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Detection of Candidal Antigens in Autoimmune Polyglandular Syndrome Type I

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Autoimmune polyglandular syndrome type I (APS I) is associated with chronic mucocutaneous candidiasis. To characterize the antibody responses in this subgroup of *Candida albicans* infections, we screened a candidal cDNA expression library with patient sera and found four cDNA clones encoding the immunopositive proteins enolase, heat shock protein 90, pyruvate kinase, and alcohol dehydrogenase. The reactivity to these antigens was studied further by immunoprecipitation assays with in vitro-transcribed and -translated proteins. Analysis of sera from 44 APS I patients showed that the highest antibody reactivity was found with enolase (80% of patients reactive), but significant serological responses were also found with heat shock protein 90 (67%), pyruvate kinase (62.5%), and alcohol dehydrogenase (64%). Overall, 95.5% of patients had detectable antibodies to at least one of these proteins. The cDNAs of enolase and heat shock protein 90 were also expressed in *Escherichia coli* and studied by immunoblotting. Again, 84% of sera reacted with enolase, whereas 44% of sera reacted with heat shock protein 90. A good correlation between the two methods was found for both enolase ($r = 0.86$; $n = 58$; $P < 0.001$) and heat shock protein 90 ($r = 0.71$; $n = 56$; $P < 0.001$). Our results indicate that the four abundant candidal proteins are the major antigens and can be used as accurate markers of candidiasis in APS I patients. The immunoprecipitation assay described here is particularly useful for the rapid analysis of a large number of samples.

Autoimmune polyglandular syndrome type I (APS I), also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy, is most often associated with chronic mucocutaneous candidiasis, hypoparathyroidism, and Addison's disease. However, many other diseases, such as vitiligo, ovarian failure, insulin-dependent diabetes mellitus, and pernicious anemia, can be present (2, 28). APS I is inherited in an autosomally recessive manner, and recently the locus for the defective gene has been assigned to the chromosome 21q22.3 region (1). The disorder is associated with a selective T-cell deficiency (3), resulting in chronic candidal infection and an inability to respond to candidal antigens (10). A defect on the T-cell level has also been described for patients with chronic mucocutaneous candidiasis without endocrinopathy (8). However, these patients have a normal B-cell response with serum antibodies to candidal antigens. This normal humoral response is considered important in preventing the further development of systemic candidiasis (12, 13). Neutrophils and macrophages have also been shown to control candidal infections because of their ability to phagocytize and kill *Candida albicans* (17, 23).

Several antigens in candidal infections have been identified. The glycolytic enzyme enolase and heat shock protein 90 (HSP90) have been reported as the immunodominant antigens in systemic candidiasis (4, 11, 15, 21). Antibodies against HSP90 are also present in patients with chronic mucocutaneous candidiasis and AIDS (12). Other glycolytic enzymes, such as pyruvate kinase and alcohol dehydrogenase, have been described as antigens in patients with oral and esophageal candidiasis (22). However, both of these enzymes appeared to be nonubiquitous immunogens that were recognized by the sera of only a few patients. In addition, *C. albicans* enolase and alcohol dehydrogenase are recognized by immunoglobulin E (IgE) antibodies in allergenic patients (7, 19).

The immune response towards *C. albicans* is complex and possibly depends on the form and duration of infection. To study the antibody responses against candidal antigens in patients with chronic mucocutaneous infection and endocrinopathy, we screened a candidal cDNA expression library with sera from patients with APS I. Four candidal proteins, i.e., enolase, HSP90, pyruvate kinase, and alcohol dehydrogenase, were found to be the major antigens in this form of candidal infection. The reactivity to these antigens was analyzed further by immunoprecipitation and immunoblotting with sera from patients and healthy controls.

MATERIALS AND METHODS

Patients. Serum samples from 44 APS I patients were used in this study. All the patients had chronic mucocutaneous candidiasis without systemic infection. Hypoparathyroidism was diagnosed in 35 patients, and Addison's disease was diagnosed in 33 patients. Serum samples from 25 healthy individuals were used as control material.

