



# Recombinant antigens as diagnostic markers for aspergillosis

Jacqueline Sarfati<sup>a</sup>, Michael Monod<sup>b</sup>, Paulette Recco<sup>c</sup>, Annie Sulahian<sup>d</sup>,  
Claudine Pinel<sup>e</sup>, Ermanno Candolfi<sup>f</sup>, Thierry Fontaine<sup>a</sup>, Jean-Paul Debeaupuis<sup>a</sup>,  
Marc Tabouret<sup>g</sup>, Jean-Paul Latgé<sup>a,\*</sup>

<sup>a</sup>Unité des *Aspergillus*, Institut Pasteur, 75015 Paris, France

<sup>b</sup>Service de Dermatologie et Vénéréologie, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

<sup>c</sup>Service de Parasitologie et de Mycologie, 31059 CHU Toulouse, France

<sup>d</sup>Service de Parasitologie-Mycologie, Hôpital St. Louis, 75010 Paris, France

<sup>e</sup>Parasitologie et Mycologie Médicale et Moléculaire, Faculté de Médecine, Université J. Fourier, 38041 Grenoble, France

<sup>f</sup>Institut de Parasitologie et de Pathologie tropicale, 67000 Strasbourg, France

<sup>g</sup>BioRad, 59114 Steenvoorde, France

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## Abstract

Eight recombinant proteins and purified galactomannan of *Aspergillus fumigatus* were tested by enzyme-linked immunosorbent assay to quantify the anti-*Aspergillus* antibodies in sera of patients with aspergilloma, allergic bronchopulmonary aspergillosis (ABPA), and invasive aspergillosis (IA). In spite of the variability observed in the immune responses of individual patients, quantification of the antibody titers against the 18-kDa ribonuclease (RNU), the 360-kDa catalase (CAT), and the 88-kDa dipeptidylpeptidase V (DPPV) was useful for the diagnosis of aspergilloma and ABPA. Differential diagnosis of ABPA was even possible among cystic fibrosis as well as noncystic fibrosis patients. In the group of immunocompromised patients with IA, no antibody response was mounted in response to the *Aspergillus* infection in any of the patients. Interestingly, about half of the patients with proven IA came to the hospital with high titers of anti-*Aspergillus* antibodies, suggesting that they were infected upon entry to the hospital. These results suggest that recombinant RNU, CAT, and DPPV have a great potential in the serodiagnosis of all forms of aspergillosis in the immunocompromised and immunocompetent patient.

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## 1. Introduction

*Aspergillus fumigatus* is an opportunistic pathogen that is responsible for a variety of infections in the immunocompetent as well as in the immunocompromised host. The successful management of the different forms of aspergillosis is often hampered by difficulties in establishing diagnosis in both types of patients. Diagnostic strategies are adapted to the immune status of the patient (Denning, 1998; Latgé et al., 1991; Singh and Paterson, 2005). In the immunocompromised host and in patients with invasive aspergillosis (IA), the “gold standard” for laboratory diagnosis is the search for circulating antigen.  $\beta$ -1,3-Glucans and galactomannan (GM) are the 2 unique antigens that

have been used to date for the diagnosis of IA (Maertens et al., 2002; Odabasi et al., 2004; Sulahian et al., 2001). In the immunocompetent host, the diagnosis of aspergillosis is based on the presence of specific anti-*Aspergillus* antibodies. It is generally agreed that a rise in the level of circulating antigen or specific antibodies would indicate mycelial extension and/or progression of the *Aspergillus* infection (Odabasi et al., 2004; Mennink-Kersten et al., 2004; Pazos et al., 2005; Styne et al., 1995).

For several reasons, a renewed interest in the development of quantitative and reproducible assays for measuring the amount of anti-*Aspergillus* antibodies has occurred because of the following reasons. i) The number of patients with preexisting lung cavities, a site of invasion by the aspergilli, has increased because of other disease processes such as tuberculosis, lung carcinoma, emphysema, and chronic obstructive bronchopneumopathy; these patients

\* Corresponding author. Tel.: +33-140613518.

E-mail address: [jplatge@pasteur.fr](mailto:jplatge@pasteur.fr) (J.P. Latgé).

are at risk for aspergilloma, and a serologic survey remains clinically relevant for these patients that contract serious but clinically silent aspergilloma (Denning, 1998; Chu et al., 2004; Meersseman et al., 2004). ii) Atopy to mold allergens worsens asthma severity; a recent study in the UK indicates that mold sensitization is associated with severe asthma attacks requiring hospital admission (Nelson et al., 1999; O'Driscoll and Hopkinson, 1998; Zureik et al., 2002). Because the value of quantification of the skin prick tests or the presence of IgE-binding allergens shared by several fungi in the crude fungal extracts used in these tests has been questioned, it is now relevant to quantify the sensitization of asthmatic patients to *Aspergillus* allergens by the measurement of anti-*Aspergillus* antibody titers. iii) Allergic bronchopulmonary aspergillosis (ABPA) is the most extreme manifestation of mould allergy occurring most often in the older cystic fibrosis patient population; because the age of this population at risk is continuously increasing and because ABPA remains a difficult complication to diagnose, a precise serologic diagnosis would be most helpful for this pathology (Stevens et al., 2003). iv) Finally, recent data suggest that antibody quantification could also be useful in patient populations at risk for IA. The peak of occurrence of IA in the immunocompromised population now apparently occurs at about the 6th month postgraft at a time when immune memory is reinitiated (Morgan et al., 2005). In addition, recent clinical surveys show an increase in the incidence of IA in patients without hematologic malignancy in intensive care units (Meersseman et al., 2004). Invasive aspergillosis in these relatively immunocompetent patients could be associated with an increase in antibody titers against *Aspergillus*. It has been also suggested (but never studied) that patients could have been grafted while being colonized by *Aspergillus*. A high titer of antibody found before immunosuppression in patients to be grafted would suggest a preexisting *Aspergillus* infection and could be an indication of mycological investigation and/or antifungal preventive treatment.

Antibody detection in patients with aspergillosis is still performed using crude antigens in semiquantitative methods such as immunoelectrophoresis (IEP), counterimmunoelectrophoresis, or hemagglutination (Latgé et al., 1991). Enzyme-linked immunosorbent assay (ELISA) methods using in-house-produced crude antigenic batches have been also introduced, but the lack of standardization between different laboratories makes comparisons of efficiency in aspergillosis diagnosis difficult to assess because of batch to batch variability. Earlier studies in particular by the group of Cramer have shown that reproducible quantification of anti-*Aspergillus* antibodies with immunochemical tests can be obtained by using recombinant antigens (Cramer, 2002; Cramer and Kodzius, 2001; Hemmann et al., 1999; Kodzius et al., 2003; Kurup et al., 2000). In the last 10 years, we characterized in our laboratory several antigens of *A. fumigatus*, among which are the classic “chymotrypsin and catalase (CAT) antigens” (Beauvais et al., 1997a; Calera

et al., 1997). These studies have shown that the production of recombinant antigens of *A. fumigatus* in *Pichia pastoris* is extremely efficient. High amounts of recombinant proteins (5–50 µg/mL) are produced extracellularly and easily purified on a Ni column after a 6× His tagging of the protein that does not interfere with the antigenic capacity of these proteins. In the present study, we investigated the antibody response of several populations of patients with aspergillosis to 8 different recombinant protein antigens and to GM and showed that 3 recombinant antigens, the 18-kDa ribonuclease (RNU), the 360-kDa CAT, and the 88-kDa dipeptidylpeptidase V (DPPV), were the most helpful in the diagnosis of aspergillosis.

## 2. Material and methods

### 2.1. Strains and plasmids

A clinical strain of *A. fumigatus* (CBS 144.89) was used in this study. *P. pastoris* GS115 and KM71 and the expression vectors pKJ111 (Monod et al., 1999), pKJ113 (Borg-von Zepelin et al., 1998), pHLS1, and pPICZαA (Invitrogen, Carlsbad, CA) were used to express recombinant antigens. All plasmid cloning experiments were performed in *Escherichia coli* XL1 blue. *E. coli* LE392 was used for the propagation of the bacteriophage λgt11 (Promega, Madison, WI).

