complement activation. First, the binding of purified MBL to the different strains of Aspergillus was analyzed in vitro. Aspergillus (10^7 conidia/ml) was incubated with purified MBL (0.5 μg/ml), and MBL levels remaining in the supernatants were determined using an ELISA. As shown in Fig. 4, MBL bound to all strains of Aspergillus. Whereas MBL showed a moderate binding to A. nidulans (12%), A. flavus (28%), and A. fumigatus (30%), the binding to A. terreus and A. niger was found to be >40 and 70%, respectively.

To confirm these results in a biological assay, MBL levels remaining in NHS after incubation with conidia were tested. A negative correlation was observed between MBL levels and the binding assay (Fig. 5A). Furthermore, a negative correlation was also found between the MBL binding assay and total hemolytic assay for alternative pathway TH50a (Fig. 5B). These data suggest that the C3 cleavage described above was induced by activation through MBL. If this was the case, the incubation of conidia from A. fumigatus strains with MBL-deficient serum should not induce alternative pathway activation. The results of this experiment showed on the contrary that activation was still observed using TH50a and TH50c assays (data not shown).

To differentiate the classical from the alternative pathway, we conducted a hemolytic C4 assay with all strains. For each strain hemolytic C4 was compared in NHS and in MBL-deficient serum. In normal MBL containing serum, conidia activate the alternative pathway of complement as indicated by the unaffected C4. In MBL-deficient serum, the data indicate that the strains studied can be divided in two groups. For one group (n = 13), no C4 activation was observed, demonstrating that the activation resulted from that alternative pathway (data not shown). This could be the result of a
the experiments. The normal value for hemolytic C4 has been determined as serum incubated with conidia from different strains of MASP complexes. The remaining hemolytic C4 activity was measured in deficient serum and in MBL-deficient serum reconstituted with MBL/MBL/FIGURE 6. Hemolytic C4 in normal human serum (NHS), in MBL-deficient serum, and in MBL-deficient serum reconstituted with purified MBL/MASP complexes. The data showed an increase of hemolytic C4 to normal levels for these five strains. This reconstitution abolished the classical activation, and reactivated the alternative pathway as indicated by the reduced TH50a for these five strains. In other words, when we add back MBL/MASP complexes in the MBL-deficient serum, we return to the dominant alternative pathway and C4 re-

direct C3 activation by conidia or a MBL-dependent C3 activation. For the second group (n = 5, Fig. 6), a C4 activation was observed, suggesting a classical pathway activation in absence of MBL. To verify the implication of MBL, we tested these five strains with MBL-deficient serum reconstituted with purified MBL/MASP complexes. The data showed an increase of hemolytic C4 to normal levels for these five strains. This reconstitution abolished the classical activation, and reactivated the alternative pathway as indicated by the reduced TH50a for these five strains. In other words, when we add back MBL/MASP complexes in the MBL-deficient serum, we return to the dominant alternative pathway and C4 re-

mains unaffected. These results demonstrated a MBL-mediated activation of the alternative pathway by these five strains of A. fumigatus.

FIGURE 7. Comparison of alternative pathway activation (TH50a) by clinical vs environmental strains of A. fumigatus. The remaining complement activity was measured using a TH50 assay in serum incubated with conidia of clinical strains isolated from patients with invasive aspergillosis (n = 10) and of environmental strains (n = 8). Bars correspond to the mean values. Differences between data sets were evaluated with the nonparametric Mann-Whitney U test.

Complement activation according to the pathogenicity of the strains of Aspergillus

Then, we correlated the pathogenicity of A. fumigatus with MBL binding and complement activation by conidia. For that we have compared strains isolated from patients with invasive aspergillosis (n = 10) with environmental strains (n = 8). We observed that conidia of clinical interest have a significantly higher potential in alternative pathway activation than the environmental strains (Fig. 7). In addition, a correlation between MBL binding to conidia and alternative pathway activation was found only for the clinical strains (p = 0.03, data not shown). This indicates a difference in complement activation according to the pathogenicity of the strains.

Discussion

Innate immunity was identified as the major host defense against aspergillosis (14, 16). However, the role of complement and particularly that of MBL in defense against fungal pathogens are poorly documented. Our data established that conidia of Aspergillus are activators of the complement alternative pathway. For several strains of Aspergillus this activation proceeds through MBL binding to conidia, using a C2 bypass mechanism.