Screening of cDNA expression library. The *C. albicans* cDNA expression library was a generous gift from R. K. Swoboda, and its preparation has been described earlier (22). The screening was performed according to standard protocols (18). Briefly, the cDNA library was plated onto Luria-Bertani agar plates (10^4 PFU/90-mm plate) with *Escherichia coli* XL-1 Blue cells which were then transferred to nitrocellulose filters (BA85; Schleicher and Schüll, Dassel, Federal Republic of Germany) and screened with a pool of sera that had been preabsorbed against the lysate of XL-1 Blue cells. The positive bacteriophage clones were purified by three or four additional subscreenings, and after a homogenous population of immunoreactive clones was obtained, the bacteriophage clones were converted into plasmid form. Restriction enzyme digestions and DNA dot blotting were used to identify similar clones (18). The sequencing was done by the dideoxy method according to the Sequenase version 2.0 kit (United States Biochemicals, Cleveland, Ohio) or with the automated ALF DNA sequencer (Pharmacia, Uppsala, Sweden). GenBank homology searches were carried out with a sequence analysis software package (Genetics Computer Group, University of Wisconsin).

Immunoprecipitation assay. The pBluescript plasmids containing *C. albicans* enolase, HSP90, pyruvate kinase, and alcohol dehydrogenase cDNAs were transcribed with T3 RNA polymerase and translated into [^{35}S]methionine (Amersham International, Aylesbury, United Kingdom)-labeled proteins with an in

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vitro transcription and translation kit from Promega (Madison, Wis.) according to the manufacturer's instructions. Trichloroacetic acid precipitation showed the incorporation of about 0.5, 8, 12, and 7% of total radioactivity to the enolase, HSP90, pyruvate kinase, and alcohol dehydrogenase translated products, respectively. The low level of radioactivity incorporation of enolase was observed in several repeated assays. In each assay, the translation mixture of the labeled protein (20,000 to 50,000 cpm of tricholoroacetic acid-precipitable material) was suspended in 50 μl of radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 1% Triton X-100, 10-μg/ml aprotinin) and incubated for 1 h at room temperature with diluted serum (1:10). Fifty microliters of Sepharose fast flow protein G (Pharmacia) diluted 1:10, was added, and the mixture was incubated for another hour at room temperature with shaking. The immune complexes were washed four times by centrifugation with RIPA buffer and counted with a scintillation counter or resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and analyzed by SDS-PAGE and autoradiography. All the assays were done in duplicate or triplicate. The intra-assay variations were below 12%. An antibody index was calculated as follows: (cpm in unknown sample − cpm in negative standard serum)/(cpm in positive standard serum − cpm in negative standard serum), where cpm is counts per minute. For the negative standard serum, a pool of five control sera was used, and for the positive standard serum, serum from the patient with the highest level of antibodies was used. The limit of detection was estimated as the mean of the antibody index ± 3 standard deviations that was obtained with sera from 25 healthy controls. For absorption, the diluted sera (1:10) were first incubated with 20 μl of purified fusion proteins for 2 h in 100 μl of RIPA buffer. After preincubation the immunoprecipitation was carried out as described above.