### 2.2. Production of recombinant polypeptides

*A. fumigatus* complementary DNAs (cDNAs) encoding the antigens described below were obtained by polymerase chain reaction (PCR) using DNA prepared from 10<sup>6</sup> clones of a λgt11 cDNA library previously constructed (Monod et al., 1993). Primers were derived from genomic DNA sequences of the genes coding for the alkaline protease (ALP) (Monod et al., 1993), metalloprotease (MEP) (Jaton-Ogay et al., 1994), aspartic protease (PEP) (Reichard et al., 1995), RNU (Paris et al., 1993), superoxide dismutase (SOD) (Holdom et al., 2000), CAT (Calera et al., 1997), dipeptidylpeptide IV (DPPIV) (Beauvais et al., 1997b), and DPPV (Beauvais et al., 1997a) (Table 1). Two hundred nanograms of target DNA, 10 µL of each sense and antisense oligonucleotides at a concentration of 42 mmol/L, and 8 L of deoxynucleotide mix (containing 10 mmol/L of each deoxynucleotide triphosphate [dNTP]) were dissolved in 100 µL of PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, and 1.5 mmol/L MgCl<sub>2</sub>). To each reaction, 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer, Boston, MA) was added. The reaction mixtures were incubated 5 min at 94 °C, subjected to 25 cycles of 0.5 min at 94 °C, 0.5 min at 55 °C, and 0.5 min at 72 °C, and finally incubated 10 min at 72 °C.

Expression plasmids were constructed by cloning cDNA PCR products in *P. pastoris* expression vectors. The PCR products were purified using a PCR purification kit (Roche Diagnostics, Mannheim, Germany) and digested by restric-

Table 1

Materials used for the production of *A. fumigatus* antigens in *P. pastoris*

| Gene  | GenBank accession no | Amplification primers <sup>a</sup>    | Orientation | Enclosed amino acid sequences <sup>b</sup> | PCR product with cloning sites <sup>c</sup> | Cloning vector with cloning and (linearization) sites |
|-------|----------------------|---------------------------------------|-------------|--------------------------------------------|---------------------------------------------|-------------------------------------------------------|
| PEP   | X85092               | GTTC/TCGAGTCCCT                       | 5'–3'       | (R)VPVVQPR                                 | PEP(61–1185)                                | pKJ113                                                |
|       |                      | GTGGTCCAGCCGCGC                       |             |                                            |                                             |                                                       |
|       |                      | TCCG/GATCCCTA(GTGATG)                 | 3'–5'       | LGFAPQAH <sub>6</sub> *                    | <i>XhoI</i> – <i>Bam</i> HI                 | <i>XhoI</i> – <i>Bam</i> HI ( <i>Sma</i> I)           |
| ALP   | Z11580               | <sub>3</sub> TGCCTGAGGGGCGAAGCCGAG    |             |                                            |                                             |                                                       |
|       |                      | CTGC/TCGAGCGCC                        | 5'–3'       | (R)APVQETR                                 | ALP(61–1209)                                | pKJ113                                                |
|       |                      | TGTCCAGGAACTCGT                       |             |                                            |                                             |                                                       |
| MEP   | Z30424               | AGCG/GATCCCTA(GTGATG)                 | 3'–5'       | LAYNGNAH <sub>6</sub> *                    | <i>XhoI</i> – <i>Bam</i> HI                 | <i>XhoI</i> – <i>Bam</i> HI ( <i>Eco</i> RI)          |
|       |                      | <sub>3</sub> AGCATTGCCATTGTAGGCAAG    |             |                                            |                                             |                                                       |
|       |                      | CTGTTT/AAACATCCCG                     | 5'–3'       | (K)HPAHQSY                                 | MEP(55–1899)                                | pKJ111                                                |
| DPPIV | U87950               | CTCACCACTTTAC                         |             |                                            |                                             |                                                       |
|       |                      | GTCTTT/AAACTA(GTGATG)                 | 3'–5'       | EVPSGVCH <sub>6</sub> *                    | <i>Dra</i> I– <i>Dra</i> I                  | <i>Sma</i> I ( <i>Bgl</i> II)                         |
|       |                      | <sub>3</sub> ACAGACACCACTGGGGACCTC    |             |                                            |                                             |                                                       |
| DPPV  | L48074               | TGCGAT/ATCGCCATTGA                    | 5'–3'       | (IA)IDVPRQP                                | DPPIV(43–2295)                              | pKJ111 <i>Sma</i> I ( <i>Bgl</i> II)                  |
|       |                      | CGTCCCTCGTCAACCA                      |             |                                            |                                             |                                                       |
|       |                      | TGCGAT/ATCCTA(GTGATG)                 | 3'–5'       | WSKKSVLH <sub>6</sub> *                    | <i>Eco</i> RV– <i>Eco</i> RV                |                                                       |
| DPPV  | L48074               | <sub>3</sub> CAGAACAGACTTCTTGCTCCA    |             |                                            |                                             |                                                       |
|       |                      | GCG/AAATCCTTACA                       | 5'–3'       | (EF)LTPEQLI                                | DPPV(55–2163)                               | pHILS1                                                |
|       |                      | CCTGAGCAGCTAATC                       |             |                                            |                                             |                                                       |
| RNU   | M55508               | GCA/GATCTA(GTGATG)                    | 3'–5'       | VPVVNYNH <sub>6</sub> *                    | <i>Eco</i> RI– <i>Bgl</i> II                | <i>Eco</i> RI– <i>Bam</i> HI ( <i>Bgl</i> II)         |
|       |                      | <sub>3</sub> GTTATAATTACAAACGGGAC     |             |                                            |                                             |                                                       |
|       |                      | TAGC/TCGAGCCT                         | 5'–3'       | (RA)SPLDA                                  | RNU(64–528)                                 | pKJ113                                                |
| CAT   | U97574               | CGCCCTCGACGCT                         |             |                                            |                                             |                                                       |
|       |                      | ACCG/GATCCCTA(GTGATG)                 | 3'–5'       | DLRLCSH <sub>6</sub> *                     | <i>XhoI</i> – <i>Bam</i> HI                 | <i>XhoI</i> – <i>Bam</i> HI ( <i>Eco</i> RI)          |
|       |                      | <sub>3</sub> AGAACACAGTCTCAAGTC       |             |                                            |                                             |                                                       |
| SOD   | AF128886             | AATGC/TCGAGTATGTCCC                   | 5'–3'       | (R)VCPYMTG                                 | CAT(46–2010)                                | pKJ111                                                |
|       |                      | TATATGACCGGC                          |             |                                            |                                             |                                                       |
|       |                      | GGTA/GATCTCTA(GTG)                    | 3'–5'       | DRFPVDH <sub>6</sub> *                     | <i>XhoI</i> – <i>Bgl</i> II                 | <i>XhoI</i> – <i>Bam</i> HI ( <i>Bgl</i> II)          |
| SOD   | AF128886             | <sub>6</sub> ATCCACGGGAAACCGGTC       |             |                                            |                                             |                                                       |
|       |                      | CAAC/TCGAGAAAAGA                      | 5'–3'       | VKAVAVL                                    | SOD(4–1386)                                 | pPICZαA <i>XhoI</i> – <i>Not</i> I ( <i>Dra</i> I)    |
|       |                      | GTCAAGGCTGTTGCTGTCTC                  |             |                                            |                                             |                                                       |
|       |                      | TAAGC/GGCCGCATTA(GTGATG) <sub>3</sub> | 3'–5'       | VIGIAAH <sub>6</sub> *                     | <i>XhoI</i> – <i>Not</i> I                  |                                                       |
|       |                      | AGCGGCGATACCAATGAC                    |             |                                            |                                             |                                                       |

