Use of Monoclonal Antibody in Diagnosis of Candidiasis Caused by *Candida albicans*: Detection of Circulating Aspartyl Proteinase Antigen

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Received 5 March 1999/Returned for modification 20 May 1999/Accepted 20 August 1999

Invasive or disseminated candidiasis is a serious and often fatal complication that can occur frequently in immunocompromised patients. The development of invasive candidiasis may also be provoked by intravascular lines that are used for a long period, by indwelling catheters, or after major surgery and antibacterial treatment (1, 2, 9, 16, 28, 34). The diagnosis of invasive candidiasis is difficult because there are no specific clinical manifestations, and conventional microbiological methods usually lack both sensitivity and specificity (19, 34). The infection can be confirmed only by organ biopsy, by aspiration of a normally sterile body fluid, or at necropsy. Blood or urine cultures are often negative or become positive only after a long delay (7). Consequently, therapy is often initiated late in the course of infection, resulting in substantial morbidity and mortality (23). Therefore, it is important to diagnose tissue invasion at an early stage, as it can be successfully treated with amphotericin B.

Much effort has been made to develop reliable tests for rapid diagnosis of invasive candidiasis leading to appropriate therapy. These diagnostic techniques include detection of fungal nucleic acid by PCR (20), detection of (1-3)-β-D-glucan and β-arabinitol (32, 46), detection of circulatory *Candida* antigens (6, 10, 12, 14, 15, 27, 30, 35, 39, 43, 45), and detection of antibodies directed against different *Candida* antigens (5, 13, 37, 48). However, each of these techniques has limitations, and none of them has obtained wide acceptance for diagnosis of invasive candidiasis (36).

Secreted aspartyl proteinase (SAP) of *Candida albicans* is well known as a virulence factor which plays an important role during invasive hyphal growth of *C. albicans*. At the initial step of invasion, production of the enzyme is increased and it may participate in degrading the surface barrier prior to hyphal formation and deeper invasion into host tissues (3, 11, 22, 29, 33, 38, 41, 42, 47). It was found that candidiasis patients have high antibody titers against the enzyme, and the enzyme was detected in the sera of candidiasis patients (18, 24, 25, 43). The advantage of using a pathogenic factor such as SAP as a direct serodiagnostic marker of candidiasis lies in its potential to differentiate invasive disease from simple colonization. Therefore, detection of SAP in sera may be indicative of active candidiasis.

In a previous study, we produced and characterized a monoclonal antibody (MAb), CAP1, which was found to be highly specific against SAP1 (30). MAb CAP1 showed high sensitivity and was capable of detecting 2 and 16 ng of the antigen per ml by enzyme-linked immunosorbent assay (ELISA) and slot blot tests, respectively. It is therefore possible to use this monoclonal antibody as a diagnostic tool for detection of SAP antigen in sera of candidiasis patients.

In this study, three different ELISAs were compared in order to develop a new method for detection of circulating SAP antigen based on the use of MAB CAP1.

**MATERIALS AND METHODS**

**SAP antigen of *C. albicans***. The SAP1 antigen was purified from the culture supernatant of *C. albicans*, as described previously (29). Analysis of purified SAP1 antigen by two-dimensional gel electrophoresis followed by staining with Coomassie blue and Western blotting with MAB CAP1 revealed only one spot. MAB CAP1, MAB CAP1 (immunoglobulin G1 [IgG1] type) was described previously (30). The antibody is highly specific for SAP1 and is able to detect 2 and 16 ng of the antigen per ml by ELISA and slot blot tests, respectively. The epitope of SAP1 recognized by the MAB was the proteinaceous part of SAP1, and the epitope of the MAB was located in the Asp⁷⁰⁰-to-Gly⁷⁰⁷ sequence. This MAB did not react with *C. albicans* whole-cell extract and mannanprotein extract in Western blot analysis.

**Sera**. A total of 53 sera of candidiasis patients were kindly provided by the Korean Institute of Tuberculosis and the Korean National Institute of Health. The sera were obtained from patients with proven or highly suspected candidiasis caused by *C. albicans* on the basis of mycological and/or serological tests (isolation by culture, API 20C Aux system, and positive serological tests). Patients were classified as leukemia patients (n = 13), cancer patients (n = 8), pneumonia patients (n = 4), and pulmonary tuberculosis patients (n = 8), according to their clinical presentations. Sera from 12 patients with serologically confirmed as-

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pergilliosis were also included. Normal sera were obtained from 13 healthy volunteers with no history of candidiasis.