cDNA cloning and expression in *E. coli*. The cDNA of enolase was digested with the restriction enzyme *Xho*I and the recessed ends were partially filled in with dCTP and dTTP deoxynucleotides by using Klenow enzyme and digested with the restriction enzyme *Bam*HI. The resulting DNA fragment was cloned into the pEX31B (20) *Bam*HI and *Bgl*II sites, the latter of which was partially filled in with dGTP and dATP deoxynucleotides (pEX31ENO). The cDNA of HSP90 was cleaved with *Bam*HI and cloned into the pEX31B *Bam*HI site (pEX31HSP). The recombinant enolase and HSP90 were expressed as bacteriophage MS2 polymerase fusion proteins and purified as described by Strebel et al. (20) with some modifications. Thirty milliliters of *E. coli* 537 cell culture, transformed with expression plasmids, was grown at 28°C overnight, and the fusion proteins were induced by adding 4 volumes of prewarmed culture medium and growing the culture for 3 h at 42°C. The bacteria were pelleted and resuspended in 10 ml of TELPA buffer (0.6× phosphate-buffered saline, 100 mM EDTA, 0.4 M L-arginine-HCl, 100-μg/ml tolylsulfonyl phenylalanyl chloromethyl ketone [TPCK], 5-mg/ml lysozyme, 10 mM Tris-Cl [pH 8.8]) and subsequently broken by sonification. The cell extracts were centrifuged (15 min, 30,000 × *g*, 0°C) and resuspended in 10 ml of guanidine buffer (2 M guanidine-HCl, 100-μg/ml TPCK, 50 mM Tris-HCl [pH 8.8]), and the mixture was then sonicated and centrifuged (15 min, 30,000 × *g*, 0°C). The guanidine wash was repeated once, and the pellets were dissolved in 0.5 ml of 7 M urea–0.4 M L-arginine-HCl (pH 8.8). The purified fusion proteins were separated by SDS-PAGE and visualized with Coomassie blue.

Immunoblotting. The proteins were separated by SDS–8% PAGE and blotted onto BA85 nitrocellulose filters. Patient and control sera were applied at a dilution of 1:100. The filters were subsequently exposed to anti-human IgG or IgA (1:500 dilution; Dako-Immunoglobulins, Glostrup, Denmark), and immunoglobulins bound to the polypeptides were visualized with 4-chloro-1-naphthol. Immunoblot reactivities were assessed as negative or positive. The anti-MS2 polymerase monoclonal antibody (kindly provided by V. Ovod), which recognizes the fusion partner of expressed proteins, was used as a control.

Statistics. Statistical analyses of the associations between antibody levels and disorders in APS I were performed with the Mann-Whitney U test, and correlation analysis was used to compare the results of the immunoprecipitation and immunoblotting assays.

RESULTS

Characterization of cDNA library clones. The cDNA expression library was screened with a pool of six serum samples from APS I patients. To identify the antigens that were recognized by IgG and IgA, both anti-human secondary antibodies were used in the screenings. Of the 100 positive clones from the IgG screening (2×10^5 cDNA clones were screened) and the 49 positive clones from the IgA screening (10^5 cDNA clones were screened) that were chosen for further analysis, 40 and 26 clones, respectively, were shown by a final subscreening to be immunoreactive. In addition, the library was screened with a pool of sera from 10 healthy individuals, but no positive reactivity was found with either IgG or IgA. The positive bacteriophage ZAPII clones were subsequently converted into pBluescriptSK plasmid form and analyzed by using restriction enzymes and DNA dot blotting. The analysis revealed that 37 clones from the IgG screening and all 26 clones from the IgA screening belonged to the same cDNA, which was identified after DNA sequencing and a GenBank homology search as that encoding the glycolytic enzyme enolase of *C. albicans* (GenBank accession no. L04943). Three clones did not have homology with enolase cDNA, and 200 to 600 nucleotides were sequenced from both ends of the cDNA clones. One of the clones had 78 and the second had 61% sequence identity with HSP90 (accession no. M26044) and pyruvate kinase (accession no. X14400) cDNA of *Saccharomyces cerevisiae*, respectively. The third clone showed 100% sequence homology to the *C. albicans* alcohol dehydrogenase gene (accession no. X81694). On the basis of the compared sequences, it was determined that all four clones contained full-length cDNAs.

