Potential of fluorescence polarization immunoassay for the detection of *Aspergillus fumigatus* galactomannan

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Galactomannan (GM) is a specific polysaccharide antigen of opportunistic mold fungi of the genus *Aspergillus*. The detection of GM in patients´ biological fluids is a reliable criterion for the diagnosis of invasive pulmonary aspergillosis, a severe disease. In this report, we evaluated the analytical capabilities of the fluorescence polarization immunoassay (FPIA) for detection of GM. Using FPIA, the high affinity of the monoclonal antibody 7B8 to the synthetic oligosaccharide β-D-Galf-[(1→5)-β-D-Galf]$_3$-(1→6)-α-D-Manp related to the galactomannan fragment was shown. The binding constant in this interaction was found to be equal to $(1.02\pm0.01)\cdot10^8$ L mol^{-1}. The detection limit determined for this oligosaccharide with the competitive FPIA method is 3 ng mL^{-1}. The data obtained indicate the possibility of using FPIA for the diagnosis of invasive aspergillosis.

Key words: *Aspergillus fumigatus*, fluorescence, antigen, antibody, galactomannan, diagnosticum.

Fungal pathogens of the genus *Aspergillus* (first of all, *A. fumigatus, A. flavus* and *A. niger*) are causative agents of various infectious diseases such as allergic bronchopulmonary aspergillosis, local (non-invasive) aspergillosis, chronic pulmonary aspergillosis and invasive pulmonary aspergillosis (IPA), with the latter being the most serious and life-threatening disease.[1,2] Early detection of lung diseases provides a significant increase in the efficacy of their treatment, which requires the development of rapid and convenient methods for their diagnostics.[3,4] The detection of galactomannan (GM) circulating in the bloodstream is currently a recognized criterion for the diagnostics of invasive aspergillosis.[4—6] Galactomannan is a polysaccharide constructed of a α-(1→2)-/α-(1→6)-linked mannose chain with short β-(1→5)-oligogalactofuranose side chains attached through the O(3) or O(6) atoms of some mannose residues.[7] Recent studies have revealed additional structural fragments of GM, in particular, the β-(1→6) linkages between galactofuranoside residues.[8,9]

Currently, the detection of GM is based on the sandwich enzyme-linked immunosorbent assay using monoclonal antibodies IgM EB-A2.[10,11] This test kit is characterized by high diagnostic sensitivity (85.7%)[12] and specificity (85.4%),[12] however, the possibility of obtaining false positive results using this test system is well documented.[13—16] In this regard, the development of a new improved diagnosticum based on alternative monoclonal antibodies,[17—19] as well as of alternative physical platforms that automate and simplify the analysis[20,21] is currently underway.

One of the promising analytical platforms is the fluorescence polarization immunoassay (FPIA), which has proven itself in solving a number of analytical and medical problems. FPIA consists in the competition of an analyte and a substance labeled with a fluorescent label (tracer) for binding with a limited number of antibodies and in determining the degree of polarization of the fluorescence of the tracer. The higher the concentration of the analyte competing with the tracer for the binding sites, the weaker the mixture fluorescence polarization. The FPIA method is quite simple and consists in adding an aliquot of a tracer and an antibody solution to a sample (usually 10—50 μL), incubation for several minutes and measuring the fluorescence polarization with a polarizing fluorimeter. The total analysis time including the sample preparation is 5—20 min. This method and examples of its use to determine biologically active species were considered in detail in a review[22] and our previous publications.[23,24]

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This work is aimed at the study of the feasibility of diagnosis of invasive aspergillosis by FPIA with the use of high-affinity mouse monoclonal antibodies IgG3 7B8 against galactomannan.

Results and Discussion

An essential requirement for conducting fluorescence polarization immunoassay is the selection of suitable highly specific antibodies and a tracer, a fluorescent-labeled antigen. Antibodies to galactomannan significantly differ depending on the structure of the epitope recognized by them.[13,17,19] In the present work, mouse monoclonal antibodies of the IgG3 7B8B isotype were used since they were shown to be highly specific with respect to galactomannan of the fungi of the genus *Aspergillus*.[17] As a tracer, fluorophore-labeled pentasaccharide **1** (see Refs. 25, 26) was used because it is a immune-determinant oligosaccharide fragment of galactomannan. For a preparation of tracer **2**, aminopropyl glycoside **1** (see Ref. 25) was treated with fluorescein isothiocyanate (FITC) in the presence of Na_2CO_3 (Scheme 1); the product was isolated using a C-18 reversed-phase cartridge. The composition of the product was confirmed with high resolution mass spectrometry. The starting pentasaccharide **1**, which is a low molecular weight model of natural galactomannan, was used as a substance to be detected in quantitative FPIA.