<sup>a</sup> Slashes indicate restriction endonuclease cleavage sites.<sup>b</sup> Amino acids (in parentheses) encoded by the recognition site sequence and added to the N-terminal extremity of *A. fumigatus* antigens; asterisks indicate stops.<sup>c</sup> The numbers in parentheses represent the nucleotide position of amplification products on *A. fumigatus* antigen cDNAs.

tion enzymes for which a site was previously designed at the 5' end of the primers (Table 1). *P. pastoris* transformation, selection of transformants, and production of recombinant enzymes in methanol medium were performed as previously described (Borg-von Zepelin et al., 1998; Beggah et al., 2000). All 6× His-tagged proteins bound to a Probond column (Invitrogen) with the exception of the DPPIV. After washing the column with a 20-mmol/L phosphate buffer, pH 6, containing 0.5 mol/L NaCl, proteins were eluted from the Ni<sup>2+</sup> column with 50 mmol/L histidine. Elution of RNU required the use of 500 mmol/L imidazole buffer pH, and the protein was dialyzed against a 50-mmol/L histidine solution. The solubilization of the different antigens in the 50-mmol/L histidine solution allowed a direct coating of the antigens on the microtiter plate, whereas imidazole interfered with coating and subsequent colorimetric reactions (data not shown).

In contrast to the other antigens, the DPPIV did not bind to the Ni<sup>2+</sup> column. Western blot analysis of the DPPIV with an antipolyhistidine clone His1 monoclonal antibody

(Sigma, St. Louis, MO) diluted 1:1000 was negative, whereas a DPPV control was positive (data not shown). This result showed that the lack of binding to the Ni column was not due to the lack of accessibility of the 6× His tag to the Ni because of a specific 3-dimensional structure of the protein but to the absence of the sequence coding for the 6× His tag in the DPPIV recombinant protein. Purification of the DPPIV protein was done as described earlier (Beauvais et al., 1997b), with some modifications. To purify the DPPIV, we precipitated the culture filtrate of *Pichia* with 4 volumes of EtOH, and the precipitate solubilized in water was dialysed against 20 mmol/L Tris–HCl buffer, pH 8.0. Proteins were loaded on a MonoQ HR 5/5 column and the protein eluted with a 0- to 500-mmol/L NaCl. Fractions containing the dipeptidylpeptidase activity measured as described previously (Beauvais et al., 1997b) were pooled and concentrated under vacuum. Final purification was performed using a Superdex 200 HR 10/30 gel permeation column using a 20-mmol/L Tris–HCl buffer, pH 8.0, containing 0.12 mol/L NaCl. We verified that the protein

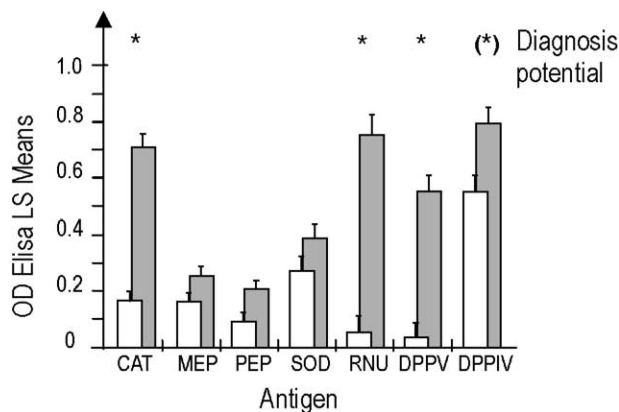


Fig. 1. Antibody response directed against 7 recombinant antigens in sera of aspergilloma and control patients. Values shown are the means  $\pm$  SEs of OD ELISA from 57 patients (in gray) and 41 controls (in white) from 3 hospitals (1 serum per patient). OD values obtained with antigens followed by an asterisk in the “diagnosis potential” line are significantly different in a 3-way analysis of variance with center, antigen, and pathology as factors. (DPPIV is only significant in one of the hospitals).

purified had the dipeptidylpeptidase activity described earlier (Beauvais et al., 1997b). The purity of the different batches of antigens was determined by overloading a sodium dodecyl sulfate polyacrylamide gel electrophoresis.

### 2.3. Purification of GM

*A. fumigatus* was grown for 40 h in a fermentor as described previously (Hearn et al., 1990). Because hydrazine is now unavailable, another protocol to purify GM was used. The culture filtrate was ethanol precipitated, and the precipitate was first digested for 3 days at 37 °C with amylase (from *Bacillus licheniformis*, Ref Sigma A4551) at 1:30 ratio (dry weight) in 100 mmol/L sodium acetate buffer, pH 5.6, and then incubated for 3 days at 37 °C with

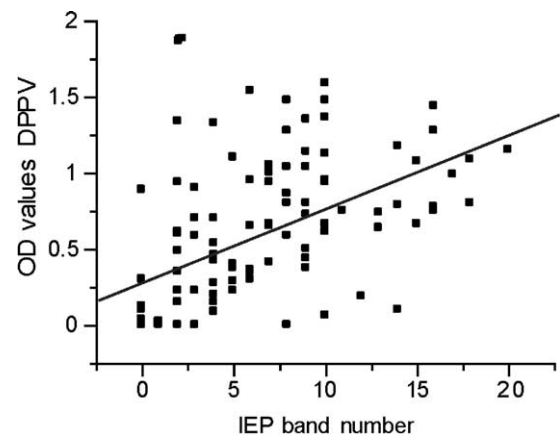


Fig. 2. Correlation between the number of IEP bands and the anti-DPPV antibody levels (expressed in OD value). The linear fit ( $OD\ DPPV = 0.3 + 0.05\ IEP\ band\ numbers$ ) is statistically significant ( $P < 0.001$  for an error  $df$  of 101). Statistically significant  $r$  values were obtained also between the number of precipitins and the OD values obtained with RNU and CAT antigens (data not shown).

pronase (from *Streptomyces griseus*, Ref Sigma P5147) at 1:6 ratio (dry weight). The reaction mixture was boiled 10 min at 100 °C and further treated for 1 h at 60 °C in presence of NaOH (1 mol/L final concentration). After neutralization, the GM was dialysed and freeze dried. The purity of GM was verified by gas chromatography as previously described (Fontaine et al., 2000; Latgé et al., 1994).

### 2.4. Patients

A total of 57 aspergilloma patients and 41 control healthy individuals were recruited from 3 hospitals (Toulouse, Grenoble, and Strasbourg, France). In addition, 2 to 9 (average, 3.9) samples were taken sequentially per patient in the Toulouse hospital. In hospitals Toulouse and Grenoble, 16 ABPA patients and 51 controls in the cystic fibrosis population were analyzed. Twelve ABPAs in a noncystic fibrosis population were compared with 27 controls. In Toulouse, sequential samples (3 to 16, average of 8.6) per patient were available for 14 ABPA patients (in a cystic fibrosis or noncystic fibrosis background). All IA patients (Ascioglu et al., 2002) were from the hospital St. Louis, Paris: 23 with proven or probable IA, 21 with possible IA, and 34 controls that were immunosuppressed and/or grafted as the IA patients. For each series of patients, 1 to 15 samples (average, 7.5), 2 to 11 samples (average, 6), and 1 to 12 samples (average, 3.9) were available per patient with proven/probable IA, possible IA, and control patients, respectively. One to 7 sera per patient were also available before the transplant or the start of immunosuppressive therapy.

