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Antibody raised against extracellular proteinases of *Sporothrix schenckii* in *S. schenkii* inoculated hairless mice

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Abstract. *Sporothrix schenckii* produces two extracellular proteinases, namely proteinase I and II. Proteinase I is a serine proteinase, inhibited by chymostatin, while proteinase II is an aspartic proteinase, inhibited by pepstatin. Studies on substrate specificity and the effect of proteinase inhibitors on cell growth suggest an important role for these proteinases in terms of fungal invasion and growth. There has, however, been no evidence presented demonstrating that *S. schenckii* produces 2 extracellular proteinases in vivo. In order to substantiate the in vivo production of proteinases and to attempt a preliminary serodiagnosis of sporotrichosis, serum antibodies against 2 proteinases were assayed using *S. schenckii* inoculated hairless mice. Subsequent to an intracutaneous injection of *S. schenckii* to the mouse skin, nodules spontaneously formed and disappeared for a period of 4 weeks. Histopathological examination results were in accordance with the microscopic observations. Micro-organisms disappeared during the fourth week. Serum antibody titers against purified proteinases I and II were measured weekly, using enzyme-linked immunosorbent assay (EIA). As a result, the time course of the antibody titers to both proteinases I and II were parallel to that of macroscopic and microscopic observations in an experimental mouse sporotrichosis model. These results suggest that *S. schenckii* produces both proteinases I and II in vivo. Moreover, the detection of antibodies against these proteinases can contribute to a serodiagnosis of sporotrichosis.

Key words: Antibody, Proteinase, Serodiagnosis Sporotrichosis, *Sporothrix schenckii*

Introduction

The ability of *Sporothrix schenckii* to infect tissues other than the skin has become strikingly apparent from the numerous published cases of articular, pulmonary, meningeal and disseminated sporotrichosis. Serodiagnosis is a valuable diagnostic tool particularly in extracutaneous sporotrichosis which is not easy to diagnose due to difficulties in culturing the organisms. Many serodiagnostic methods have been attempted using complement fixation [1, 2], precipitation [1, 3], agglutination [1, 2, 4–7] and immunoassay [7, 8] tests. Crude culture media and organisms from both yeast and mycelial forms have been used as antigens, however, none were satisfactory in terms of either sensitivity or specificity. Excellent sensitivity has been obtained by immunoassays such as RIA (radioimmunoassay) and EIA (enzyme-linked immunosorbent assay) [7, 8]. Specificity of the test depends on the antigen used. In general, yeast antigens are preferred to hyphal antigens [9]. For reliable diagnostic and prognostic values, it is desirable to use more specific antigens.

Pathogenic fungi produce extracellular protein-

ases which contribute particularly to their growth and invasion [10–18]. Proteinases, therefore, can be useful as antigens for serodiagnosis as they are specific and reliable markers of fungus activity.

S. schenckii produces two extracellular proteinases in albumin- or collagen-supplemented liquid medium [16]. Proteinase I had an optimal pH of 6.0, and its activity was strongly inhibited by chymostatin, while proteinase II had an optimal pH of 3.5, and its activity was strongly inhibited by pepstatin [16]. Both proteinases hydrolyze natural substrate sources such as stratum corneum, collagen and elastin [16]. The addition of either pepstatin or chymostatin to the culture medium did not inhibit the cell growth of *S. schenckii*, although the simultaneous addition of both inhibitors strongly inhibited growth [17]. Accordingly, it is suggested that the proteinases play an important role in fungal invasion and growth. There is, however, no evidence as yet suggesting that those proteinases are produced in vivo.

In order to substantiate an in vivo production of the proteinases, and furthermore to evaluate a new serodiagnosis in sporotrichosis, serum antibodies against purified proteinases I and II in *S. schenckii* inoculated hairless mice were investigated.

Materials and method

Mice. Six female homozygous type 1 hairless (hr/hr) mice backcrossed to ICR strain, 10 weeks of age, were obtained from a stock of defined flora mice (Sakyo Laboratories, Tokyo, Japan).

Organism and infection. A fresh clinical isolate of *S. schenckii*, the same strain used in our previous study [16], was obtained from a localized type of sporotrichosis and identified by standard morphological studies [19]. Precultivation was carried out with 3.7% brain heart infusion liquid medium (Eiken, Tokyo, Japan) in a shaking water bath (80 cycles/min) at 27 °C for 1 week. A suspension of the yeast form of the organism was prepared to give 10^4 cells per ml in culture medium after washing with sterilized water. The liquid culture medium, which was sterilized with a GS membrane filter (0.22–μm pores; Millipore Corp, Bedford, MA), was prepared with the following contents in 1 liter of distilled water: 10 g of yeast carbon base (Difco Laboratories, Detroit, MI), 50 mg of inositol (Sigma Chemical Co., St Louis, MO). 10 mg of thiamine (Sigma), and 2.5 g of bovine serum albumin (A 7030; Sigma). Liquid medium (70 ml) in 200–ml Erlenmeyer flasks was incubated in a shaking water bath (80 cycles/min) at 27 °C for 10 days. Cell counts were done with a hemacytometer. A yeast form of the organism was prepared to give 10^9 cells/ml in phosphate buffered saline solution (PBS) after three washes with sterilized PBS.

