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In Vitro Release by *Aspergillus fumigatus* of Galactofuranose Antigens, 1,3-β-D-Glucan, and DNA, Surrogate Markers Used for Diagnosis of Invasive Aspergillosis

Monique A. S. H. Mennink-Kersten,* Dorien Ruegebrink, Nazhat Wasei, Willem J. G. Melchers, and Paul E. Verweij

Department of Medical Microbiology and Nijmegen University Center for Infectious Diseases, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

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***Aspergillus* markers are becoming increasingly important for the early diagnosis of invasive aspergillosis. The kinetics of release of these surrogate markers, however, is largely unknown. We investigated the release of β-(1-5)-galactofuranosyl (gal*f*) antigens (Platelia *Aspergillus*), 1,3-β-D-glucan (BG) (Fungitell), and DNA (PCR) in an in vitro model of *Aspergillus fumigatus*. The results showed that release is correlated to the growth phase of the fungus, which depends on available nutrients. Whereas gal*f* antigens and BG are released during logarithmic growth, DNA is released only after mycelium breakdown. During early logarithmic growth, gal*f* antigens seem to be released somewhat earlier than BG. Furthermore, gal*f* antigen concentrations of more than 120,000 times the serum cutoff value (0.5 ng/ml) can be measured, while BG concentrations reach a value only 978 times the serum cutoff value (60 pg/ml). During lytical growth, release of gal*f* antigens further increased to a maximum level, which depended on pH. After that, the concentration of gal*f* antigens stayed high (pH 7.4) or decreased to zero within 4 days (pH 5.0). In contrast to gal*f* antigens, BG concentration decreased after 1 day of growth. The decrease of gal*f* components seems to be due to the enzyme β-galactofuranosidase, which is able to destroy gal*f* epitopes and whose activity fluctuates in the culture filtrates in parallel with gal*f* antigen concentration. Fungal DNA seems to be released only due to autolysis caused by nutrient limitation. In conclusion, several factors clearly influence the release of surrogate markers in vitro. These same factors might also play a role at the infection site of *Aspergillus* disease in humans.**

Invasive aspergillosis (IA) has become a leading cause of death among immunocompromised hosts, including transplant patients, those treated for hematological malignancy, and those treated with high-dose corticosteroids (41, 49). In addition, IA is increasingly observed in the nonneutropenic phase after hematopoietic stem cell transplantation and in nonclassic settings, such as intensive care units with critically ill patients (13, 16). The high mortality is due partly to the difficulty in establishing a diagnosis at an early stage of infection, since presenting symptoms are nonspecific and sensitivity of cultures is low. Techniques to improve timely diagnosis have focused on the detection of circulating surrogate markers released by the fungus (43, 45, 50). With the development of nonculture-based methods, such as PCR and antigen detection, circulating markers can be detected at an early stage of infection in patients with invasive disease (12, 27, 28).

The commercially available sandwich enzyme-linked immunosorbent assay (ELISA) (Platelia *Aspergillus* [PA]; Bio-Rad, Marnes-la-Coquette, France) is based on the rat immunoglobulin M monoclonal antibody EB-A2, which binds the β-(1-5)-galactofuranosyl (gal*f*) side chains of the *Aspergillus* galactomannan (GM) molecule (24, 50, 51). In addition to GM, fungal glycoproteins also react with the EB-A2 antibody, including phospholipase C and phytase, which were shown to have only one terminal galactofuranose unit that was essential for binding with the EB-A2 antibody (24, 48). These findings implicate that the so-called "GM antigen" is not a single molecule but a family of molecules for which expression could be modulated by the immediate fungal environment (34). However, the actual gal*f* antigens that circulate in vivo in the body fluids of patients have not been characterized. Furthermore, there are several problems related to antigen detection. In some patients with proven IA, circulating antigen is not detected despite repeated sampling (false negative) (55). Also, the performance of antigen detection might be reduced in patients receiving antifungal prophylaxis. These and other factors that influence the release of gal*f* antigens, including their leakage from the site of infection into the blood and their bonding to substances present in the blood, were recently explored (31).

In contrast to the PA ELISA, there is no standardized PCR method for the detection of *Aspergillus* DNA for early diagnosis of IA. Whereas most methods show good sensitivity and specificity in experimental settings, unresolved issues, such as the optimal specimen (e.g., whole-blood, serum, plasma, and bronchoalveolar lavage specimens) and DNA extraction method, are probably major causes of variability in performance in clinical studies (5, 6, 18, 26). False-negative results are especially often found in patients with proven IA (6), which might be correlated with antifungal drug treatment.

A third promising nonculture-based diagnostic test detects circulating 1,3-β-D-glucan (BG). BG is a cell wall component of most medically important fungi, including *Aspergillus fumigatus* (35, 36). Furthermore, *A. fumigatus* releases BG into

* Corresponding author. Mailing address: Department of Medical Microbiology, Radboud University Nijmegen Medical Center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Phone: 31-24-3613514. Fax: 31-24-3540216. E-mail: m.mennink@mmb.umcn.nl.

the culture fluid in parallel with in vitro fungal growth (33) and can be quantified spectrophotometrically by activation of factor G, a coagulation factor of the horseshoe crab (35). A recently FDA-approved commercial method for the determination of BG has become available (Fungitell, formerly Glucatell; Associates of Cape Cod) (38). There are only a limited number of clinical studies published, and a definitive cutoff value for the diagnosis of IA remains to be established (37, 38, 42).