### 2.5. Immunoelectrophoresis

Fast IEP was performed using the Beckman-IEP Paragon system (Beckman Instruments, Fullerton, CA) according to the manufacturer's instructions. Briefly, migration of the

Table 2

Efficiency of the RNU, CAT, and DPPV antigens in the diagnosis of aspergilloma by ELISA

|                                     | RNU | DPPV | CAT | CAT +<br>RNU | CAT +<br>DPPV | RNU +<br>DPPV | RNU +<br>DPPV +<br>CAT |
|-------------------------------------|-----|------|-----|--------------|---------------|---------------|------------------------|
| True negative                       | 40  | 40   | 40  | 39           | 39            | 39            | 38                     |
| False positive                      | 1   | 1    | 1   | 2            | 2             | 2             | 3                      |
| True positive                       | 46  | 45   | 44  | 53           | 53            | 52            | 54                     |
| False negative                      | 11  | 12   | 13  | 4            | 4             | 5             | 3                      |
| Sensitivity (%)                     | 81  | 79   | 77  | 93           | 93            | 91            | 95                     |
| Specificity (%)                     | 98  | 98   | 98  | 95           | 95            | 95            | 93                     |
| Negative<br>predictive<br>value (%) | 78  | 77   | 75  | 91           | 91            | 89            | 93                     |
| Positive<br>predictive<br>value (%) | 98  | 98   | 98  | 96           | 96            | 96            | 95                     |

The number of patients is presented in the upper part of the table; sensitivity, specificity, and positive and negative predictive values are estimated in percentage calculated from the patients' response. Data from 3 hospitals were pooled; 1 serum per patient randomly selected when several sera were available per patient.

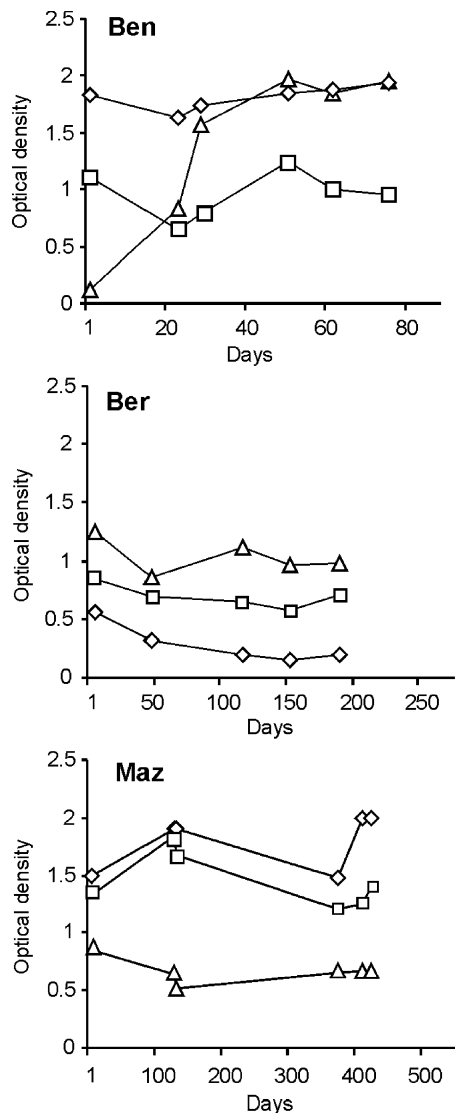


Fig. 3. Follow-up of antibody levels directed against CAT, RNU, and DPPV in the sera of 3 aspergilloma patients (Ben, Ber, and Maz). Antirecombinant antigen antibody levels are expressed in OD values (CAT = □; DPPV = ◇; RNU = Δ).

somatic and metabolic *Aspergillus* antigens (Sanofi Pasteur Diagnosis, Marnes la Coquette, France) was performed at 100 V for 12 min. Incubation with the patient serum lasted for 18–24 h. After gels were washed with sodium citrate, the presence of a precipitin with CAT activity was visualized with 3% H<sub>2</sub>O<sub>2</sub>, and the total number of precipitins was counted after Coomassie blue or Paragon violet acid staining of the dried gel.

## 2.6. Antigen coating and ELISA

All protein antigens and GM were coated on ELISA plates (Greiner Ref 762070) at a concentration of 1 µg/mL of 50 mmol/L histidine solution for all antigens except CAT, which was coated at 4 µg/mL. After coating, the wells were emptied and filled with 300 µL of phosphate-buffered saline (PBS) supplemented with 2% Tween 20. After a 1-h

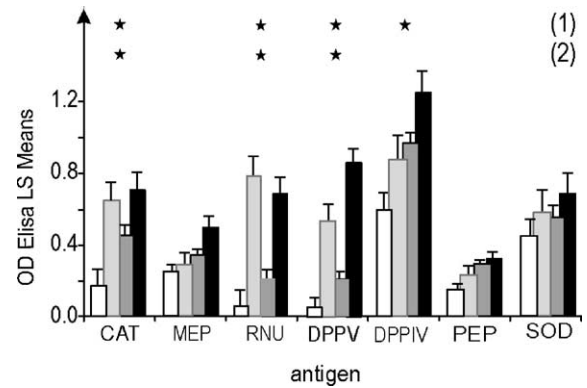


Fig. 4. Antibody response against 7 recombinant antigens by ABPA patients and their respective atopic or cystic fibrosis control cohorts. OD values shown are means ± SEs of 12 ABPA (in dark gray) and 16 ABPA with cystic fibrosis (in light gray) and 51 control cystic fibrosis (in white) (1 serum per patient, 2 hospitals). OD values obtained with antigens followed by an asterisk in the “diagnostic potential” lines are significantly different in a 3-way analysis of variance with center, antigen, and pathology as factors 1) population of patients with cystic fibrosis and 2) population of patients without cystic fibrosis. Note that DPPIV is only significantly discriminant for ABPA in the cystic fibrosis population.

incubation at room temperature (RT), the PBS–Tween solution was replaced with 300 µL of PBS-containing 5% glucose and 2.5% defatted milk, and the plates were incubated for 2 min at RT. All wells were emptied and dried in an oven at 60 °C for 10 min. After drying, plates were stored frozen. Protocols used to coat antigens were based on preliminary experiments that indicated which antigen concentration ensured optimal optical density (OD) readings

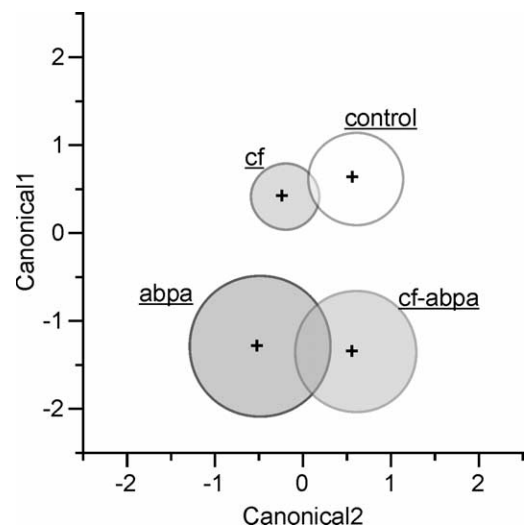


Fig. 5. Canonic plot showing the separation of the ABPA populations with their respective controls. For the discriminatory analysis, the OD values obtained with all recombinant antigens have been used. The canonic plot shows the multivariate means in the 2 dimensions (canonic 1 and 2) that best separate the groups of patients. These multivariate means are surrounded by a 95% confidence ellipse that appears circular in the canonic space. Groups that are significantly different (ABPA versus control) tend to have non-intersecting circles.

with a pool of sera from aspergilloma patients known to contain high amounts of anti-*Aspergillus* antibodies. Patient sera were diluted 1:500 in PBS-containing 0.05% Tween 20 and 1% bovine serum albumin. The sera from Toulouse and St. Louis were analyzed in our laboratory. The sera from Grenoble and Strasbourg were tested on site according to the protocol followed at Pasteur, which included the following steps: incubation for 1 h at 37 °C with the patient serum; 5 washings with PBS–Tween 20 0.05%; incubation with a secondary anti-IgG (H + L) antibody conjugated to peroxidase (Sigma); and 5 washings and incubation with *O*-phenylene diamine (OPD) for OD readings estimating the level of specific antibodies (Ab) directed against *Aspergillus*. Experiments were done in duplicate and repeated at least once.