When we evaluated complement activation by conidia of the different strains of Aspergillus, our results showed that the classical pathway was not involved. Despite the reduction in TH50c observed in NHS incubated with most Aspergillus strains, no C4 activation was detectable, hence no formation of the C4bC2a convertase. However, one report has suggested that anti-Aspergillus Abs present in normal human serum could induce the activation of the classical pathway (17). We have checked that the NHS and the MBL-deficient sera used in this study did not contain anti-Aspergillus Abs, eliminating this possibility.

Aspergillus conidia are able to activate the alternative pathway in normal serum. This activation was evidenced using a TH50a assay and confirmed by analysis of C3 cleavage products. This confirms with 29 strains the results previously obtained with one strain of A. fumigatus reporting that complement activation by rest-

FIGURE 5. Correlation between MBL binding and consumption and MBL binding and alternative pathway activation (TH50a). A, MBL binding correlated to consumption of MBL expressed as percentage of remaining MBL in serum incubated with conidia. B, MBL binding correlated to complement hemolytic assay for alternative pathway (TH50a), expressed as the remaining complement activity in the serum incubated with conidia. Correlation coefficients between variables and statistical significance of the association were determined with Spearman rank order correlation analysis.
described by Selander et al. (12). This pathway is dependent on MBL, bypassing C2 and directly leading to C3 activation. MBL consumption in sera incubated with conidia, and a significant correlation between in vitro binding to MBL and complement activation by the alternative pathway were observed. These results support that MBL is involved in the alternative pathway activation by conidia. Experiments with MBL/MASP3 complexes in MBL-depleted serum showed that MBL is necessary for activation of alternative pathway by conidia of five strains of Aspergillus. For the 13 other strains tested, alternative pathway was still activated independently of MBL. However, because it was not possible to specify the activators of the C3 cleavage, i.e., conidia alone or conidia opsonized by MBL, we cannot exclude the involvement of MBL in the alternative pathway activation.

In the lectin pathway, the binding of MBL to ligands induces activation of the serine protease MASP-2 with the following cleavage of C4 and C2 and the formation of the C3 convertase (Fig. 1). Because no C4 activation was observed when conidia were incubated in NHS, this lectin pathway was not activated by Aspergillus. How to reconcile the fact that MBL is necessary for activation of alternative pathway and that the activation observed did not induce C4 cleavage? Selander et al. (12) have recently described a MBL dependent pathway bypassing C2 and directly leading to C3 activation. The data obtained for five strains demonstrated the validity of this pathway. In addition, we have tested one strain of each species A. niger, A. terreus, A. flavus, and A. nidulans with similar results suggesting that this activation pathway could be extend to the Aspergillus genus.

We have no clear hypothesis to explain the difference of complement activation according to the Aspergillus strains. MBL is a lectin that has high affinity for N-acetyl-D-glucosamine, D-mannose and l-fucose (30). The conidial cell wall consists of two layers: an external protein layer and an internal polysaccharide layer (31). However, the molecular structures are not yet well characterized. Therefore, the ligands of MBL onto the surface of conidia remain uncertain. In addition, the composition might be different depending of the A. fumigatus species and related to the pathogenicity (32). For example, galactomannan of A. fumigatus was shown to be species specific (33) and differences in sialic acid density was reported in A. fumigatus and in other Aspergillus species (34, 35). Furthermore, a recent report highlights the role of galactofuranose in A. Fumigatus growth and virulence (36).

Finally, we observed that strains isolated from patients, A. fumigatus as well as the other species, have a significantly higher level of activation than environmental strains. We are permanently confronted with inhaled conidia of A. fumigatus from the environment. Therefore, why do those strains that cause invasive infection led to stronger complement activation compared with environmental isolates? This is in apparent contradiction with the hypothesis that MBL-deficient patients could have an increased incidence of fungal infections. This increased susceptibility to fungal infection has been reported in MBL-deficient patients under immunosuppressive therapy (37). These MBL defects could result either from protein levels and/or genotypes (38, 39).

It is not clear whether the ability of Aspergillus to activate complement is an intrinsic property facilitating aspergillosis, or is acquired from its replication within human tissues. We can hypothesize that the strains that replicated in humans have adapted to their host, and that MBL-mediated complement activation results in Aspergillus dissemination, rather than its expected limitation. It could be that the conidia inhaled activate complement leading to local inflammation that participate in the dissemination of hyphae. The environmental conidia that are less inflammatory are eliminated by other innate defense molecules, such as surfactant proteins. Further studies in murine models or in vitro assays would be useful to explore these possibilities.

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Disclosures

The authors have no financial conflict of interest.

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