**Standard ELISA.** Polystyrene 96-well plates were coated with 1 μg purified SAP1 antigen diluted in carbonate buffer (pH 9.4) per well. The plates were incubated overnight at 4°C. After incubation, the plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) and blocked with 200 μl of 1% bovine serum albumin (BSA) in PBST per well for 2 h at 37°C. After three additional washes with PBST, 100 μl of 1:100 diluted patients’ sera were added to each well and incubated at 37°C for 2 h. After being washed as described above, the plates were incubated at 37°C for 2 h with 100 μl of peroxidase-conjugated anti-human IgG (Sigma Chemical Co., St. Louis, Mo.) diluted 1:3,000 in PBST. After incubation at 37°C for 2 h, the plates were washed three times with PBST and 100 μl of substrate solution was added. The substrate solution was prepared immediately before use by dissolving 0.4 mg of o-phenylenediamine (Sigma) per ml in 0.05 M citrate buffer (pH 5.2) and then adding hydrogen peroxide at a final concentration of 0.005%. The plates were incubated for 20 min in darkness. The reaction was terminated with 50 μl of 4 N H₂SO₄, and optical densities at 490 nm (OD₄₉₀) were measured with an ELISA reader (Microplate Reader 450; Bio-Rad, Richmond, Calif.).

**Antigen capture ELISA.** Polystyrene 96-well plates were coated with 100 μl of purified MAB CAP1 (10 μg/ml) diluted in carbonate buffer (pH 9.4) overnight at 4°C. Then, the plates were washed three times with PBST and blocked with 1% BSA in PBST by incubation at 37°C for 2 h. After three washes with PBST, 100 μl of patients’ sera pretreated with 2% trichloroacetic acid (TCA) and neutralized with carbonate buffer, pH 9.6, were added to each well, and the plates were incubated at 37°C for 2 h. Plates were again washed three times with PBST, and 100 μl of mouse anti-SAP1 polyclonal antibody diluted at 1:1,000 in PBST was added. The mouse anti-SAP1 polyclonal antibody was prepared by immunizing mice with purified SAP1 as described previously (3). After 2 h of incubation at 37°C, the plates were washed three times with PBST and 100 μl of peroxidase-conjugated anti-mouse IgG (Sigma) diluted at 1:3,000 in PBST was added. After 2 h of incubation at 37°C, the plates were washed three times with PBST and substrate solution was added. Subsequent steps were identical to the standard ELISA described above.

**Inhibition ELISA.** Purified SAP1 (10 μg/ml) diluted in carbonate buffer, pH 9.4, was added to each well of polystyrene 96-well plates and incubated overnight at 4°C. After being coated, the plates were washed three times with PBST and blocked with 1% BSA in PBST by incubation at 37°C for 2 h. After three washes with PBST, mixtures of patients’ sera and MAB CAP1 (10 μg/ml) were added to each well and incubated at 37°C for 2 h. Mixtures of patients’ sera and MAB CAP1 were prepared by incubating patients’ sera in PBST with MAB CAP1 at 4°C overnight or at 37°C for 2 h. Plates were washed three times with PBST, and peroxidase-conjugated anti-mouse IgG (Sigma) diluted at 1:3,000 in PBST was added, followed by incubation at 37°C for 2 h. After three washes with PBST, substrate solution was added; subsequent steps were identical to the standard ELISA described above.

**Comparison of the three ELISA procedures.** The results obtained from the three different ELISA procedures are summarized in Table 2. As expected, the standard ELISA showed a relatively low sensitivity, 69.7%, and a specificity of 76.0%. The positive predictive value, negative predictive value, and efficiency of this method were 79.3, 65.5, and 72.4%, respectively. For the antigen capture ELISA and the inhibition ELISA, the values for all categories which are meaningful for determining the usefulness of diagnostic procedures were improved significantly. The sensitivity was 93.9% for both procedures. However, the other categories, including specificity, positive predictive value, negative predictive value, and efficiency, were better for the inhibition ELISA than for the antigen capture ELISA. In the inhibition ELISA, the specificity, positive predictive value, negative predictive value, and efficiency were 96.0, 96.9, 92.3, and 94.8%, respectively. These results suggested that these methods for detecting circulating SAP antigen in the sera of candidiasis patients are more reasonable in their specificity and sensitivity than are classical methods which detect antibodies produced by the patient against various antigens. In this study, the inhibition ELISA using purified SAP and MAB CAP1 showed the highest sensitivity and specificity. Therefore, the inhibition ELISA method developed here might be useful for diagnosis of candidiasis.