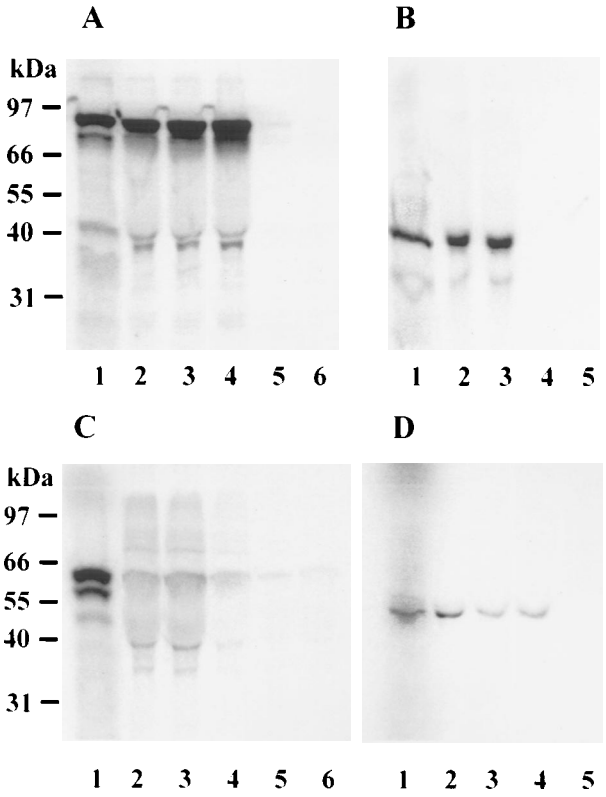

FIG. 1. SDS-PAGE and autoradiography of in vitro-translated and immunoprecipitated candidal proteins HSP90 (A), alcohol dehydrogenase (B), pyruvate kinase (C), and enolase (D). (A) In vitro-translated HSP90 (lane 1) and immunoprecipitation with three patient serum samples (lanes 2 to 4) and two control serum samples (lanes 5 and 6); (B) in vitro-translated alcohol dehydrogenase (lane 1) and immunoprecipitation with two patient serum samples (lanes 2 and 3) and two control serum samples (lanes 4 and 5); (C) in vitro-translated pyruvate kinase (lane 1) and immunoprecipitation with three patient serum samples (lanes 2 to 4) and two control serum samples (lanes 5 and 6); (D) in vitro-translated enolase (lane 1) and immunoprecipitation with three patient serum samples (lanes 2 to 4) and one control serum sample (lane 5).

Immunoprecipitation. [^{35}S]methionine-labeled enolase, HSP90, pyruvate kinase, and alcohol dehydrogenase were produced by in vitro transcription and translation assays. SDS-PAGE and autoradiography reproducibly indicated the correct sizes of the major products; however, bands with lower molecular masses were also present in the HSP90 and pyruvate kinase preparations (Fig. 1). Patient sera with high titers of antibodies were able to precipitate over 60% of translated