At present, there is insufficient understanding of the in vitro and in vivo kinetics of circulating gal*f* antigens, BG, and fungal DNA. Recently, an in vitro study of the release of gal*f* antigens by *A. fumigatus* in relation to the fungal biomass (20) showed that the amounts of gal*f* antigens released in the culture supernatant were not different for strains from circulating antigen-positive (CAG^+) and circulating antigen-negative (CAG^-) IA patients. Other causes for negative antigenemia could be the reduced release of gal*f* antigens due to environmental factors, such as pH, and reduced availability of nutrients at the site of infection. Because the kinetics of release of surrogate markers is largely unknown, we used the same in vitro model to study and compare the release of gal*f* antigens, DNA, and BG by *A. fumigatus* strains from different patients.

MATERIALS AND METHODS

Fungal strains and culture conditions. *A. fumigatus* strains from a CAG^+ and a CAG^- patient were cultured in flasks containing yeast nitrogen base (YNB) liquid medium with different glucose concentrations (5, 25, and 100 mM) and at different pHs (5.0 to 7.4). The medium was buffered with MOPS (morpholinopropanesulfonic acid) (170 mM) or MES (morpholineethanesulfonic acid) (100 mM) or not buffered. Each growth experiment was performed in duplicate and with one CAG^+ (AZN 4684) and one CAG^- (AZN 4565) strain. The cultures were incubated for a maximum of 9 days at 37°C and 160 rpm after inoculation with spores to a final concentration of 10^6 to 10^7 spores/ml. For each time measurement, one Erlenmeyer flask (500 ml) containing 200 ml of culture was used. The mycelium was harvested by filtration on a filter (0.8 μm; Schleicher & Schuell) and freeze-dried to determine the biomass. The culture filtrate was used for the PA ELISA, pH and glucose measurements, the β-galactofuranosidase assay, and detection of DNA and BG. Furthermore, the release of gal*f* antigens, BG, and DNA was also measured in one Erlenmeyer flask by taking (sterile) samples at serial time points, followed by filtration of the sample (0.2 μm cellulose acetate; Schleicher & Schuell).

PA ELISA. The culture filtrate samples were serially diluted in water for detection of reactivity by the PA ELISA. Each plate contained a standard calibration curve (0 to 15 ng/ml purified GM) in order to use the ELISA quantitatively. The PA ELISA was performed according to the manufacturer's instructions. Briefly, 50 μl of a reaction mixture containing horseradish peroxidase-conjugated anti-GM monoclonal antibody EB-A2 was added to each well of a microtiter plate coated with the same monoclonal antibody EB-A2, followed by the addition of 50 μl of pretreated sample. After 90 min of incubation at 37°C, the plates were washed five times with washing buffer before 200 μl of buffer containing tetramethylbenzidine solution was added. Then, the plates were incubated for another 30 min in the dark at room temperature, followed by the addition of 100 μl of 1.5 N sulfuric acid to stop the reaction. The optical density (OD) was read at 450 and 620 nm. A test sample was considered positive when the OD at 450 nm was higher than that of the cutoff sample (i.e., 0.5 ng GM). When testing different dilutions, the OD value closest to the OD value of the cutoff sample (i.e., 0.5 ng GM; R4) was interpolated in the calibration curve, and the total PA reactivity was expressed as the amount of GM in ng/ml (in addition to GM, actual PA reactivity in culture filtrates comes from several gal*f* components; however, only purified GM was available for quantification).

Fungitell BG assay. The culture filtrate samples were serially diluted in glucan-free reagent grade water (RGW) for detection of BG by the Fungitell test kit (Associates of Cape Cod, Falmouth, Mass.) in a kinetic, chromogenic format as recommended by the manufacturer. All assays were performed in duplicate. Briefly, 25-μl samples of the standards (100 to 6.25 pg/ml pure pachyman, a linear BG) and unknown pretreated samples were added to each well of a microtiter plate. Unknown samples (5 μl) were pretreated for 10 min at 37°C with an alkaline reagent (20 μl; 0.125 M KOH/0.6 M KCl). Fungitell reagent (lyophilized BG-specific *Limulus* amebocyte lysate) was reconstituted with 2.8 ml of RGW, followed by 2.8 ml of pyrosol reconstitution buffer (2 M Tris HCl, pH 7.4), and 100 μl of this mixture was added to each sample. An OD_{405} was read for 40 min at 37°C, using an Anthos HT-3 plate reader and MikroWin software (Mikrotek Laborsysteme GmbH), and the concentration in each unknown sample was calculated using the calibration curve after the standards were multiplied by 5, so that the range was from 500 to 31.25 pg/ml. When absorbance was outside the range of the standard curve, the samples were diluted in RGW and tested again.

Glucose assay. Glucose concentration was measured enzymatically by coupling the glucose oxidase reaction to the peroxidase reaction. Culture filtrate samples (serially diluted in water) and glucose standard samples (0.05 to 1.2 mM glucose) were analyzed by taking 100 μl of sample and by mixing this with 1 ml of reagent containing $Na_2HPO_4 \cdot 2H_2O$ (13.6 g/liter), $NaH_2PO_4 \cdot H_2O$ (7.2 g/liter), glucose oxidase (750 U), peroxidase (150 U), and *o*-dianisidine dihydrochloride (39.4 mg/liter). After 45 min of incubation at room temperature, the formed brown-colored product (oxidized *o*-dianisidine) was measured spectrophotometrically at 450 nm. The A_{450} of the unknown samples was corrected for the blank (diluted) culture medium without glucose, and the amount of glucose was calculated from the standard curve.