**RESULTS**

**Standard ELISA.** In the standard antibody detection ELISA, a cutoff value was determined by calculating an average OD for 13 normal serum samples (0.124) plus 3 standard deviations (0.095). The cutoff OD was 0.219. As shown in Table 1, based on this cutoff value, 23 serum samples of the 33 candidiasis patients were positive, resulting in 69.7% true positivity. However, 6 of 12 serum samples (50.0%) obtained from patients with aspergilliosis also were positive reactions (Table 1). These results indicated that the standard anti-SAP antibody detection ELISA is not useful for diagnostic purposes due to low sensitivity and cross-reactivity with other fungal infections.

**Antigen capture ELISA.** Figure 1 shows the standard curve of the amount of SAP detected by the antigen capture ELISA. This system is able to detect as little as 1 ng of SAP per ml in PBS. The results of the inhibition ELISA using sera are shown in Table 1. The cutoff value, 0.157, was calculated from average OD for 13 normal sera (0.082) plus 3 standard deviations (0.075). Thirty-one of the 33 serum samples obtained from patients with candidiasis were determined to be positive, and 2 of the 12 aspergilliosis patients’ sera (sera 34 and 36) were determined to be positive even though the OD were 0.175 and 0.182, respectively. Sera from all candidiasis patients had detectable circulating SAP antigen, ranging from 6.3 to 19.0 ng/ml, except for two sera (sera 16 and 26) which were determined to be negative. Twenty-three of 31 (74.2%) serum samples had detectable antigen, ranging from 10.0 to 19.2 ng/ml, and 8 of 31 (25.8%) serum samples had antigen levels of less than 10 ng/ml. The mean SAP antigen concentration detected in the sera of candidiasis patients was approximately 12 ng/ml.

**DISCUSSION**

There is increasing interest in the use of reliable serological tests for rapid diagnosis and prophylactic treatment of invasive candidiasis in immunocompromised patients. Serological detection of antibodies to *Candida* antigens in patients with invasive candidiasis can fail because patients cannot produce an adequate immune response, especially immune-deficient patients, or because the first serum sample is taken before antibodies have been formed (4). Moreover, high titers of antibody may be the results of simple colonization of *Candida* spp. or a related fungal infection (8). In fact, antibody titers against *Candida* antigens are not useful in diagnosis of candidiasis, probably because a fairly high frequency of healthy people also have antibodies against *Candida* antigens, as *Candida* spp. are commensals in the human mouth, vaginal mucosa, and gastro-

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### TABLE 1. Results of three different ELISAs