To estimate sensitivity, specificity, and negative and positive predictive values, the cutoff OD values per antigen per center and per pathology selected gave the best positive likelihood ratio [LR+ = sensitivity/(1 – specificity)]. LR+ were calculated from receiver operating characteristic curves using the Medcalc software (<http://www.medcalc.be>). Based on the highest value of the sera samples when several samples were available per patient, 4 group of patients were identified: true negatives (A, no pathology, Ab negative), false positives (B, no pathology, Ab positive), true positives (C, pathology, Ab positive), and false negatives (D, pathology, Ab negative). Sensitivity was  $C/C + D \times 100$ ; specificity was  $A/A + B \times 100$ ; negative predictive value was  $A/A + D \times 100$ ; and positive predictive value was  $C/B + C \times 100$ .

### 2.7. Statistical analysis

Statistical analysis of the data was performed using the JMP software (SAS, Cary, NC). Mean values were calculated and the SE computed. Variance analysis was

Table 3

Results of the antibody determinations against the 3 antigens, RNU, CAT, and DPPV, indicated either individually or in combination in ABPA and control patients with cystic fibrosis

|                                     | RNU | DPPV | CAT | CAT +<br>RNU | CAT +<br>DPPV | RNU +<br>DPPV | RNU +<br>DPPV +<br>CAT |
|-------------------------------------|-----|------|-----|--------------|---------------|---------------|------------------------|
| True negative                       | 50  | 48   | 50  | 49           | 48            | 47            | 47                     |
| False positive                      | 1   | 3    | 1   | 2            | 3             | 4             | 4                      |
| True positive                       | 8   | 12   | 6   | 9            | 12            | 14            | 14                     |
| False negative                      | 8   | 4    | 10  | 7            | 4             | 2             | 2                      |
| Sensitivity (%)                     | 50  | 75   | 38  | 56           | 75            | 88            | 88                     |
| Specificity (%)                     | 98  | 94   | 98  | 96           | 94            | 92            | 92                     |
| Negative<br>predictive<br>value (%) | 86  | 92   | 83  | 88           | 92            | 96            | 96                     |
| Positive<br>predictive<br>value (%) | 89  | 80   | 86  | 82           | 80            | 78            | 78                     |

The number of patients is presented in the upper part of the table; sensitivity, specificity, and positive and negative predictive values are estimated in percentage calculated from the patients' response. Data from 2 hospitals were pooled; 1 serum per patient randomly selected when several sera were available per patient.

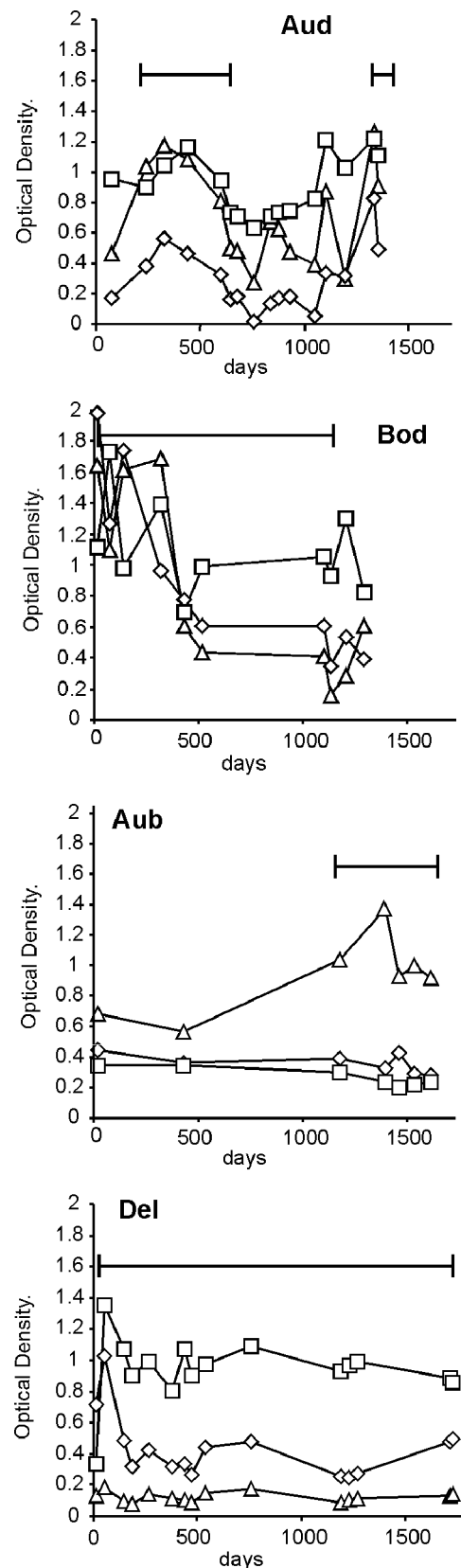


Fig. 6. Follow-up of antibody levels directed against CAT, RNU, and DPPV in the sera of 4 ABPA patients (Aud, Bod, Aub, and Del). Antirecombinant antigen antibody levels are expressed in OD values. Bars indicate the ABPA period as defined by the clinician. (CAT = □; DPPV = ◇; RNU = △).

Table 4  
Efficiency of the RNU, CAT, DPPV, and GM in the diagnosis of IA patients

|                               | RNU | DPPV | CAT | GM  |
|-------------------------------|-----|------|-----|-----|
| True negative                 | 35  | 34   | 26  | 35  |
| False positive                | 0   | 1    | 9   | 0   |
| True positive                 | 3   | 7    | 18  | 4   |
| False negative                | 20  | 16   | 5   | 19  |
| Sensitivity (%)               | 13  | 30   | 78  | 17  |
| Specificity (%)               | 100 | 97   | 74  | 100 |
| Negative predictive value (%) | 64  | 68   | 84  | 65  |
| Positive predictive value (%) | 100 | 88   | 67  | 100 |

Calculations were made based on the antibody response of the patients upon entry to the hospital and before immunosuppressive therapy. The number of patients is presented in the upper part of the table; sensitivity, specificity, and positive and negative predictive values are estimated in percentage calculated from the patients' response.

used followed by ranking of the means using the least-square means differences analyzed by the Student's *t* test. Bivariate analysis to analyze the distribution of 1 continuous variable to another one was done through linear fitting using the Pearson correlation *r* value for significance. Discriminant analysis classified OD values for antigens into groups of patients predefined on the basis of their pathology. Analysis was presented as a canonic plot showing the multivariate least-square means in 2 dimensions that best separate the groups.

### 3. Results

#### 3.1. Aspergilloma

Fig. 1 shows that among the protein antigens, the DPPV, CAT, and RNU antigens produced the best discrimination between patients and controls. In spite of high levels of antibodies in the control sera, DPPV was also useful to identify aspergilloma patients. In contrast, the proteases (aspartic protease, metalloprotease, and serine proteases) and the SOD did not induce a specific humoral response in aspergilloma patients (data not shown for serine protease). A 3-way variance analysis showed a significant response ( $P = .03$ ) for the interaction factor [hospital  $\times$  antigen  $\times$  pathology]. This result was due to the response toward DPPV that was discriminative with patients from Toulouse but was not in other hospitals (data not shown). In contrast, the discriminative efficiency of the RNU, dipeptidylpeptidase, and CAT was good in 3 hospitals. Anti-RNU, anti-CAT, and anti-DPPV antibody levels were significantly higher in the patient populations than in the controls at all hospitals tested. Table 2 shows that the sensitivity of the test increased with the number of antigens included in the test; a maximal value of 95% was reached when the OD values obtained with the 3 antigens were computed. In contrast, the specificity values slightly decreased when multiple antigens were used. The predictive positive value for the test did not increase with the increase in the number of antigens considered, whereas the negative predictive value significantly

