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A New Sensitive Sandwich Enzyme-Linked Immunosorbent Assay To Detect Galactofuran in Patients with Invasive Aspergillosis

D. STYNEN,[1]† A. GORIS,[1]† J. SARFATI,[2] AND J. P. LATGÉ[2]*

Sanofi Diagnostics Pasteur, B-3600 Genk, Belgium,[1] and Unité de Mycologie, Institut Pasteur, 75724 Paris Cedex 15, France[2]

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A double-direct sandwich enzyme-linked immunosorbent assay that uses a rat anti-galactomannan monoclonal antibody as the acceptor and detector antibody was designed. This immunoassay, which detects less than 1 ng of galactomannan per ml, was assessed in a retrospective study with samples from patients with invasive aspergillosis. Serum is more appropriate than urine for use in the search for circulating galactomannan. Antigenemia does not have a transient character. Galactomannan can be detected at least 39 days before the death of the patients.

Invasive infections by *Aspergillus fumigatus* and other *Aspergillus* species have emerged as major threats to immunocompromised patients (2, 4). Effective therapy of invasive aspergillosis (IA) is only possible in the case of an early diagnosis. At present, the diagnosis of IA, which is mainly based on clinical symptoms, is made at a time when the fungal burden is usually high (2, 4). Serodiagnosis has been of very little help in the diagnosis of IA. The presence or absence of specific anti-*Aspergillus* antibodies has no diagnostic value since it reflects more a preexisting humoral situation rather than a pathological situation related to IA (1). The only other promising alternative is the search for circulating antigens in serum and urine (3, 4, 9).

Among the antigens found in the biological fluids of patients with IA, carbohydrates are the most suitable antigens for the diagnosis of this mycosis (3, 9). They are stable over time and withstand the harsh treatments used to dissociate antigens from immune complexes in serum specimens. Indeed, most tests for antigenemia have used antibodies directed against galactomannan (GM) or carbohydrate-containing antigens as the detector antibodies. Although antigen detection appears to be highly specific, the sensitivities of all tests used so far have been quite low (6, 19).

The anti-GM monoclonal antibody (MAb) EB-A2, which was characterized previously (16), binds to an epitope composed of four β(1→5)-linked galactofuranose residues. The recent elucidation of the chemical structure of the *A. fumigatus* GM has shown that one GM molecule contains more than 10 galactofuran epitopes (10). The presence of multiple epitopes recognized by the MAb on one GM molecule made possible the development of a double-direct sandwich enzyme-linked immunosorbent assay (ELISA) with EB-A2 as the capture and detector (after conjugation to peroxidase) antibody. This report describes the performance of this sandwich ELISA in the detection of *Aspergillus* GM in vitro as well as in the sera and urine of patients with IA.

GM was isolated from a culture filtrate of *A. fumigatus* (10). The amount of galactofuranose (Galf) of GM was quantified as trimethylsilylated derivatives by gas-liquid chromatography (10). Serum and urine samples were from leukemic immunosuppressed patients of the bone marrow transplantation and the pediatric hematology units of St. Louis Hospital (Paris, France) and were kindly donated by F. Derouin. Fifty-nine serum and 54 urine samples were taken sequentially from nine patients with aspergillosis. Additionally, 164 serum and 222 urine samples from immunosuppressed leukemic patients without aspergillosis in the same hospital units were used as controls. Three hundred microliters of serum was mixed with 100 μl of 4% EDTA treatment solution, and the solution was boiled for 3 min (12). After centrifugation, the supernatant was recovered for further testing. Urine samples were not submitted to the heat treatment but were only cleared by centrifugation at 5,000 × *g* for 5 min. The anti-GM immunoglobulin M MAb EB-A2 was produced and purified as described previously (16).

Overnight coating of microtiter plates at 16 to 20°C in 0.1 M carbonate buffer (pH 9.4) with MAb EB-A2 at 1 μg of immunoglobulin M per ml and postcoating with 0.15% (wt/vol) bovine serum albumin (BSA)–5% (wt/vol) sucrose were carried out automatically in an industrial setting. Fifty microliters of horseradish peroxidase-conjugated EB-A2 in conjugate buffer (0.1 M Tris [pH 7.4], 0.17 M NaCl, 0.05% [vol/vol] Tween 20, 50% [vol/vol] rat ascitic fluid) was added to the wells; this was followed by the addition of 50 μl of the sample. Samples were (i) Tris-buffered saline (TBS; pH 7.4) or the serum or urine of control patients containing a range of GM concentrations and (ii) serum and urine samples from different control and IA patients. All samples were treated as described above for the clinical samples. Plates were incubated for 90 min at 37°C. After extensive washings, the immunological reactions were revealed by a 30-min incubation in darkness with 100 μl of revelation buffer containing *o*-phenylenediamine. The optical density (OD) results are shown as obtained or were converted into galactose or GM concentrations as deduced from a calibration curve.