| Serum | Underlying disease | Standard ELISA result | Antigen capture ELISA Result | SAP concn (ng/ml) | Inhibition ELISA Result | SAP concn (ng/ml) |
|-------|---------------------|-----------------------|-------------------------------|------------------|-------------------------|------------------|
| 1     | AML                 | P                     | P                             | 14.0             | P                       | 15.0             |
| 2     | AML                 | N                     | P                             | 16.7             | P                       | 16.4             |
| 3     | CML                 | N                     | P                             | 15.7             | P                       | 15.5             |
| 4     | CML                 | N                     | P                             | 16.7             | P                       | 16.0             |
| 5     | CML                 | P                     | P                             | 14.6             | P                       | 15.0             |
| 6     | ALL                 | N                     | P                             | 11.2             | P                       | 11.7             |
| 7     | ALL                 | P                     | P                             | 10.8             | P                       | 10.4             |
| 8     | ALL                 | P                     | P                             | 12.7             | P                       | 12.6             |
| 9     | CLL                 | N                     | P                             | 11.6             | P                       | 11.4             |
| 10    | CLL                 | N                     | P                             | 8.7              | P                       | 7.7              |
| 11    | CLL                 | P                     | P                             | 12.6             | P                       | 13.5             |
| 12    | Lymphoma            | P                     | P                             | 10.7             | P                       | 11.4             |
| 13    | Lymphoma            | N                     | P                             | 18.1             | P                       | 18.5             |
| 14    | Stomach cancer      | P                     | P                             | 19.2             | P                       | 19.0             |
| 15    | Stomach cancer      | P                     | P                             | 6.6              | P                       | 6.6              |
| 16    | Duodenal cancer     | P                     | N                             | ND               | N                       | ND               |
| 17    | Liver cancer        | P                     | P                             | 12.0             | P                       | 12.9             |
| 18    | Liver cancer        | P                     | P                             | 6.3              | P                       | 6.3              |
| 19    | Liver cancer        | P                     | P                             | 6.9              | P                       | 8.0              |
| 20    | Lung cancer         | N                     | P                             | 12.3             | P                       | 12.5             |
| 21    | Lung cancer         | P                     | P                             | 10.0             | P                       | 11.5             |
| 22    | Pneumonia           | N                     | P                             | 17.4             | P                       | 16.9             |
| 23    | Pneumonia           | P                     | P                             | 13.3             | P                       | 13.6             |
| 24    | Pneumonia           | P                     | P                             | 9.7              | P                       | 10.9             |
| 25    | Pneumonia           | P                     | N                             | ND               | N                       | ND               |
| 26    | Tuberculosis        | P                     | N                             | ND               | N                       | ND               |
| 27    | Tuberculosis        | P                     | P                             | 12.1             | P                       | 12.4             |
| 28    | Tuberculosis        | P                     | P                             | 8.7              | P                       | 9.4              |
| 29    | Tuberculosis        | P                     | P                             | 10.0             | P                       | 10.7             |
| 30    | Tuberculosis        | P                     | N                             | 14.9             | P                       | 15.2             |
| 31    | Tuberculosis        | P                     | P                             | 12.8             | P                       | 12.3             |
| 32    | Tuberculosis        | P                     | P                             | 7.4              | P                       | 7.2              |
| 33    | Tuberculosis        | P                     | P                             | 10.4             | P                       | 10.9             |
| 34    | Aspergillosis       | P                     | P                             | 4.4              | N                       | ND               |
| 35    | Aspergillosis       | N                     | N                             | ND               | N                       | ND               |
| 36    | Aspergillosis       | P                     | P                             | 4.6              | P                       | 4.6              |
| 37    | Aspergillosis       | N                     | N                             | ND               | N                       | ND               |
| 38    | Aspergillosis       | N                     | N                             | ND               | N                       | ND               |
| 39    | Aspergillosis       | P                     | N                             | ND               | N                       | ND               |
| 40    | Aspergillosis       | N                     | N                             | ND               | N                       | ND               |
| 41    | Aspergillosis       | P                     | N                             | ND               | N                       | ND               |
| 42    | Aspergillosis       | P                     | N                             | ND               | N                       | ND               |
| 43    | Aspergillosis       | N                     | N                             | ND               | N                       | ND               |
| 44    | Aspergillosis       | P                     | N                             | ND               | N                       | ND               |
| 45    | Aspergillosis       | N                     | N                             | ND               | N                       | ND               |
| 46    | N                   | N                     | N                             | ND               | N                       | ND               |
| 47    | N                   | N                     | N                             | ND               | N                       | ND               |
| 48    | N                   | N                     | N                             | ND               | N                       | ND               |
| 49    | N                   | N                     | N                             | ND               | N                       | ND               |
| 50    | N                   | N                     | N                             | ND               | N                       | ND               |
| 51    | N                   | N                     | N                             | ND               | N                       | ND               |
| 52    | N                   | N                     | N                             | ND               | N                       | ND               |
| 53    | N                   | N                     | N                             | ND               | N                       | ND               |
| 54    | N                   | N                     | N                             | ND               | N                       | ND               |
| 55    | N                   | N                     | N                             | ND               | N                       | ND               |
| 56    | N                   | N                     | N                             | ND               | N                       | ND               |
| 57    | N                   | N                     | N                             | ND               | N                       | ND               |
| 58    | N                   | N                     | N                             | ND               | N                       | ND               |

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*Sera used in this study were kindly provided by the Korean Institute of Tuberculosis and the Korean National Institute of Health. Serum samples are as follows: 1 to 33, sera from patients with candidiasis; 34 to 45, sera from patients with aspergillosis; 46 to 58, negative control sera from healthy volunteers. Each assay was repeated three times for at least two independent assays. Test results were considered positive if the OD exceeded the mean OD ± 3 SDs obtained with the negative control sera.*

AML, acute myeloid leukemia; CML, chronic myeloid leukemia; ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia.

*P* positive; *N* negative.

determined by comparing the OD to the standard curve shown in Fig. 1.

determined by comparing the OD to the standard curve shown in Fig. 2.

| ND | Not determined. |
intestinal tract. In addition, serological tests for antibodies are of limited diagnostic value because antibody titers may remain elevated for long periods of time during and after therapy. Therefore, detection of *Candida* circulating antigens in body fluids or sera could be more reliable for diagnosis of active invasive candidiasis and to facilitate the early diagnosis of the mycosis and confirm a preliminary diagnosis when antibody detection is nonconclusive.

Several attempts have been made to detect circulating antigens of the fungus by various biochemical and immunological techniques (6, 12, 14, 19, 21, 26, 27, 31, 35, 39, 44, 48). Some of these diagnostic assays are commercially available, but their clinical usefulness remains controversial due to their low sensitivity and specificity.

In this study, we developed a method for diagnosis of candidiasis by means of the detection of circulating SAP antigen. The novelty of the method developed in the present study was the use of a MAb (MAb CAP1) directed against SAP.