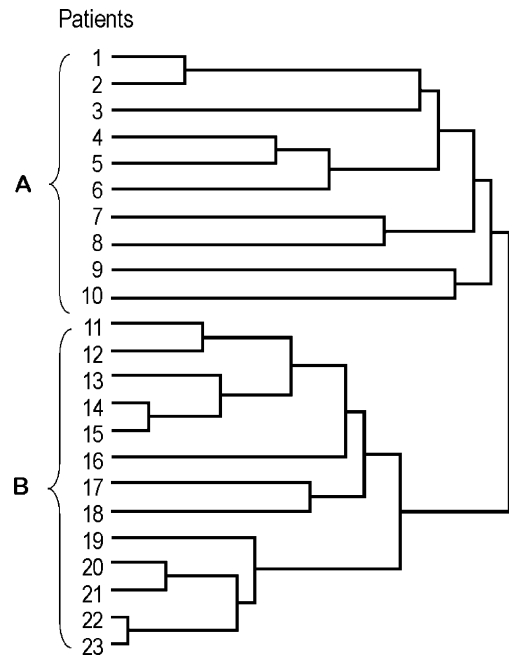


Fig. 7. Hierarchical clustering (Ward method) of 23 patients with proven IA. Clustering is based on the OD values obtained by ELISA with CAT and DPPV antigens in sera taken at the entrance of patients at the hospital; A, patients with high antibody titers; B, patients with low antibody titers. Distance of clustering is 80% for the 2 clusters identified in the dendrogram.

increased from 75% to 78% to 93% if single or triple antigens data were compared, respectively.

A detailed analysis of the patients of the Toulouse hospital was undertaken because we had multiple sequential samples per patient. Under the conditions tested, average OD values of  $0.82 \pm 0.05$ ,  $0.95 \pm 0.08$ , and  $0.71 \pm 0.04$  were obtained, respectively, with RNU, DPPV, and CAT in the sera of the aspergilloma patients, whereas these values

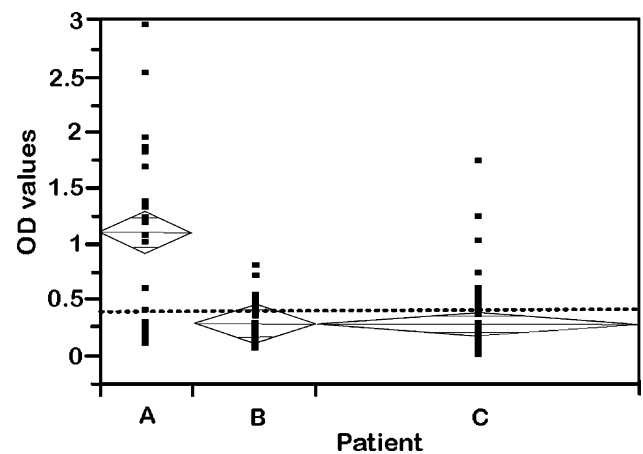


Fig. 8. One-way variance analysis showing that the subpopulation (A) of IA patients with high titer of antibodies before graft was different from the subpopulation (B) of IA patient and control (C) population with low antibody titers (see Fig. 7 for the clustering of the patients). Diamond indicates the sample mean (based on OD values stacked from the response toward DPPV and CAT) and 95% confidence interval.

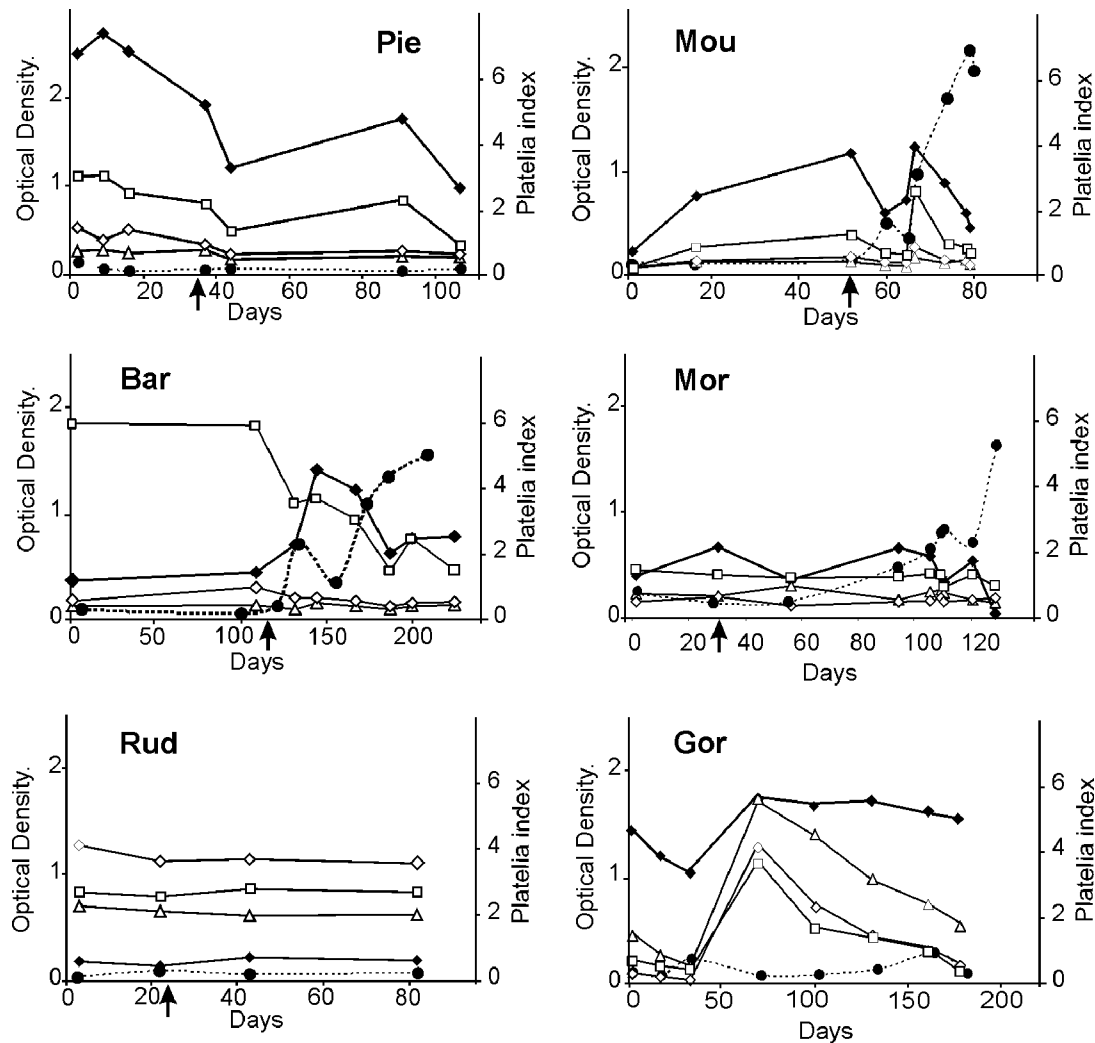


Fig. 9. Evolution of antibodies titers against RNU, CAT, DPPV, GM, and GM antigen in 6 patients with proven IA. Day 0 is the first sample taken at the entrance of the patient to the hospital. Graft (when performed) is indicated by an arrow. Patients PIE, BAR, and RUD belong to the A cluster (see Fig. 7) characterized by high antibody levels directed against DPPV and CAT. Patients MOU, MOR, and GOR belong to the cluster B of patients with low levels of antigens against CAT and DPPV. CAT =  $\square$ ; DPPV =  $\diamond$ ; RNU =  $\triangle$ ; GM =  $\blacklozenge$ ; GM Ag =  $\bullet$ .

were  $0.26 \pm 0.07$ ,  $0.25 \pm 0.06$ , and  $0.47 \pm 0.09$  in the sera of the control patients (average values on all sera analyzed). This result indicated that diagnosis was possible whenever the serum was taken. Moreover, bivariate analysis showed a statistically significant correlation between the numbers of IEP bands and the OD values obtained with the 3 recombinant antigens. The best correlation with the number of precipitin bands was obtained with the DPPV (Fig. 2).