A range of 0.2 to 2 ng of galactofuran per ml was detected by the double-direct sandwich ELISA when GM was added to TBS or spiked into negative serum and urine specimens (Fig. 1 and 2).

Serum and urine samples from immunocompromised patients without evidence of aspergillosis were tested in order to study the ELISA background. A cutoff value was calculated from a total of 164 serum samples (OD values between 0 and 0.700), distributed according to a Gaussian distribution, as the

* Corresponding author. Mailing address: Unité de Mycologie, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France. Phone: 1-45 68 82 25. Fax: 1-45 68 84 20.

† Present address: NV Innogenetics, Haven 1009, B-2070 Zwijndrecht, Belgium.

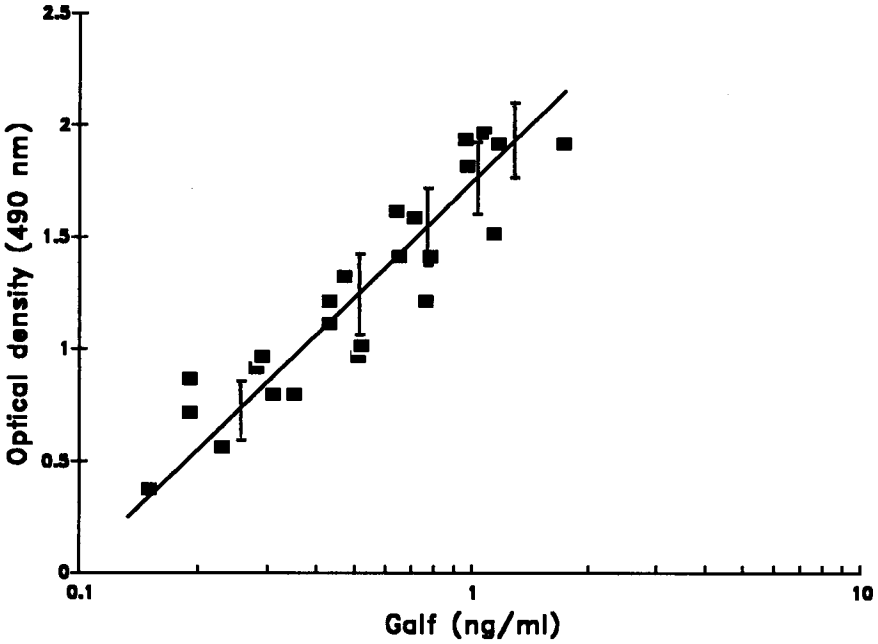

FIG. 1. Amount of galactose (Galf) detected by sandwich ELISA (average value calculated from duplicate OD values obtained with five different batches in vitro-produced GM diluted in TBS). The standard deviations were artificially calculated from calibration curves constructed with OD values corresponding to different GM concentrations. The regression curve is based on all values and is OD = 1.49 log (10 × nanograms of Galf per milliliter) + 0.17 (correlation coefficient, 0.92).

average OD value of the selected negative control samples (0.23) plus 4 standard deviations. This yielded a cutoff OD value of 0.79. The background in urine samples was much lower. For 222 samples tested the OD values were between 0 and 0.3, with an average OD of 0.03. The cutoff OD value for urine samples, calculated as described above for serum samples, was 0.19. Although zero values were always lower with the urine samples than with serum samples, the slopes of the calibration curves constructed after the addition of different amounts of GM were always steeper with urine than with serum. The slopes calculated from equations expressing OD as a function of log (10 × nanograms of Galf per milliliter of urine or serum) were 2.0 ± 0.1 and 1.4 ± 0.3 (five replicates), respectively.

Table 1 shows the results obtained with nine patients deceased from proven or suggested invasive aspergillosis. GM was detected in the serum samples of all patients. Urine samples were also available from seven of these nine patients. GM was detected in the urine of only five of these seven patients. When it was detected, the amount of antigen present in the urine was always lower than that in serum. In addition, GM was always detected earlier in serum than in urine; the maximal numbers of days separating patient death and the first detection of GM in patient serum and urine were ≥39 and ≤12, respectively. In contrast to previous reports (6, 13), it is more appropriate to use serum than urine for the detection of GM because of the lower degree of specificity and the later diagnosis obtained with urine samples.