The kinetics of the interaction of antibody 7B8 with tracer **2** was studied first. Irrelevant antibody 5H5, which is specific to β-glucan, another polysaccharide component of the fungus cell walls,[27] was used as a negative control. As expected, tracer **2** well associated with the monoclonal antibody 7B8,[17] resulting in a substantial increase in the fluorescence polarization (m*P*) with time (Fig. 1). At the same time, addition of irrelevant antibody 5H5 had no effect on the fluorescence polarization, indicating that there was no non-specific interaction between the tracer and immunoglobulin.

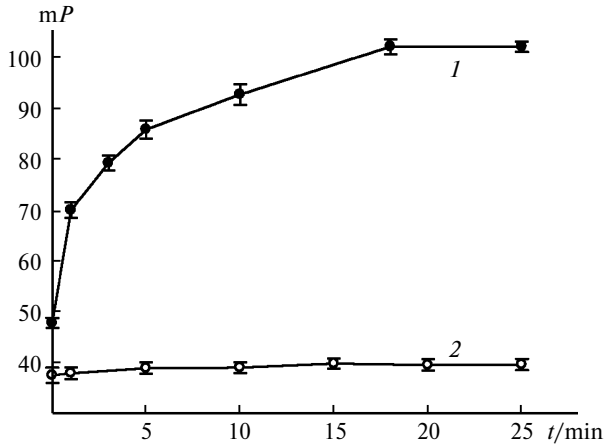
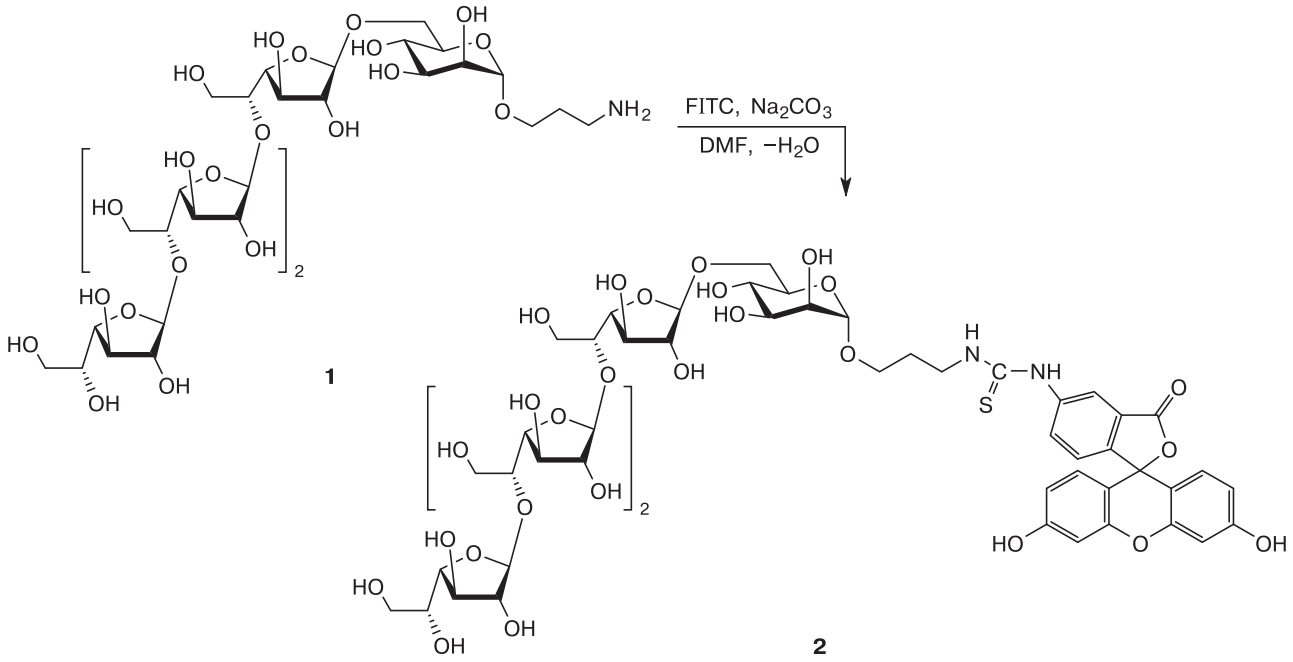


Fig. 1. Kinetics of the binding of tracer **2** with antibody 7B8 (*1*) and non-relevant antibody 5H5, which was used as a negative control (*2*). The fluorescence polarization (m*P*)[22] is in arbitrary units. The measurements were repeated three times for each antibody. The data are presented as mean ± standard deviation.