The analysis of 13 patients from Toulouse hospital illustrated the variations seen among patients in the antibody titers against the same 4 antigens (Fig. 3 as an example of 4 patients). Although overall, there was a significant difference between the aspergilloma and control patients (Fig. 1), the antibody response depended both on the patient and on the antigen. First, the levels of antibody directed against individual antigens varied with the patient. For example, for patient Maz, the greatest response was directed against CAT

and DPPV, whereas the lowest response was directed against these 2 antigens in patient Ber. Second, the level of antibody also varied over time depending on the antigen and on the patient. For example, in patient Ben, the levels of antibody against antigen RNU increased, whereas in the same patient, the level of antibody against the other antigens tested remained constant over time (Fig. 3).

### 3.2. Allergic bronchopulmonary aspergillosis

Allergic bronchopulmonary aspergillosis is a major complication of both cystic fibrosis patients as well as noncystic fibrosis patients. Cystic fibrosis patients are often colonized by *Aspergillus*, and this colonization may be associated with high levels of anti-*Aspergillus* antibodies, which makes difficult the differentiation between patients who were colonized from true ABPA patients in this cystic fibrosis population. For this reason, we analyzed 4 popula-

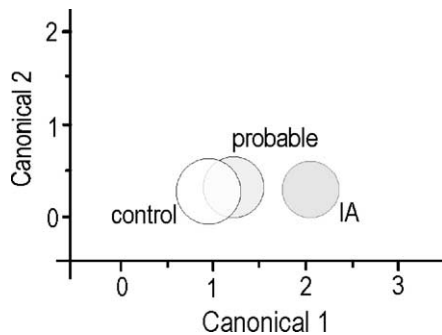


Fig. 10. Canonic plot showing the 3 populations of patients (proven IA, probable IA or negative) discriminated by antibody levels at their entrance in the hospital. For the discriminatory analysis, the OD values obtained with DPPV, GM, RNU, and CAT antigens have been used. Canonic plot shows the multivariate means in the 2 dimensions (canonic 1 and 2) that best separate the groups of patients. The size of the circle corresponds to a 95% confidence limit for the mean. Groups that are significantly different tend to have nonintersecting circles.

tions of patients: control patients without cystic fibrosis, control patients with cystic fibrosis, ABPA patients without cystic fibrosis, and ABPA patients with cystic fibrosis.

Fig. 4 shows that our ELISA was able to diagnose ABPA in populations of patients with or without cystic fibrosis. As with the aspergilloma patients, the 3 most discriminative antigens were RNU, CAT, and DPPV in all hospitals. Dipeptidylpeptide IV was discriminative only in the cystic fibrosis population (Fig. 4). Pooling all antibody data together in a canonic plot showed the stringent separation of the ABPA and control populations whatever was the underlying condition of the patient (Fig. 5).

This study confirmed that cystic fibrosis patients had high levels of anti-*Aspergillus* antibodies against all antigens and especially against the DPPV. This result was in agreement with the fact that all cystic fibrosis patients are colonized with *A. fumigatus*, although the antibody titers remained lower than those found in the ABPA patients. These data suggest that the monitoring of antibody titers of the cystic fibrosis patients during follow-up visits could be used to identify progression of the colonization and/or the development of ABPA. In Table 3, the specificity, the sensitivity, as well as the negative and positive predictive values for ABPA for the cystic fibrosis population are described. When each of the 3 antigens was considered, the lowest sensitivity value (38%) was obtained with CAT, whereas the highest diagnostic values were obtained with a combination of RNU and DPPV. Accordingly, these 2 antigens were considered the most valuable antigens for the diagnosis of ABPA. Similar data were found when the ABPA population was analyzed in a noncystic fibrosis background (data not shown).

As for the aspergilloma patients, significant variation in antibody titers against an antigen was seen among patients and over time in the same patient (Fig. 6). For example, patient Aud had a low anti-DPPV titer and a high anti-RNU titer, whereas patient Del had a low anti-RNU titer during the

ABPA period; in contrast, patient Aub had only high levels of antibody titers against RNU. Levels of antibodies could be variable or during the ABPA period, but an increase in antibody titers seemed to be more often associated with ABPA episodes, whereas a decrease in the antibody titer was correlated with an improvement of the patient (Fig. 6).

### 3.3. Invasive aspergillosis

Because the most discriminant antigens that indicated disease in the immunocompetent or atopic population were RNU, CAT, and DPPV, these 3 antigens were tested to investigate antibody responses in IA. In addition, antibodies directed against GM were analyzed because they have been suggested to be responsible for false negatives obtained with the Platelia kit used for the diagnosis of IA that is based on the detection of GM antigenemia (Hearn et al., 1995; Herbrecht et al., 2002). The antibody levels against these 4 antigens were measured in a cohort of immunocompromised patients with proven and probable IA and controls that were submitted to the same clinical treatment but did not have a fungal infection.

Comparison of the serum titers in the same patients after graft or immunosuppression and after clinical IA diagnosis showed a lack of increase in antibody levels during IA in these patients ( $P$  value of .83 for an error  $df$  of 118). This result confirms previous data that immunocompromised patients with IA did not produce detectable antibodies directed against the fungus during the development of the disease. In contrast to the antibody levels toward CAT, RNU, DPPV, or GM, the level of circulating GM in the same sera was predictive of IA with an increase in the amount of antigen released during IA progression over time ( $P$  value  $<.01$  for an error  $df$  of 118).

Although the level of antibody did not change during the disease, the analysis of the antibody response in the population of patients with proven IA showed that a high level of antibodies against the 4 antigens was positively correlated with the occurrence of proven/probable IA. Table 4 showed that the most discriminating antigen was CAT and, to a lesser extent, DPPV. Ribonuclease and GM were poorly discriminatory (see the low sensitivity values for these 2 antigens in Table 4). Fig. 7 shows the hierarchical clustering based on OD values of patient sera obtained with CAT and DPPV antigens: 2 groups of patients containing 10 (group A) and 13 patients (group B) were clearly differentiated. The OD values noted with the group B patients with low titers were not significantly different from the controls (group C). In contrast, the OD values in group A patients with high antibody titers were significantly different from the other groups, groups B and C (Fig. 8). The best sensitivity and specificity values obtained for group A (10 patients) versus control population (34 patients) was, respectively, 89% and 70% for DPPV and 100% and 97% for CAT. Anti-*A. fumigatus* antibody detection in this subpopulation of patients was extremely predictive of IA.

The presence of high levels of antibodies in the sera of these patients before immunosuppression suggests that they were infected before graft or immunosuppressive treatments. Using the partition platform of the JMP software on the OD values obtained under our experimental ELISA conditions and with the population sampled here, the OD values indicative of a negative serum for the 4 antigens were 0.21, 0.12, 0.44, and 0.54 for RNU, DPPV, CAT, and GM, respectively. Values above 0.36, 0.35, 1.08, and 1.05 for the same antigens were indicative of an *Aspergillus* infection. Logistic regression data have confirmed that the most discriminating antigens ( $P < .001$ ) were CAT and DPPV (data not shown).

As with the immunocompetent patients, an important variability was seen in the response of each patient to the different antigens. Fig. 9 shows examples of the evolution of the antibody and antigen response in 6 different patients. In patient PIE, BAR, and RUD from group A as defined above, the antigens inducing the highest reactivity were GM, CAT, and DPPV, respectively. Patient GOR had high levels of antibody against RNU and GM and low levels against CAT and DPPV. Although patients GOR or PIE (Fig. 9) had low levels of GM associated with high anti-GM antibody titers, the correlation coefficient obtained between anti-GM antibodies and the Platelia index in patients with proven IA was  $-.04$  with a nonsignificant  $P$  value of .22 for a  $df$  of 172. In the present study, negative results in IA Platelia were not directly associated with the presence of anti-GM antibodies.

These data show that a sensitive measure of the level of antibody in the patient sera before immunosuppression had a good positive predictive value for IA (Fig. 10). This conclusion is seen when proven and control patients were analyzed. There is also a trend toward the separation of the probable and control populations based on antibody levels suggesting that some of the probable patients have IA.