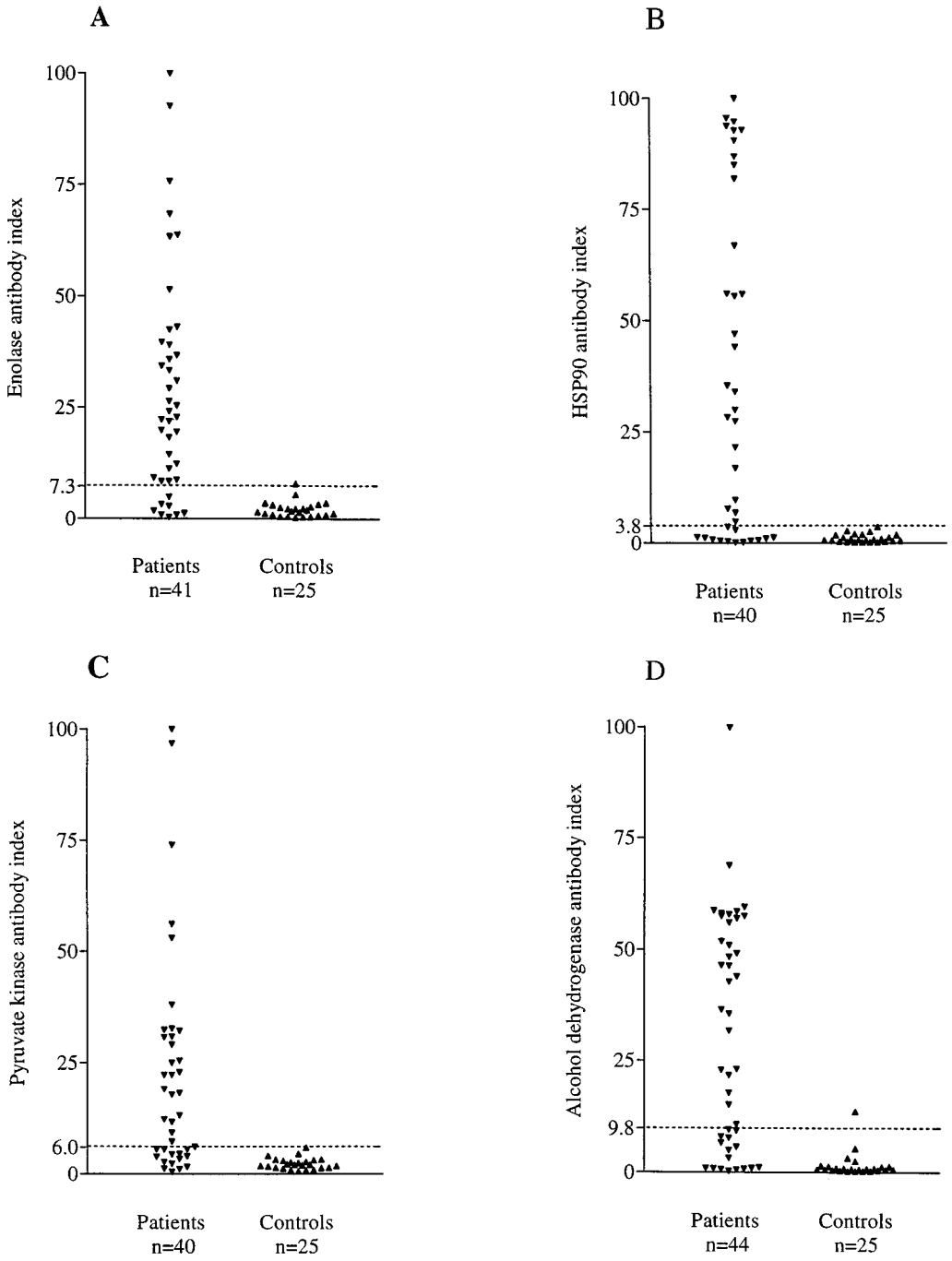

FIG. 2. Antibodies to candidal proteins among sera from APS I patients with chronic mucocutaneous candidiasis and healthy controls in immunoprecipitation assays. Dotted lines show the detection limits.

enolase, HSP90 and alcohol dehydrogenase and about 30% of the pyruvate kinase, as determined by total product radioactivity.

Immunoprecipitation analyses of the sera from APS I patients and control sera with four proteins are shown in Fig. 2. The study revealed significant associations between antibodies to candidal proteins and chronic mucocutaneous candidiasis in APS I. The highest reactivities were found with enolase, as an overall 80% (33 of 41 tested; $P < 0.001$) of patients were positive for antienolase antibodies. The other three proteins were also extensively immunoprecipitated by the sera from APS I patients. HSP90 was recognized by 67% of the sera from APS I patients (27 of 40; $P < 0.001$), whereas 62.5% of the sera from patients (25 of 40; $P < 0.001$) reacted with pyruvate kinase and 64% of the sera from patients (28 of 44; $P < 0.001$) reacted with alcohol dehydrogenase. A total of 95.5% of patients had detectable antibodies to at least one of the four antigens. Two patients, although clinically diagnosed with chronic mucocutaneous candidiasis, had no detectable antibodies to any of the four proteins. Among the controls, serum from one

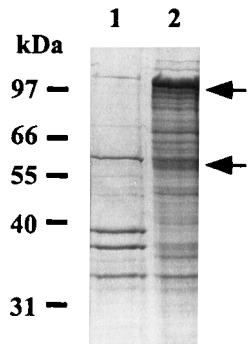

FIG. 3. SDS-PAGE and Coomassie blue staining of purified *C. albicans* enolase (lane 1 and lower arrow) and HSP90 (lane 2 and upper arrow) fusion proteins.

individual was found to immunoprecipitate enolase and serum from one individual was found to immunoprecipitate alcohol dehydrogenase at a level above the detection level. There was no association of antibodies with other disorders in APS I, such as hypoparathyroidism or Addison's disease.