As expected, a standard ELISA based on detection of antibody against SAP revealed a relatively low sensitivity, 69.7%, and a specificity of 76.0%. Six of 12 serum samples obtained from aspergillosis patients showed false-positive reactions. It is known that *Aspergillus* spp. also secrete aspartic proteinase (17, 40). Therefore, this result was due to cross-reactivity of antibodies against aspartic proteinase of *Aspergillus* spp. with SAP. This suggests that antibody detection is not reliable, since a great number of pathogenic fungi also produce aspartic proteinase. In an antigen capture ELISA and an inhibition ELISA using MAb CAP1, the values for categories that are meaningful for evaluating the usefulness of diagnostic procedures were improved significantly. For both of the procedures, 31 of 33 serum samples obtained from candidiasis patients were determined to be positive, and the specificity was 93.9%. Two serum samples (sera 16 and 26) which were positive by the standard ELISA were negative by the antigen capture ELISA and the inhibition ELISA. This suggests that a detectable amount of SAP antigen was not contained in those sera even though antibodies against the antigen were detectable. Although not clear, these data suggest the possibility that the two serum samples were not derived from patients with true active or progressive candidiasis. The two ELISA procedures can evaluate true active candidiasis that may be difficult for the antibody detection standard ELISA. Furthermore, 10 serum samples (sera 2, 3, 4, 6, 9, 10, 13, 20, 22, and 30) were determined to be negative by the standard ELISA but positive by the antigen capture ELISA and the inhibition ELISA. This suggested that antibodies were not produced despite a significant amount of SAP present in the sera, which may be explained by the possibility that the patients were immunocompromised and that sufficient amounts of antibodies were not produced. This also suggested another possibility: that the two ELISA procedures could be useful for early diagnosis, especially for immunocompromised patients and those with the juvenile form of mycosis. However, false-positive results due to minor cross-reactions with another mycosis, aspergillosis, were also observed for both of the procedures. Two serum samples were positive with the antigen capture ELISA, and one serum sample was positive with the inhibition ELISA. However, the mean antigen concentrations observed in these heterologous sera were much lower than that recorded in the sera of candidiasis patients.

All of the sera of candidiasis patients had detectable amounts of circulating SAP antigen except for two samples (sera 16 and 26). The predicted SAP concentrations in positive sera for the antigen capture ELISA and the inhibition ELISA

| Parameter | Standard | Antigen capture | Inhibition |
|-----------|----------|-----------------|-----------|
| Sensitivity | 69.7 | 93.9 | 93.9 |
| Specificity | 76.0 | 92.0 | 96.0 |
| Positive predictive value | 79.3 | 93.9 | 96.9 |
| Negative predictive value | 65.5 | 92.0 | 92.3 |
| Efficiency | 72.4 | 93.1 | 94.8 |

# Table 2. Comparison of three different ELISAs

![Graph 1](image1.png)

**FIG. 1.** Amount of SAP detected by antigen capture ELISA. Data are averages calculated from triplate OD obtained with three different batches of in vitro-produced SAP diluted in PBS. Standard deviations were artificially calculated from calibration curves with OD corresponding to different SAP concentrations.

![Graph 2](image2.png)

**FIG. 2.** Amount of SAP detected by inhibition ELISA. Data are averages calculated from triplate OD obtained with three different batches of in vitro-produced SAP diluted in PBS. Standard deviations were artificially calculated from calibration curves with OD corresponding to different SAP concentrations.
were similar. This supports the concept that these two ELISA methods are more reliable than antibody detection ELISA. Although the sensitivity of both procedures was 93.9%, the other categories, including specificity, positive predictive value, negative predictive value, and efficiency, were better for the inhibition ELISA than for the antigen capture ELISA. In the inhibition ELISA, the specificity, positive predictive value, negative predictive value, and efficiency, were better for the antigen capture ELISA than for the antigen capture ELISA. In the other categories, including specificity, positive predictive value, negative predictive value, and efficiency, were better for the inhibition ELISA than for the antigen capture ELISA. In conclusion, detection of SAP antigen in sera by an inhibition ELISA using MAb CAP1 appears to be of value for the diagnosis and treatment monitoring of Candida patients. Detection of SAP in the sera of patients allowed early recognition of Candida infection, especially in patients with tissue-proven invasive candidiasis, for which the serological diagnosis was obtained several days before the microbiological diagnosis was made. However, a larger prospective study with a larger number of specimens and different concentrations of samples are needed to generalize this method for clinical use.

ACKNOWLEDGMENTS

This work was supported by a grant from Chung-Ang University (1999).

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