A significant heterogeneity was seen in the follow-up of each patient (see examples in Fig. 3). The maximal concentration of galactofuran varied for each patient from 3.5 to 13 ng of Galf per ml of serum and 1.2 to 2.4 ng of Galf per ml of urine. In addition, the slope of the rise in GM concentration during the progression of the disease also depended on the patient (Table 1; Fig. 3). However, it must be stressed that the calculated concentrations of GM present in vivo are only estimates for several reasons: (i) the exact nature of the galactofuran-bearing antigens present in the sera of patients with IA is unknown, and (ii) the batches of GM produced in vitro have different galactose:mannose ratios as well as variable reactivities toward the MAb because of a reduction in the size of the galactofuran

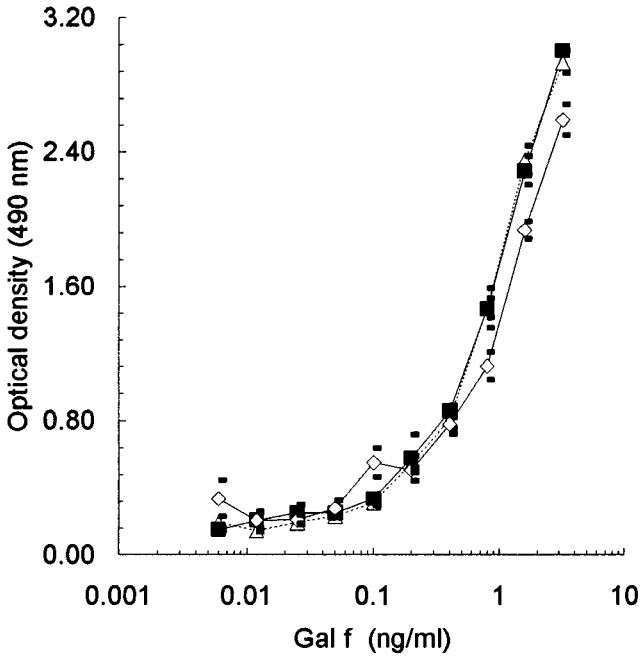

FIG. 2. Detection of GM spiked in TBS (△) and in the urine (■) and sera (◇) of patients without IA (average value and standard deviation calculated from duplicates in two different experiments).

TABLE 1. Application of the sandwich ELISA method for the detection of GM to patients with proven or suspected invasive aspergillosis

Patient no.	Sex/age (yr)[a]	Underlying disease[b]	IA[c]	Serum				Urine			
				No. of samples tested	No. positive after first positive/ total no.[d]	Days before death and GM detection	Max Galf concn (ng/ml)	No. of samples tested	No. positive after first positive/ total no.	Days before death and GM detection	Max Galf concn (ng/ml)
1	F/28	CML	PPP	15	10/10	19	5	15	8/9	12	1.2
2	M/42	CLL	PPP	5	4/5	≥7[e]	5.3	6	0	0	0
3	M/30	Lymphoma	PPP	3	2/2	≥4[e]	5.3	ND[f]	ND	ND	ND
4	M/29	ALL	PPP	4	4/4	≥39[e]	7	ND	ND	ND	ND
5	M/22	ALL	PPP	1	1/1	NA[g]	5.3	2	2/2	>2	NA
6	F/38	Aplastic anemia	PP	8	5/5	9	5.7	9	8/8	8	2.4
7	M/5	ALL	PP	1	1/1	NA	3.7	1	1/1	NA	NA
8	F/14	Aplastic anemia	P	8	7/7	10	13	9	6/7	7	2.3
9	M/49	Lymphoma	P	14	7/7	30	3.5	12	0	0	0

[a] F, female; M, male.
[b] CML, chronic myeloid leukemia; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia.
[c] PPP, IA proven at autopsy; PP, probable IA on the basis clinical symptoms including computed tomography scans of different organs and positive culture of *A. fumigatus*; P, possible IA on the basis of radiological arguments without positive cultures.
[d] A sample was considered positive when the OD value was greater than the cutoff value calculated on the basis of either the mean of negative control sera + 4 standard deviations or an early negative serum sample from the same patient spiked with 0.5 ng of GM per ml.
[e] ≥ *x*, the first sample, which was available *x* days before the death of the patient, was positive.
[f] ND, not done because samples were not available.
[g] NA, not appropriate because a limited number of samples were tested per patient.