Expression in *E. coli* and immunoblotting. The cDNAs of enolase and HSP90, two immunodominant antigens, were expressed as fusion proteins with bacteriophage MS2 polymerase (Fig. 3). The fusion proteins were purified from bacterial sonic extracts and used in immunoblotting. Of the 43 serum samples, 84% (from 36 patients) showed reactivity to enolase and 44% (from 19 patients) showed reactivity to HSP90 (Fig. 4), as detected with the secondary antibody against IgG class antibodies. Two of 17 control serum samples gave weak immunopositive staining with enolase, but no antibody responses among controls were found with HSP90. The anti-MS2 polymerase antibody was used to verify the expressed proteins. In studies with the secondary antibody against IgA class antibodies, 83% of patients had antibodies against enolase and 13% of patients had antibodies against HSP90 (of 40 patients studied). All patients having IgA class antibodies against enolase and HSP90 also had IgG class antibodies to these proteins.

A good correlation between immunoprecipitation and immunoblotting was observed. Comparison of the two methods showed an agreement, with an r of 0.86 ($n = 58$; $P < 0.001$) for enolase and an r of 0.71 ($n = 56$; $P < 0.001$) for HSP90. Furthermore, the immunoprecipitations of in vitro-transcribed and -translated enolase and HSP90 were inhibited by *E. coli*-expressed fusion proteins in three serum tested samples tested (data not shown).

DISCUSSION

We screened the expression library to identify the candidal antigens recognized by sera from APS I patients with chronic mucocutaneous infection. In addition to screening for IgG antibodies, screening for IgA antibodies was included because of their role in mucosal immunity. Thirty-seven of 40 immunopositive clones from the IgG screenings and all clones from the IgA screenings carried the cDNA of the glycolytic enzyme enolase. This enzyme is known to be abundant in *C. albicans*, and the main reason for the observed preferential reactivity with enolase is probably the high level of expression of the corresponding mRNA (21). Enolase has been shown to circulate in the blood of patients with systemic infection (26), and antibodies against enolase have been found in patients with systemic infection but not, or to a lesser extent, in patients with superficial infection or in healthy individuals (16, 25). Our results also show that the majority of patients with chronic mucocutaneous infection connected with APS I have antibodies against enolase. Eighty percent of patients had antibodies to enolase, as indicated by either immunoprecipitation assay or immunoblotting with purified enolase protein. The fact that the clones obtained by IgA screening expressed enolase also indicates a mucosal immune response toward this enzyme.

Three other antigens were recognized by the sera of the majority of APS I patients with chronic mucocutaneous candidiasis. Although a relatively good agreement between the immunoprecipitation and immunoblotting assays with HSP90 was observed, the former assay showed a higher antibody frequency (67 versus 44%). A likely explanation for this difference is that different HSP90 epitopes, predominantly conformational by immunoprecipitation or linear by immunoblotting, are detected with these approaches. In addition to enolase, two other glycolytic enzymes, pyruvate kinase and alcohol dehydrogenase, were immunoprecipitated by sera from a large number of APS I patients. Previously, Swoboda et al. (22), using immunological dot blotting, identified both enzymes as rare antigens among patients with superficial candidiasis and argued that antibody reactions against these proteins cannot be used as proper diagnostic criteria. The results of this study, obtained by using immunoprecipitation assays, suggest that these enzymes are major antigens. Unfortunately, we failed to express pyruvate kinase and alcohol dehydrogenase cDNAs in our *E. coli* expression system, and therefore the reason for the discrepancy in our results and those of Swodoba et al. remains unclear. The discrepancy may be due to the difference between the approaches or between the study populations.