#### 4. Discussion

Our study of antibody responses in different types of aspergillosis patients using an ELISA format has shown the important diagnostic potential of 3 recombinant antigens: RNU, DPPV, and the mycelial CAT 1 previously characterized in our laboratory (Calera et al., 1997; Paris et al., 1993; Beauvais et al., 1997b; Latgé, 1999; Kobayashi et al., 1993). Cramer (2002) and Cramer and Kodzius (2001) have also described the value of recombinant antigens in the diagnosis of allergic aspergillosis. The 3 antigens shown here to be the most discriminatory were previously recognized as important in the diagnosis of aspergillosis 20–30 years ago; however, at that time, they were not biochemically and genomically characterized. Ribonuclease was the Ag 3/C of Longbottom, and the DPPV and CAT were, respectively, the “chymotrypsin” and CAT antigens of Biguet (Arruda et al., 1990, 1995; Biguet et al., 1967; Longbottom, 1986; Tran Van Ky et al., 1968). The data shown here confirmed also

that *A. fumigatus* was the major pathogenic *Aspergillus* spp. because the RNU was a discriminatory antigen for these populations, and this enzyme is only produced by *A. fumigatus* among all other pathogenic species such as *Aspergillus nidulans*, *Aspergillus flavus*, or *Aspergillus terreus* (Hearn et al., 1990). Originally described biochemically, the absence of RNU in *Aspergillus oryzae* (*A. flavus*) and *A. nidulans* has been now confirmed by the analysis of their respective genome sequences (data not shown). The major problem with the earlier studies of these antigens was that these were performed using immunologic methods, such as IEP or counterimmunoelectrophoresis, that have been replaced by methods that offer more accurate quantitation of each antigen. In addition, recombinant technology standardizes the quality and reproducibility of antigen production. Although nobody knows accurately how “precipitin” bands were formed in the IEP or counterimmunoelectrophoresis tests, results reported here showed that the number of precipitin bands is directly correlated with OD values noted with the different recombinant antigens. This result validates the use of these recombinant antigens to identify aspergillosis diseases using an ELISA format.

If the 3 antigens can be used in the diagnosis of aspergilloma, ABPA, and IA, the respective potential of each of these 3 antigens depends on the population analyzed. Catalase was the most useful antigen in both immunocompetent and immunocompromised populations. In aspergillosis patients, RNU and DPPV antigens were as or more discriminatory than CAT. In ABPA patients, the most discriminatory antigens were RNU and DPPV. In patients with IA, RNU was not an appropriate antigen, whereas DPPV and overall CAT were the antigens recognized in most cases. In addition, follow-up analysis of patients with aspergillosis showed that the level of antibodies directed against every antigen varied with the patient and that the major diagnostic antigen was often different in different patients. It is unknown to date if this result was due to a genetic variation in the B-cell population of the different patients or if it is associated with the infective fungal strains that would produce different amounts of the respective antigens in vivo. Lower sensitivity and positive predictive values were obtained with ABPA patients than in aspergillosis patients. This result was in agreement with the difficulty to diagnose this pathology. The association of at least 2 and, better yet, 3 antigens (RNU, CAT and dipeptidylpeptidase) will be optimum for the diagnosis of the most important and life-threatening aspergillosis, that is, aspergillosis, ABPA, and invasive pulmonary aspergillosis.

In the cystic fibrosis control population, high titers of anti-*Aspergillus* antibodies were found. This result was in agreement with the common continuous colonization of these patients with *A. fumigatus*. Interestingly, the level of antibody against the DPPV protein was higher in the cystic fibrosis control population than in the ABPA patients in the noncystic fibrosis population, suggesting that this antigen, that is, homologous to the CD26, may play a role

in the cystic fibrosis pathology. Our study showed that ABPA could be diagnosed either in an atopic or in a cystic fibrosis population only by the quantification of specific anti-*Aspergillus* antibodies. Cramer et al. did not identify the same antigens for the diagnosis of ABPA because antigen selection was based on a phage display system such that CAT and DPPV antigens may have been underrepresented because of their high Mr (114, 20). A recent collaborative study with Cramer has shown that ABPA patients from Davos, Switzerland, react significantly with RNU, CAT, and DPPV (Cramer, personnel communication). In addition, in Cramer's studies, primary selection was based on IgE binding, whereas our analysis considered IgG levels. It is known for ABPA that specific anti-*Aspergillus* IgE and IgG may recognize different epitopes in these patients. An analysis of the specificity of the isotype response of ABPA patients toward the RNU, CAT and DPPV is presently underway.

The most important discovery of our study was the presence of high titers of anti-*Aspergillus* antibodies in about half of the patients before transplantation. A few previous publications have also reported the presence of anti-*Aspergillus* antibodies in immunocompromised patients with IA (Hearn et al., 1995; Herbrecht et al., 2002; Chan et al., 2002; Pinel et al., 2003; Woo et al., 2002). The lack of increase of antibody levels during the disease in the patients examined in this study suggested that the occurrence of antibodies is not a response of the patient to the fungal infection. In contrast, it suggested that a significant portion of the patients to be immunosuppressed was indeed harboring an *Aspergillus* infection upon hospitalization. Interestingly, earlier epidemiologic studies by our group (Chazalet et al., 1998; Debeaupuis et al., 1995) have shown that the origin of IA was nosocomial in about 50% of the cases. Our current antibody detection study suggests that about 50% (10/23 patients) of the patients with IA were infected at the entrance at the hospital. These results are of the most interest for patients at risk for IA because they suggest the possibility of colonization before immunosuppression and that this hidden *Aspergillus* colonization can now be suspected as an origin of IA for a significant number of patients. Looking for antibodies will become then an essential screening to perform before starting an intensive immunotherapy and subsequent graft. All these findings will influence greatly the management of the patients before immunosuppression because attempts can be made to eliminate the fungus from the lungs. A multicenter study will be undertaken soon to see if similar findings are found in other hospitals. The importance of an anti-*Aspergillus* antibody test in the immunocompromised setting is also reinforced by the displacement of the peak of IA toward the 6th month after graft (Morgan et al., 2005), and by the occurrence of IA in immunocompetent patients from intensive care units (Meersseman et al., 2004).

Previous studies have reported that the presence of high amounts of antibodies could be associated with the clearance

of circulating antigens in fungal infections of the immunocompromised patient. Such findings have been repeatedly seen in *Candida* infections where low antigen levels could be associated with high levels of antimannan antibodies (Sendid et al., 1999, 2003). In *Aspergillus* infections, several studies have also suggested that false-negative antigenemia tests could be due to the presence of a high level of anti-GM antibodies in the serum (Herbrecht et al., 2002; Pinel et al., 2003; Marr et al., 2004). Although in our study, the presence of antibodies against GM seems correlated with a low titer of antigens in a few sera, no statistically significant negative correlation could be demonstrated between the GM antigen and anti-GM antibody concentrations. For some IA false-negative patients, low level of GM and anti-GM antibodies were both found. Such false-negative data remain unexplained, but it excludes or at least minimizes the contribution of antibodies to false negatives in the Platelia kit.

These studies have been performed only in a limited number of hospitals using an anti-IgG (H + L) conjugate. The detection of other anti-*Aspergillus* Ig isotypes or subtypes against these major antigens must be undertaken because IgE or IgG4 is important in ABPA and allergy diagnosis (Stevens et al., 2003), and IgA levels may be a useful determinant in IA diagnosis (Latzg , unpublished data). This analysis of the humoral response of patients toward *Aspergillus* antigens should now be extended to multicenter trials with the 3 recombinant antigens of interest to confirm that this new serologic approach correlates well with clinical findings in different hospital situations. A standardized antibody test based on the association of the recombinant RNU, CAT, and DPPV that has shown the highest level of specificity in this study is currently under preparation for such a multicenter study.

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