To assess the applicability of FPIA to galactomannan detection, an approach was used, which is based on the

Scheme 1



competition between the targeted and fluorescein-labeled antigens for a restricted number of specific binding centers of antibodies and measurement of the degree of polarization of the reaction mixture fluorescence. The experimentally obtained dependence of the fluorescence polarization on the antigen **1** concentration was non-linear (Fig. 2, *a*). The concentration range within which the dependence was linear (Fig. 2, *b*) was from 3 to 20 ng mL^{-1}; the detection limit was 3 ng mL^{-1}. The latter value corresponds to the point at which the m*P* signal recorded in the presence of pentasaccharide **1** differs from the fluorescence polarization in the absence of antigens by three standard deviations.

To obtain the binding constant *K*, the Scatchard plot was built for the linear dependence region (Fig. 3). The Scatchard plot represents the dependence of the ratio between the concentrations of the antigen—antibody immune complex (*B*) and the free antigen (**1**) (*F*) on the immune complex concentration (*B*), *i.e.*, the dependence of *B*/*F* from *B*. The slope of the plot is equal to the complexation constant.[28]

The diagnostic sensitivity of an immunochemical analysis method is known to be determined by the affinity

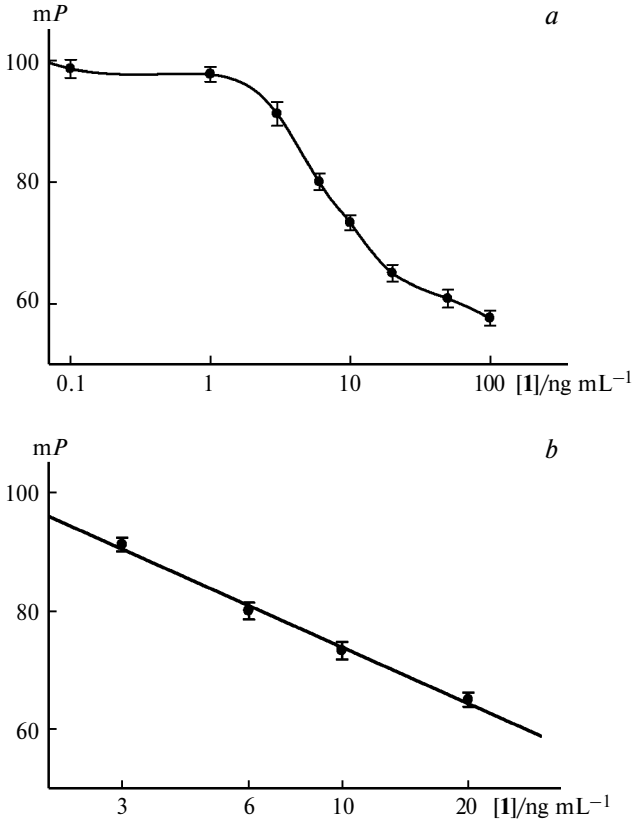


Fig. 2. Calibration curve (*a*) and its linear range (*b*) with tracer **2** for detection of pentasaccharide **1**. Two independent measurements were performed in triplicates for each antigen concentration. The data are presented as mean ± standard deviation.

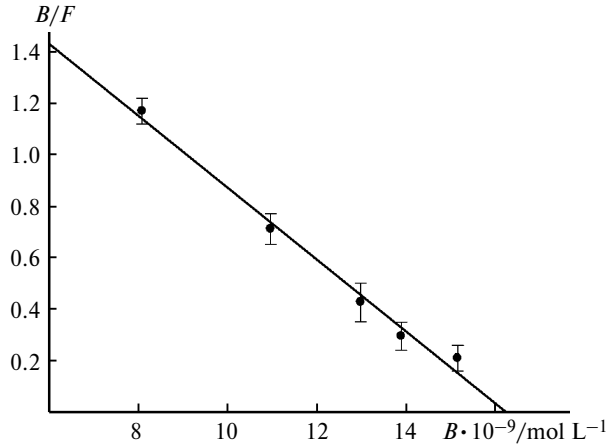


Fig. 3. The Scatchard dependence used to obtain the constant of binding of antigen **1** with antibody 7B8. The straight line equation is $y = (5.99\pm0.2) - (1.02\pm0.01)\cdot10^8x$. The calculated binding constant (*K*) is $(1.02\pm0.01)\cdot10^8$ L mol^{-1}.