β-Galactofuranosidase assay. β-Galactofuranosidase activity was determined by measuring (in microtiter plates) the hydrolysis of *p*-nitrophenyl-β-D-galactofuranoside (PNP-gal*f*; Sigma) following incubation at 37°C and pH 4.0 for 1.5 h. The reaction was started by the addition of 60 μl of culture filtrate to 1 mM of substrate in a total volume of 100 μl 100 mM sodium acetate buffer, pH 4.0. The reaction was terminated with 200 μl of stop solution containing 0.25 M Na_2CO_3 and 0.25 M $NaHCO_3$. The yellow-colored product (*p*-nitrophenol) was measured spectrophotometrically at 405 nm. One enzyme unit is defined as the amount required to hydrolyze 1 μmol of substrate min^{-1} under these conditions, and *p*-nitrophenol (Sigma) was used for the standard curve. Different blanks were used, including water and culture medium.

DNA detection. DNA extraction of 1-ml culture filtrate samples was performed with and without physical disruption, using MagNA lyser green 1.4-mm ceramic beads (MagNA lyser; Roche), for two cycles of 20 s at 6,500 rpm, followed by isolation with the MagNA Pure LC apparatus (Roche Biochemicals). The total nucleic acid large-volume isolation kit (Roche Biochemicals) was used. We used a starting volume of 1,000 μl eluted in 50 μl, and 10 μl was used for the PCR. *A. fumigatus* DNA was detected with a previously published PCR protocol which amplifies a 363-bp fragment of the *Aspergillus* 18S rRNA gene (30).

RESULTS

General growth characteristics. Growth on glucose resulted in an associated drop in pH when the medium was not buffered, followed by a pH increase when glucose became limited (Fig. 1A to C). During the logarithmic growth phase, glucose consumption was correlated to biomass production. Glucose became limited within 17 h (5 mM) or after 1 and 3 days (25 and 100 mM glucose, respectively). In order to study the effect of glucose limitation, the 5 mM concentration (also human blood values) was used for further studies. The effects of different pHs at this glucose concentration were tested (Fig. 2 and 3). In all cases, the maximum dry weight was about 0.4 to 0.5 mg/ml, which was reached within 17 h, the same time needed for glucose consumption. CAG^+ and CAG^- *A. fumigatus* strains gave similar results. Glucose limitation initiated the lytical phase, which is shown by the decrease of dry weight (Fig. 2 and 3; glucose concentrations are not shown but were 0 within 1 day of growth). Nine days after glucose became limited, the mycelium seemed to be broken down entirely in most cultures. However, the fungus was still viable after being transferred to a fresh medium.

Release of gal*f* antigens. During the logarithmic growth phase, when glucose was consumed (values not shown), gal*f* antigens were released in the culture supernatant (Fig. 2 and

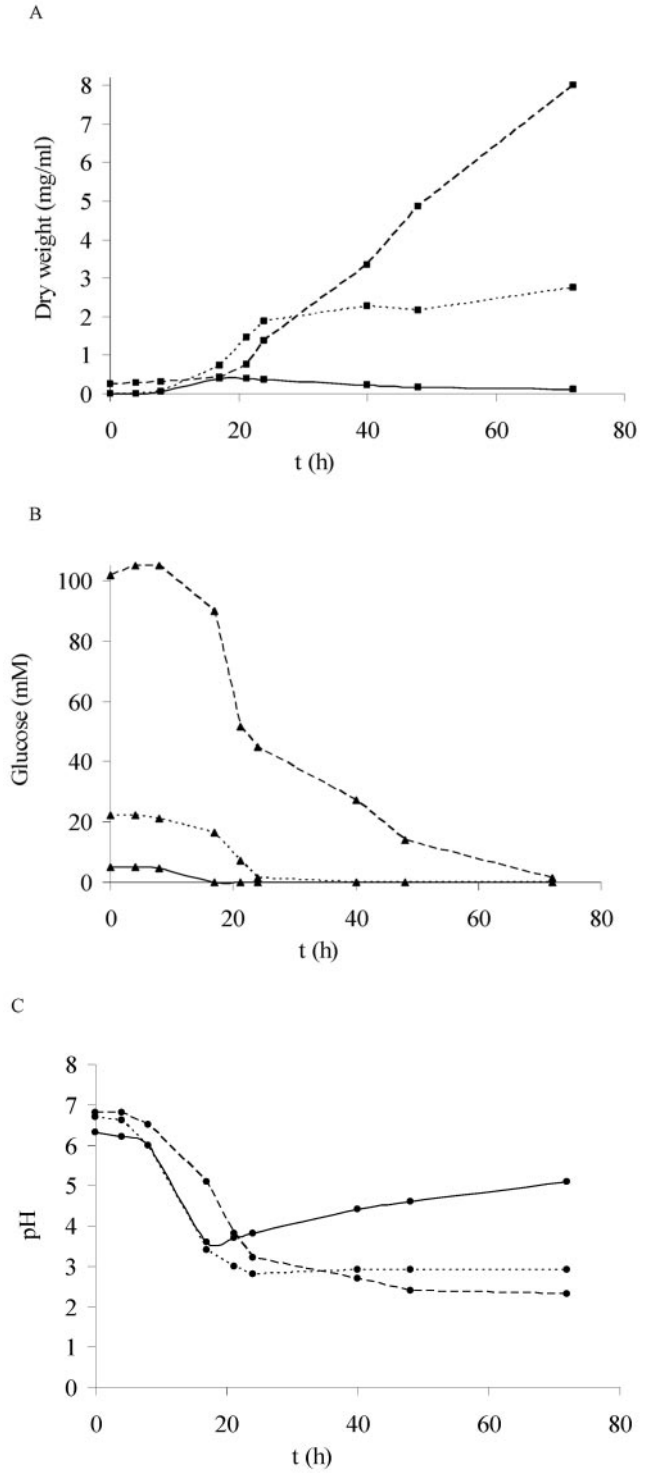

FIG. 1. Growth of *A. fumigatus* (CAG$^-$ strain) on YNB supplemented with 5 mM (—), 25 mM (- - -), or 100 mM (– –) of glucose as the carbon source. The medium was not buffered, and the initial pH was 6.3 to 6.8. (A) ■, dry weight (mg/ml). (B) ▲, glucose concentration in culture filtrate (mM). (C) ●, pH of culture filtrate.