side chains in some GM samples (9a). In contrast to a generally accepted postulate, antigenemia does not have a transient character. In every patient studied, all serum samples taken after the first positive sample was obtained were also positive, and the concentration of GM increased with the progression of the disease. The rapidity with which the disease progresses and the antigen concentration in the serum at the time of death varied with each patient. As also shown by the studies of Fujita et al. (7), a decrease in the detection limit allows for an earlier diagnosis. For instance, when it was positive, agglutination of the commercial Latex Pastorex Aspergillus coated with the same EB-A2 MAb, which occurs only at a concentration of 15 ng of GM per ml, detected the presence of the antigen only a few days before the death of the patient, at a time when antifungal therapy became useless (9). In contrast, the improvement of the detection limit by the sandwich ELISA now offers the possibility of treating patients with IA on the basis of a positive antigenemia result. A decrease in the GM concentration in response to antifungal treatment is indicative of the efficiency of the therapy (13a).

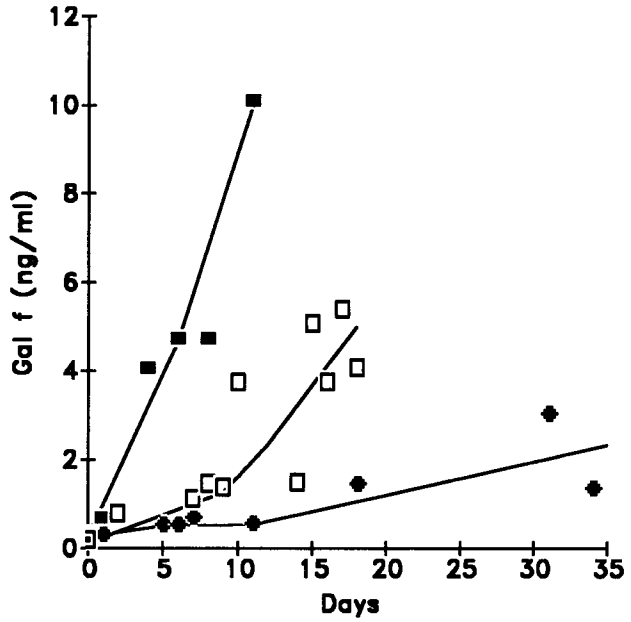

FIG. 3. Examples of monitoring of antigenemia in three patients, patients P1 (□), P8 (■), and P9 (●), with IA (average values of duplicate test). The three patients are described in Table 1.

Since the initial report of Reiss and Lehmann (11), several studies have demonstrated the presence of antigens in the serum and urine of patients with IA (5, 8, 13–15, 17, 20–22). A comparative review of all results reported has shown that several factors are critical in the detection of antigens in serum and urine (9): (i) the treatment of the serum samples to dissociate the immune complexes; (ii) the use of MAbs, which is an important asset for the development of a commercial test for the detection of antigens; and (iii) the choice of the test procedure, which appears to be the most critical factor. The range of GM concentrations detected by latex agglutination, radioimmunoassay, ELISA inhibition, and sandwich ELISA are 15, 10, 4 to 5, and 1 to 2 ng/ml, respectively. The double-direct sandwich ELISA described in this report is the most sensitive method developed until now since it can detect 0.5 to 1 ng of GM per ml serum.

The increase in the sensitivity of GM detection was associated with the occurrence of false-positive results. In a survey of seven control patients with no evidence of *Aspergillus* infections, 8% of serum samples (7 of 88 samples) and 8% of urine samples (8 of 97 samples) gave OD values above the calculated cutoff OD value, with respective calculated means ± standard deviations of 1.9 ± 2.2 and 8.1 ± 8.9, respectively. Until now, the reasons for these false-positive results remained unexplained. The presence of false-positive results is not due to successive freezing and thawing of the samples; similar values were obtained when the sample was tested twice after a cycle of freezing-thawing (verified for 15 false-positive samples as well as for 18 positive and 12 negative serum samples). It also is not due to the selection of patients with particular pathologies since a similar percentage of false-positive results was seen in a population of patients with *Candida* or *Cryptococcus* in-

fections (6% false-positive samples for 37 serum samples tested) (3a). In addition, the molecules responsible for this false positivity interacted at the epitope-binding domain of the MAb since preincubation of the serum with a rat MAb not directed against GM did not suppress the reactivity, whereas the addition of EB-A2 did. Minimization of the number of false-positive results could result from the exact identification of the antigen(s) present in the serum of patients with IA, which could then lead to the development of a more specific serum treatment. It is still unknown if these galactofuran-bearing molecules are a pure polysaccharide, glycoproteins, or a peptidophospholipogalactomannan like the one described for *Penicillium charlesii* (18).

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