of antibodies, the concentrations of labeled antigen and antibodies, as well as by the type of the detection method and the data accuracy. A high antibody affinity provides high analysis sensitivity. The binding constant obtained from the Scatchard plot constructed from the FPIA data is equal to $1.02\cdot10^8$ L mol^{-1}. This indicates a high affinity between the antigen and monoclonal antibody 7B8 and, in addition, well agrees with previous data. The constant of association between a biotinylated derivative of pentasaccharide **1** with monoclonal antibody 7B8 obtained by a surface plasmon resonance (SPR)[17] is $1.9\cdot10^8$ L mol^{-1}. As can be seen, both values are of the same order, but differ by about half, which can be considered a good result. This difference can be caused by obvious fundamental differences in the detection methods: firstly, SPR is a heterogeneous method (*i.e.*, antibodies interact with an antigen-modified surface), while FPIA is a homogenous method (*i.e.*, the antigen-antibody interaction occurs in a solution), secondly, a modified antigen was used in the SPR experiment, thirdly, the buffer solutions used in the SPR and FPIA studies had different pH values.

Thus, in the course of the present study, a tracer and antibodies suitable for detection of *A. fumigatus* galactomannan by FPIA were chosen. The high affinity of the 7B8 antibody under the experimental conditions was confirmed by determination of the binding constant, which was found to be of the same order as the constant obtained previously by SPR. The detection limit measured for oligosaccharide **1** was found to be equal to 3 ng mL^{-1}, which is comparable with the sensitivity of the sandwich enzyme-linked immunosorbent assay. All of these findings make it possible to consider fluorescence polarization immunoassay as a promising physical platform for the development of efficient diagnostic systems for the detection of diseases caused by fungi of the genus *Aspergillus*.

Experimental

Reagents and equipment. We used a synthetic pentasaccharide **1** related to the galactomannan fragment of *A. fumigatus* and obtained by us earlier[25,29] by a pyranoside-furanoside rearrangement.[30,31] Monoclonal mouse antibodies 7B8 were also obtained previously[17] using synthetic immunogens based on pentasaccharide **1**. The following commercially available reagents were used: fluorescein isothiocyanate (Sigma-Aldrich), sodium carbonate (Chimmed, puriss.), acetic acid (Avilon-Companychim, glacial, puriss.), dimethylformamide (Sigma-Aldrich, 99.8%), phosphate buffered saline tablets (Sigma). Isolation of conjugates was carried out using reversed-phase Sep-Pak C18 cartridge. Deionized water was prepared with the use of a Simplicity Millipore water purification system (Merck). Phosphate buffer (0.01 *M* sodium phosphate, 0.0027 *M* KCl, 0.137 *M* NaCl, pH 7.4) was prepared by dissolution of one saline tablet in 200 mL of distilled water.

High resolution mass spectra were obtained using a Bruker micrOTOF II spectrometer with electrospray ionization (ESI). Both positive ion (capillary voltage of −4500 V) and negative ion spectra (capillary voltage of 3200 V) were recorded. The used mass range (m/z) was 50—3000 Da, external and internal calibrations were applied (Electrospray Calibrant Solution, Fluka). Acetonitrile, methanol or water solutions of analytes were injected through a syringe injector, flow rate was 3 μL min^{-1}. Nitrogen was used as a nebulizer gas (4 L min^{-1}), interface temperature was 180 °C.

Fluorescence polarization measurements were carried out at room temperature (20 °C) with Sentry 200 fluorescence polarization instrument (Ellie, USA). A glass cuvette was placed into a specially designed cell, and fluorescence polarization (m*P*) and fluorescence intensity (in relative units) were measured. The obtained data were treated using the Sigma Plot 11 program (Systat Software Inc., USA).