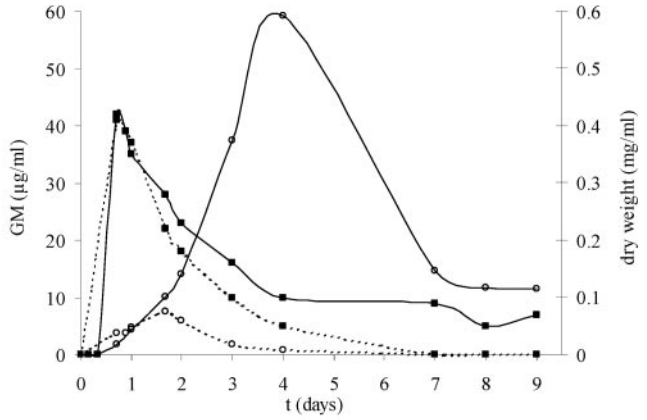

FIG. 2. Growth of *A. fumigatus* on YNB medium supplemented with 5 mM of glucose as the carbon source. Each line is the mean value of a CAG$^+$ and a CAG$^-$ strain. Media were not buffered, and the initial pH was 7.4 (—) or pH 6.3 (- - -). (■) Dry weight (mg/ml); (○) GM (μg/ml), i.e., PA ELISA reactivity due to gal*f* antigens.

3). After glucose became limited (after 1 day of growth), PA ELISA reactivity further increased, followed by a decrease. The maximum PA ELISA reactivities varied between the different culture conditions. At these maximum levels, the pH was 7.1 for the culture that started at pH 7.4 and pH 4.4 for the culture that started at pH 6.3 (values not shown). PA ELISA reactivity was the highest when pH was 7.1 to 7.4 (about 60 μg/ml) (Fig. 2 and 3) and lower when pHs were 4.4 (7.5 μg/ml) (Fig. 2) and 5.0 (10.9 μg/ml) (Fig. 3). During this lytical phase, PA ELISA reactivity reached the maximum value after 40 h (pH 5 and lower) or after 4 days (pH 7.1) and more than 7 days (pH 7.4), followed by a decrease. Gal*f* antigens became undetectable within 4 days at low pH. In contrast, at pH 7.4, PA ELISA reactivity (expressed as GM concentration) decreased only slightly (Fig. 3).

Release of *Aspergillus* DNA. *Aspergillus* DNA was not detected in the culture supernatant during logarithmic growth (Tables 1 and 2 and Fig. 2 and 3), indicating that DNA was not

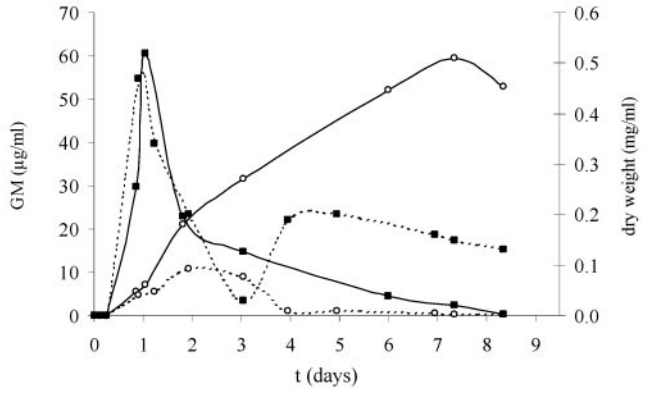

FIG. 3. Growth of *A. fumigatus* on YNB medium supplemented with 5 mM of glucose as the carbon source. Each line is the mean value of a CAG$^+$ and a CAG$^-$ strain. Media were buffered with MES at pH 7.4 (—) or pH 5.0 (- - -). (■) Dry weight (mg/ml); (○) GM (μg/ml), i.e., PA ELISA reactivity of the supernatant due to gal*f* antigens.

TABLE 1. Release of *Aspergillus* markers during in vitro growth under different conditions[a]

Medium[b]	*t* (h/days)	GM (μg/ml) (*n* = 2)	BG (pg/ml) (*n* = 2)	GM (ng/ml)[c]	BG (pg/ml)[d]	DNA (PCR) (CAG^+/ CAG^-)[e]
I	0	0.01	539	0	0	Neg/neg
	16/0.7	3.70	1,351	7,380	14	Neg/neg
	24/1.0	4.85	1,351	9,680	14	Neg/neg
	40/1.7	7.47	664	14,920	2	Neg/pos
	48/2.0	5.89	539	11,760	0	Neg/pos
	72/3.0	1.80	1,039	3,580	8	Neg/pos
	96/4.0	0.73	664	1,440	2	Pos/pos
	168/7.0	0.01	40	0	0	Pos/pos
	192/8.0	0.02	165	20	0	Pos/pos
	216/9.0	0	102	0	0	Pos/pos
II	0	0.015	40	0	0	Neg/neg
	4/0.2	0.025	290	20	4	Neg/neg
	8/0.3	0.068	352	106	5	Neg/neg
	16/0.7	1.71	914	3,390	15	Neg/neg
	24/1.0	4.40	477	8,770	7	Neg/pos
	40/1.7	10.10	477	20,170	7	Neg/pos
	48/2.0	14.10	914	28,170	15	Neg/pos
	72/3.0	37.50	914	74,970	15	Neg/pos
	96/4.0	59.30	102	18,570	1	Neg/pos
	168/7.0	14.70	0	29,370	0	Pos/pos
	192/8.0	11.60	165	23,170	2	Pos/pos
	216/9.0	11.40	40	22,770	0	Pos/pos

[a] Results are the mean values from two individual growth experiments (CAG^+ and CAG^- strains).
[b] Medium I, YNB medium with 5 mM of glucose. The medium was not buffered and started at pH 6.3. Medium II, YNB medium with 5 mM of glucose. The medium was not buffered and started at pH 7.4. See also Fig. 2.
[c] GM amount divided by the Platelia *Aspergillus* cutoff value (0.5 ng/ml) after correction for value at time zero.
[d] BG amount divided by the Fungitell cutoff value (60 pg/ml) after correction for value at time zero.
[e] Neg, negative; pos, positive.