The inability of T cells to respond to candidal antigens has been shown for APS I patients with chronic mucocutaneous infection. Although few cases have been reported in the literature (5), patients with chronic mucocutaneous candidiasis rarely suffer from invasive candidal infections. Similarly, AIDS patients, having deficient cell-mediated immunity, usually have a superficial candidiasis, further suggesting that a normal T-cell-mediated response is needed to control superficial infection whereas anticandidal antibodies may be responsible for preventing the systemic spread of infection. Primarily, the protective role has been attributed to antibodies against HSP90, because anti-HSP90 antibodies have been shown to correlate with the course of the infection and because in patients with fatal cases of systemic infection low-level or no antibody responses have been found (14, 15). The physiological chaperone function of HSP90 is to interact with a variety of cellular pro-

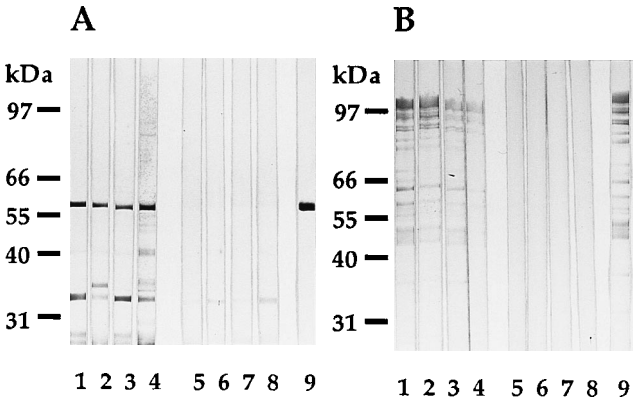

FIG. 4. Immunoblots of sera from APS I patients (lanes 1 to 4) and controls (lanes 5 to 8) and anti-MS2 polymerase monoclonal antibody (lane 9) against *C. albicans* enolase (A) and HSP90 (B) fusion proteins.

teins, keeping them inactive. Therefore, antibodies to HSP90 would protect serum proteins from such possibly harmful interaction with circulating *C. albicans* HSP90. However, 13 of 40 patients with long-standing mucocutaneous infections were negative for anti-HSP90 antibodies, as determined by both immunoprecipitation and immunoblotting, suggesting that other protective mechanisms or proteins are also involved. If the anti-HSP90 antibodies protect patients from systemic invasion, antibodies to other abundant candidal antigens, such as enolase, pyruvate kinase, and alcohol dehydrogenase, may have a similar role in these patients.

Autoantibodies to three cytochrome P-450 steroidogenic enzymes—21-hydroxylase, 17α-hydroxylase, and side chain cleavage enzyme—are present in APS I patients with Addison's disease (9, 24, 27). Candidal infection, often starting within the first years of life, usually precedes the onset of Addison's disease and therefore could form a basis for a molecular mimicry between candidal and human antigenic proteins, resulting in Addison's disease. *C. albicans*, like other yeasts, has an active steroidogenic metabolism expressing at least one cytochrome P-450 steroidogenic enzyme, lanosterol 14α-demethylase. Although patient sera with high titers of autoantibodies to human cytochrome P-450 steroidogenic enzymes were used in the library screening, none of the immunopositive clones contained candidal counterpart enzymes. Such a lack of humoral reactivity towards candidal cytochrome P-450 enzymes suggests that Addison's disease is likely not the result of molecular mimicry between candidal and human steroidogenic enzymes but rather the consequence of a genetic defect altering specific T-cell function.

The immunoprecipitation assay employing in vitro-synthesized and [^{35}S]methionine-labeled proteins is simple and suitable for the analysis of a large number of patients, and the results are reproducible. It also allows the study of native conformational epitopes, which by contrast are not always detectable by the immunoblotting assay (6). The immunoprecipitation assay with specific antigens should be useful for the diagnosis of candidal infections.

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