Synthesis of fluorescein-labeled antigen 2 (tracer). 1 *M* aqueous solution of Na_2CO_3 (17 μL) and a DMF solution (100 μL) of fluorescein isothiocyanate (1 mg, 2.57 μmol) were added to a solution of aminopropyl glycoside **1** (2.0 mg, 2.26 μmol) in distilled water (300 μL). The obtained mixture was vigorously mixed and kept at 60 °C for 2 h. The reaction mixture was concentrated in vacuo, dissolved in water (400 μL), a 0.1 *M* solution of acetic acid (400 μL) was added, and the mixture was loaded onto a Sep-Pak C-18 cartridge, which was preliminary washed with methanol and then by water excess. The cartridge was washed with 2 mL portions of solutions of 0—55 vol.% methanol in water, with the methanol concentration being changed in increments of 5 vol.%. The product was collected in the range of eluent concentrations of 25—45 vol.%, the eluate was concentrated with a rotary evaporator, and the residue was lyophilized to give a light orange product. R_f 0.55 ($CHCl_3$—CH_3OH—H_2O, 10 : 5 : 1). MS (ESI): found m/z 1297.3446 $[M + Na]^+$; $C_{54}H_{70}N_2NaO_{31}S$; calculated: 1297.3575.

Fluorescence polarization immunoassay. A tracer solution in 0.05 *M* borate buffer (pH 8) with the fluorescence intensity of ~$(2.5\pm0.2)\cdot10^5$ rel. unit and concentration of $7\cdot10^{-9}$ mol L^{-1} was used in the study of kinetics of the association of tracer **2** with antibodies 7B8 and 5H5. An antibody solution (10 μL, 0.3 mg mL^{-1}) was added to 1 mL of a tracer solution; the final concentration of antibodies was $2\cdot10^{-8}$ mol L^{-1}. Fluorescence polarization was measured for 20—25 min. Three independent measurements were carried out for each antibody.

A tracer working solution with an intensity of $(4.0\pm0.2)\cdot10^5$ rel. unit was used to construct a calibration curve and calculate the antigen—antibody binding constants. The concentration of the working solution of antibody 7B8 ($1.75\cdot10^{-8}$ mol L^{-1}) was chosen so that the fluorescence polarization of the mixture of the standard solution with the zero antigen concentration (0.5 mL of the tracer working solution + 0.5 mL of the antibody working solution) was approximately equal to 70% of the maximum m*P* value observed when antibodies and tracers were tested. Standard solutions with different concentrations (from 0.1 to 100 ng mL^{-1}) were prepared by dilution of the initial solution of pentasaccharide **1** in deionized water.

Plotting the calibration curve. To plot the calibration curve, 10 μL of a standard solution of pentasaccharide **1** of particular concentration, 500 μL of the working solution of tracer **2** and 500 μL of the working solution of antibodies 7B8 were sequentially added into a cuvette. The solutions were left in air at 20 °C for 15 min, then, the degree of the solution fluorescence polarization was measured. Two independent measurements were performed in triplicates for each antigen concentration. The calibration curve was constructed on the basis of the obtained data.

Calculation of the binding constant of the antigen-antibody complex. The Scatchard method[32] was used to determine the binding constant. With this purpose, the dependence in the Scatchard coordinates, *viz.*, the dependence of the ratio of the concentrations of the antigen-antibody immunocomplex (*B*) to the free-form antigen concentration (*F*) on the concentration of the immunocomplex (*B*), *i.e.*, the dependence of *B*/*F* from *B*, was constructed. The slope of this curve corresponds to the complexation constants.

Based on the fluorescence polarization detection, the ratio of the bound and free antigen forms (*B*/*F*) was obtained by the equation

$$\left(\frac{B}{F}\right)=\frac{P_i-P_{\min}}{P_{\max}-P_i}, \qquad (1)$$

where *B* is the concentration of the antigen in the bound form, *F* is the concentration of the antigen in the free form, P_i is a measured fluorescence polarization, P_{max} is the fluorescence polarization at the maximum antigen-antibody binding, P_{min} is the fluorescence polarization at the minimum antigen-antibody binding.

The concentration of the antigen bound form (*B*) was calculated by the equation

$$B=T\frac{X}{X+1}, \qquad (2)$$

where *T* is the total amount of labeled and unlabeled antigen in the system (*B* + *F*), *X* is the ratio of the bound and free antigen forms (*B*/*F*). The total amount of labeled and unlabeled antigen *T* in the fluorescence polarization measurements, in particular, in the FPIA measurements, represents a sum of concentrations of the free antigen added to the system and the tracer. The linear approximation of the obtained data in the Scatchard coordinates was carried out using the Sigma Plot 11 software package (Systat Software Inc., USA).

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