TABLE 2. Release of *Aspergillus* markers during in vitro growth under different conditions[a]

Medium[b]	*t* (h/days)	GM (μg/ml) (*n* = 2)	BG (pg/ml) (*n* = 2)	GM (ng/ml)[c]	BG (pg/ml)[d]	DNA (PCR) (CAG^+/ CAG^-)[e]
III	0	0.015	44	0	0	Neg/neg
	6/0.3	0.080	180	130	2.3	Neg/neg
	22/0.9	4.67	588	9,307	9.1	Neg/neg
	30/1.3	5.61	180	11,190	2.3	Neg/neg
	47/1.9	10.90	1,064	21,770	17.0	Neg/pos
	73/3.0	9.01	1,268	17,990	20.4	Neg/pos
	95/4.0	1.08	112	2,130	1.1	Pos/pos
	119/5.0	1.08	44	2,130	0	Neg/pos
	167/7.0	0.570	248	1,110	3.4	Pos/pos
	177/7.4	0.250	248	470	3.4	Neg/pos
	200/8.3	0.150	44	270	0	Neg/pos
IV	0	0.010	215	0	0	Neg/neg
	4/0.2	0.020	44	20	0	Neg/neg
	6/0.3	0.052		84		Neg/neg
	21/0.9	5.40	594	10,779	6	Neg/neg
	25/1.0	7.08	1,177	14,140	16	Neg/neg
	44/1.8	21.09	581	42,160	6	Pos/neg
	73/3.0	31.50	492	62,980	5	Neg/neg
	144/6.0	52.03	1,177	104,040	16	Neg/neg
	177/7.4	59.43	819	118,840	10	Neg/neg
	200/8.3	52.80	492	105,580	5	Neg/neg

[a] Results are the mean values from two individual growth experiments (CAG^+ and CAG^- strains).
[b] Medium III, YNB medium with 5 mM of glucose. The medium was buffered at pH 5.0. Medium IV, YNB medium with 5 mM of glucose. The medium was buffered at pH 7.4. See also Fig. 3.
[c] GM amount divided by the Platelia *Aspergillus* cutoff value (0.5 ng/ml) after correction for value at time zero.
[d] BG amount divided by the Fungitell cutoff value (60 pg/ml) after correction for value at time zero.
[e] Neg, negative; pos, positive.

released by the fungus. DNA was detected only during the lytical phase, which corresponded with autolysis of the fungus due to lack of nutrients (i.e., carbon source). These positive samples were negative after mechanical disruption with beads (MagNA lyser; Roche), which indicates that pretreatment results in loss of "free" DNA. Furthermore, DNA was detected in pretreated mycelial samples (positive control) but not in any untreated mycelial samples, indicating that pretreatment was required to release the fungal DNA from the mycelium.

Release of BG. Like PA ELISA-reactive components, BG was released during logarithmic growth in the culture medium, as shown in Table 1. BG increased to a maximum, ranging from 914 to 1,351 pg/ml, after about 1 day of growth with 5 mM of glucose and at different pHs (mediums I to IV) and was followed by a decrease. After the first decrease, the BG level showed a second fluctuation at all pHs. However, these absolute values are not much higher than background values at time zero. Furthermore, BG levels reached approximately 16 times the serum cutoff value of 60 pg/ml while gal*f* antigen levels reached up to 118,840 times the serum cutoff value of 0.5 ng/ml GM (Table 1).

Release of BG compared to gal*f* antigens and DNA. In order to study the release of surrogate markers during early logarithmic growth under nutrient-sufficient conditions (i.e., 100 mM glucose), BG, gal*f* antigens, DNA, and the consumption of glucose were measured during the first 40 h of growth of the fungus in one Erlenmeyer flask. Compared to 5 mM of glucose, the BG levels in the supernatant were much higher when the fungus was grown at 100 mM of glucose, as shown in Fig. 4. Whereas PA ELISA reactivity continued to increase to more than 60,000 ng/ml GM or 120,000 times the serum cutoff value of 0.5 ng/ml after 3 days of growth, when 100 mM glucose is consumed (results shown only for first 40 h), BG reached a maximum of 58,915 pg/ml or 978 times the serum cutoff value after 1 day of growth, when about 30 mM of glucose had been consumed, and decreased after that (Fig. 4A and B). Furthermore, the results of the PA ELISA, Fungitell assay, and PCR are also shown in Table 3. The first indication of PA ELISA reactivity was shown by an increase of the ELISA index (EI) of 0.5 after 2 h of growth. After 4 h of growth, the EI was three times the EI at time zero. After 6 h of growth, the EI was 42 times higher than the value at time zero. The first indication of BG release was more difficult to determine because the BG background at time zero was 213.4 pg/ml (i.e., the mean of 0, 0, 76.1, and 777.5 pg/ml). After 2 h of growth, the BG value was just above the highest background value, and the BG values after 4 and 6 h of growth were below the highest background value. Definitive release was measured after 8 h of growth (Table 3). *Aspergillus* DNA was not detected in the culture supernatant within the first 40 h of growth (Table 3).

In order to confirm that the decrease of BG was caused by an enzyme that was released into the culture medium, the following experiment was performed. A filtered supernatant

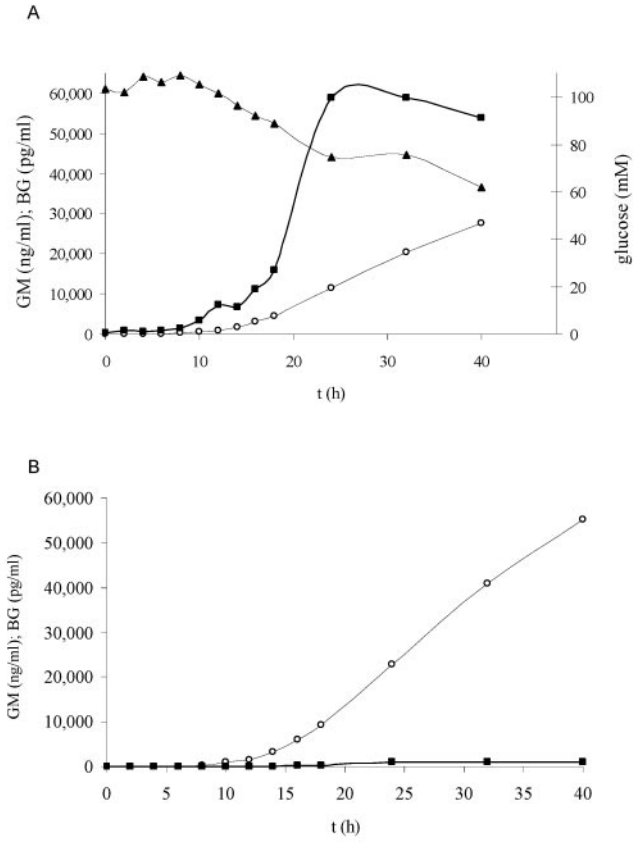

FIG. 4. (A) Release of gal*f* antigens (expressed as the amount of GM) and BG during growth of *A. fumigatus* on YNB medium supplemented with 100 mM of glucose as the carbon source in an Erlenmeyer flask. Each line is the mean value of a CAG^+ and a CAG^- (GM) strain. The medium was buffered with MOPS at pH 7.4. (▲) Glucose concentration in culture filtrate (mM); (○) GM (ng/ml); (■) BG (pg/ml). (B) Same as panel A, but the amounts of GM and BG are divided by serum cutoff values (CV) 0.5 ng/ml and 60 pg/ml, respectively.

containing 51,814 pg/ml BG was boiled to reduce the enzyme activity and then incubated for 24 h at 37°C. The remaining BG concentration was measured at 4, 8, and 24 h. There was a 60% (24 h) decrease in BG concentration in nonboiled supernatant but no decrease of BG in the boiled supernatant.

β-Galactofuranosidase activity. The galactofuranosidase activity of the same culture filtrates as those shown in Fig. 2 and 3 was determined (Fig. 5 and 6). The enzyme activity was low during logarithmic growth (about 0.2 to 0.4 U/liter) and increased during the lytical phase, when the carbon source was limited. Maximal activity was the lowest at higher pHs, being 1.4 U/liter at pH 7.1 to 7.4 (Fig. 5) and 2.6 U/liter at pH 7.4 (Fig. 6), and higher at low pHs, being 5.4 U/liter at pH 3.6 to 4.4 (Fig. 5) and 4.9 U/liter at pH 5.0 (Fig. 6). Activity was even higher when grown on 25 mM of glucose at pH 5.0, being 11.6 U/liter. Furthermore, a strong increase in enzyme activity seems to be correlated with a decrease in PA ELISA reactivity. This phenomenon is most clearly seen with 25 mM of glucose (Fig. 6). In order to confirm that β-galactofuranosidase was released into the culture medium and reduced the amount of PA ELISA-reactive material, a filtered supernatant with a galactofuranosidase activity of 10 U/liter and a PA ELISA reactivity of 2.1 μg/ml GM was boiled to reduce the enzyme activity and then incubated for 24 h at 37°C. The remaining enzyme activity and PA ELISA reactivity were measured at 4, 8, and 24 h. The enzyme activity showed a 100% reduction due to the boiling of the supernatant. There was a 69.8% (24 h) decrease in PA ELISA reactivity in the nonboiled supernatant but no reduction in PA ELISA reactivity in the boiled supernatant.

TABLE 3. Release of *Aspergillus* markers during in vitro growth in Erlenmeyer flask on YNB with 100 mM of glucose buffered at pH 7.4[a]

Time of growth (h)	PA ELISA result (EI)[b]	Fungitell result (BG in pg/ml)[c]	PCR (*Aspergillus* DNA)[d]
0	0.470	213.4	Neg
2	0.970	827.6	Neg
4	1.570	527.0	Neg
6	21.0	727.4	Neg
8	157.5	1,278.5	Neg
10	506.0	3,274.2	Neg
12	791.5	7,278.1	Neg
14	1,663.5	6,773.0	Neg
16	3,031.0	11,282.0	Neg
18	4,594.0	15,791.0	Neg
24	11,440.0	58,914.9	Neg
32	20,430.0	58,914.9	Neg
40	27,669.5	53,904.9	Neg

[a] Results are the mean values from two individual growth experiments (CAG^+ and CAG^- strains).
[b] Cutoff value, 0.5 ng/ml GM.
[c] Cutoff value, 60 pg/ml BG. The values at time zero ranged from 0 to 777 pg/ml. Values at 2, 4, and 6 h were within the same range as those at time zero.
[d] Neg, negative.

DISCUSSION

In this study, the glucose concentration and buffering capacity of the medium determined the pH during fungal growth. In *Aspergillus* species, growth in the presence of glucose is normally associated with organic acid production and an associated drop in pH (21). In addition, growth in the presence of ammonium, the main nitrogen source of YNB, also decreased medium pH because uptake of ammonia is accompanied by the

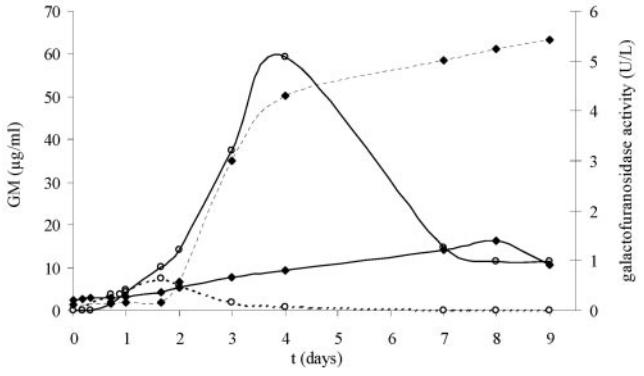

FIG. 5. Release of GM and β-galactofuranosidase during growth of *A. fumigatus* on YNB medium supplemented with 5 mM of glucose as the carbon source. Each line is the mean value of a CAG^+ and a CAG^- strain. Media were not buffered and started at pH 7.4 (—) or pH 6.3 (- - -). (◆) β-Galactofuranosidase activity (U/liter); (○) GM (μg/ml).

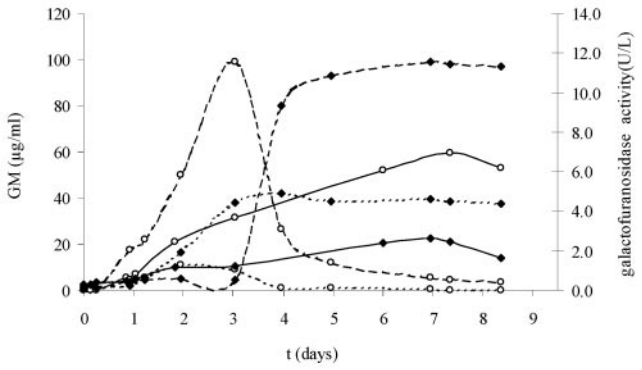

FIG. 6. Release of GM and β-galactofuranosidase during growth of *A. fumigatus* on YNB medium supplemented with 5 mM (— and - - -) or 25 mM (– –) of glucose as the carbon source. Each line is the mean value of a CAG^+ and a CAG^- strain. Media were buffered with MOPS (170 mM) at pH 7.4 (—) or with MES (100 mM) at pH 5.0 (- - - and – – –). (◆) β-Galactofuranosidase (U/liter); (○) GM (μg/ml).

release of a proton from the cell, as shown for other fungi (1, 17). This study shows that the release of GM and other gal*f* antigens by *A. fumigatus* during the logarithmic growth phase corresponds with the increase in fungal biomass and the glucose concentration used. The same is true for BG release during the first 24 h of logarithmic growth. However, BG was detected somewhat later than gal*f* antigens. Furthermore, BG showed a decrease after 24 h, which is not due to nutrient limitation (i.e., glucose). A decrease of BG after 24 h of in vitro growth has already been shown by Miyazaki et al. (33) for *Candida albicans* and was suggested to be caused by the enzyme β-1,3-glucanase. Those authors also measured a maximum BG release by *A. fumigatus* of about 10,000 pg/ml after 48 h of growth at 30°C. *A. fumigatus* has been shown to produce cell wall-associated exo-1,3-β-glucanases and an endo-1,3-β-glucanase, which might have a role in cell wall morphogenesis (14, 15). In other filamentous fungi, exocellular β-glucanases have also been found and seem to have a role in hydrolyzing exocellular BGs for fungal catabolism. However, these enzymes are expressed only under glucose limitation (15). The BG decrease in this study is clearly enzyme related, but it is not clear which of the glucanases is responsible.

When glucose becomes limited, mycelium is broken down. This results in a further release of gal*f* antigens (expressed as GM concentration with equivalent PA ELISA reactivities). A part of these antigens probably originate from the cell wall, including GM and peptidoGM (25); however, other gal*f* antigens might be induced due to nutrient limitation, e.g., glucose and phosphate. Phospholipase C, a possible virulence factor, is repressed by phosphate (34) and might be induced by phosphate limitation. Together with phytase and alkaline phosphatase, this enzyme might be part of a phosphate-scavenging system as suggested by Morelle et al. (34). The exact cause of increase of PA ELISA reactivity in the culture supernatants remains unknown because not all in vitro gal*f* antigens and their contributions to the total PA ELISA reactivity are characterized. In addition to glucose, pH also has an influence on the release of gal*f* antigens. More PA ELISA reactivity is found in the medium when it is cultured at a higher pH than at a low pH. Galactomannan is a cell wall component that is also released in the environment, probably as a carbon overflow mechanism (31, 40). The role of some extracellular polysaccharides of fungi may be to reserve nutrient sources of carbon. During C limitation, GM and other gal*f* epitopes might be broken down by the enzyme exo-β-D-galactofuranosidase to produce galactose, which can then be used as a secondary C source, as suggested for *Aspergillus niger* (57) and *Penicillium fellutanum* (40). The production of this enzyme seemed to be medium dependent, with glucose as a repressor (10). Furthermore, a galactofuranosidase was purified from *A. niger* with an optimum pH of 3 to 4 (56). The results of this study suggest that β-galactofuranosidase activity is involved in the decrease of gal*f* epitopes in the culture supernatant. The activity rises after glucose becomes limited and seems to be influenced by the pH of the culture medium. As a consequence, the maximum gal*f* antigen concentration is lower at a low pH than at a higher pH. Furthermore, lowering the pH gives a fast decrease of gal*f* antigen concentration compared to buffering the medium at pH 7.4 (Fig. 2 and 3). The β-galactofuranosidase activity in the culture filtrates is probably even higher because the assay uses synthetic PNP-gal*f* as a substrate. The purified galactofuranosidase from *Trichoderma harzianum* showed a low activity on synthetic PNP-gal*f*, indicating that the enzyme needs more than one gal*f* residue for binding (53). Furthermore, the presence of gal*f* antigens as a concurrent substrate in the culture supernatant might also decrease the measured enzyme activity.

A specific β-D-galactofuranosidase has been detected in only a few species of fungi: *P. fellutanum* (44), *Penicillum* and *Aspergillus* species (10), *T. harzianum* (53), *Helminthosporium sacchari* (11), and *A. niger* (56). A comparison between enzyme levels shows that *A. fumigatus* (this study) produced about the same level as the *Penicillium* species (5 to 50 U/liter) but not as much as *A. niger* (250 U/liter) (56). However, growth conditions and substrates differed between different studies. The β-D-galactofuranosidase in the culture supernatant probably has an autocatalytic role because activity increased under starvation conditions. A polyclonal antibody against the enzyme of *P. fellutanum* showed that the enzyme was present throughout the hyphae, including the walls (32). The production of extracellular β-D-galactofuranosidase by *Aspergillus* species could interfere with their ability to be detected in immunoassays because the enzyme degrades the epitopes. Similar activity could also be used in vivo against gal*f* components from competing microorganisms, as suggested by Wallis et al. (56). This study shows no difference in galactofuranosidase activity between CAG^+ and CAG^- *A. fumigatus* strains, which argues against a role of this enzyme in the cause of false-negative reactivity.

Since galactofuranosyl residues and galactofuranosidases are absent in mammalian species, it was suggested that gal*f* residues may have a role in the survival of fungi by preventing the action of hosts' glycosidases on fungal gal*f* components, including GM and the majority of extracellular proteins (52). Marino et al. showed that the *P. fellutanum* peptidophosphogalactomannan could not be detected in vitro after the addition of galactofuranosidase inhibitors (29). Furthermore, the content of galactofuranose in the cell wall was significantly decreased, and cell structure was strongly disturbed. This suggests that the metabolism of gal*f* components might be a target

for development of therapeutic agents, for instance, UDP-galactopyranose mutase, which converts UDP-galactopyranose to UDP-gal*f*. This mutase enzyme is essential for the viability of mycobacteria (46), and activity of the enzyme was shown some time ago in penicillin fungus (39).

As opposed to gal*f* components and BG, *Aspergillus* DNA was not detected during the logarithmic growth phase of this fungus in vitro. Fungal DNA seemed to be released only after mycelium breakdown, due to autolysis caused by nutrient limitation. However, not all PCR samples were positive during the lytical phase. DNA might be below the detection limit of the PCR test, and DNases secreted by the fungus could lower the DNA level in the culture filtrate. The results suggest that during invasive infection, damage of hyphae is required in order to release fungal DNA, for instance, by human host defense or autolysis. The actual form in which DNA circulates in the blood of an IA patient is not known. Blood cultures are rarely positive and could result only from viable hyphal fragments because conidia are not present in the blood. So the most likely circulating form seems to be free DNA (i.e., naked DNA or DNA bound to fungal cell walls), also suggested by Costa et al. (9). Furthermore, Bougnoux and coworkers showed that intravenously injected *Candida* DNA is detectable in rabbit serum for hours, which argues against a rapid degradation of foreign DNA in the blood (3).

The form that circulates has a consequence for the DNA isolation method used for PCR. Our results showed that the method normally used for pretreatment of fungal mycelium in order to release DNA resulted in the loss of free DNA after pretreatment of PCR-positive culture filtrate samples. Furthermore, many DNA extraction methods result in decanting of free DNA. This could explain the fact that especially antifungal therapy, which probably releases much free DNA, is often combined with negative PCR results (7, 22, 23). In contrast, Kami et al. used an extraction method that includes free DNA and found no negative PCRs in sera from patients receiving antifungal therapy (18). In conclusion, two extraction methods should be used on EDTA whole-blood samples, one setup to isolate free DNA and another to isolate DNA from fungal cells, in order to include all possible forms of circulating DNA.

During an early stage of fungal infection, detection of circulating gal*f* antigens seems to be superior to that of PCR and BG because of much higher amounts that are released. However, other factors play a role, for instance, the level of host defense, the ease by which the antigen reaches the circulation, and its circulating half-lives. Many studies have been performed to compare the different diagnostic methods for detection of circulating markers. While some studies show that the PA ELISA was more frequently positive (2, 8, 47, 54), other studies seem to be in favor of DNA detection (6, 18). The combination of PCR with the PA ELISA should improve the diagnosis of IA, as also suggested by other authors (4, 7, 8, 47). Only a limited number of comparative studies have been performed with the BG test (18, 19, 42). In a prospective comparison between the diagnostic potentials of real-time PCR, the PA ELISA, and the BG test for IA in hematological patients, the PA ELISA was the most sensitive, using a reduced cutoff of 0.6 ng/ml GM (19). Another study retrospectively compared the BG test with the PA ELISA and showed improved specificity when the two tests were combined (42). However, the usefulness of the BG test for early diagnosis of IA remains to be determined.

This study suggests that during invasive infection, the conditions at the infection site may determine the actual amounts of gal*f* antigens, DNA, and BG that are released. Further studies will be focused on in vitro experiments with nutrients that mimic the clinical situation, for instance, lung proteins, and in vivo (animal) experiments in order to determine the conditions at the infection site and their impact on the release of surrogate markers.

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