Six mice were injected intracutaneously at 6 points on each animal's back with 0.1 ml of the fungus suspension. The mice were observed daily for macroscopic evidence of inflammation, crusting and scaling. The macroscopic, microscopic, microbiological and immunological observations were recorded weekly for 7 weeks. Two mice were reserved for purely macrosopic observation, histology or culturing. A further two, which were subjected to sporotrichin skin tests, were also separately studied for macroscopic changes, histology or culturing. Antibody studies were performed on the remaining two. The aforementioned last pair were not subjected to any biopsy or sporotrichin testing, so as to avoid any influence on the immune system and antibody production.

Histopathology and culturing. In 4 mice, regardless of whether or not sporotrichin testing was performed, the skin of each inoculated site was biopsied weekly for histology and culturing on Sabouraud's dextrose agar plates. Tissue sections were stained with either hematoxylin & eosin (H&E) or periodic acid-Schiff (PAS).

Sporotrichin skin test. Sporotrichin, prepared from culture supernatants of the hyphal form of *S. schenckii*, was kindly donated by Dr Masataro Hiruma, Department of Dermatology, National

Defense Medical College. Macroscopic abdominal skin changes 48 hours after intracutaneous injection of 0.05 ml of sporotrichin were evaluated at weekly intervals.

Preparation of antigens: purification of proteinases I and II from S. schenckii. Purification procedures of proteinase I and II are described elsewhere [16]. Briefly, extracellular proteinases extracellularly produced in culture medium containing albumin, were ultrafiltrated with a YM-5 membrane filter (Amicon Corp., Lexington, MA) and chromatographed on DEAE-Sepharose CL-6B (Whatman BioSystems Ltd., Maidstone, KY) column in NaCl linear gradient elution, resulting in good separation of proteinase I and II activities. Each fraction was then separately applied on gel filtration using Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) and repeatedly chromatographed.

Enzyme-linked immunosorbent assay (EIA). Fifty to a hundred microlitres of mouse blood was collected from the mouse's tail prior to the inoculation of the organisms and thereafter weekly. Sera separated by centrifugation were assayed for antibody titers to proteinases I and II, using EIA. EIA was performed using Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Briefly, 0.5 micrograms of proteinases I and II were absorbed to the bottom of 96-well microtiter plates (Dynatech, Alexandria, VA) in 0.05 M bicarbonate buffer, pH 9. 6 and left for at least 24 h at 4 °C. Before using and between each step of the EIA procedure, the plates were washed three times with 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5 containing 0.1% Tween 20. After blocking with normal horse serum, samples from serial dilutions of the control (preimmunized mouse serum) and the sample sera from 1:50 to 1:3.28 million in a volume of 100 microliters were incubated with proteinases I or II for 15 min. The plates were then washed and incubated for 15 min with biotinylated anti-mouse IgG diluted 1:200, then incubated with a mixture of avidin DH and biotinylated peroxidase (Vectastain ABC Reagent, Vector) with color being revealed by the addition of 0.015% hydrogen peroxide and 0.1 mg/ml of 2,2'-azino-di(3–ethylbenzthiazoline-6–sulfonic acid) (Sigma) in 0.1 M citrate-phosphate, pH 5.3. The absorbency of the product was measured in a micro-ELISA autoreader (Dynatech) at 450 nm. Positive titers of the sample sera were determined as the highest, absorbency values of which were over those of the control sera.

Results

Macroscopic and histopathological observations after inoculation of S. schenckii onto hairless mouse skin. A time course study on the development of cutaneous lesions is shown in Fig. 1. The lesions' size in the hairless mice increased remarkably during the first week, and then at 3 weeks they began to reduce in size almost disappearing over the next 6 weeks. The time course curve of the residual nodule number (ratio of nodule numbers to inoculation sites at weekly intervals) was almost parallel to that of the lesion size. A positive fungal culture was obtained by 6 weeks. Histopathologically, the presence of abscesses consisting of neutrophils and nuclear dusts surrounding large numbers of organisms were observed during the initial 3 weeks. The number of PAS positive yeast cells then decreased in number and disappeared at 8 weeks. In accordance with the decrease in organisms, histiocytes began to take the place of polymorphonuclear leukocytes and were finally surrounded by lymphocytes.

Sporotrichin tests and serum antibodies against proteinase I and II. During the course of observation, there was no skin reaction following the intradermal sporotrichin injection. In contrast, serum antibody titers to proteinase I and II rose sharply until one week after inoculation in accordance with the development of nodules. High titers of 1:0.2 million (Fig. 1, experiment 1) to 0.8 million (Fig. 1, experiment 2) were maintained until the disappearance of nodules. One

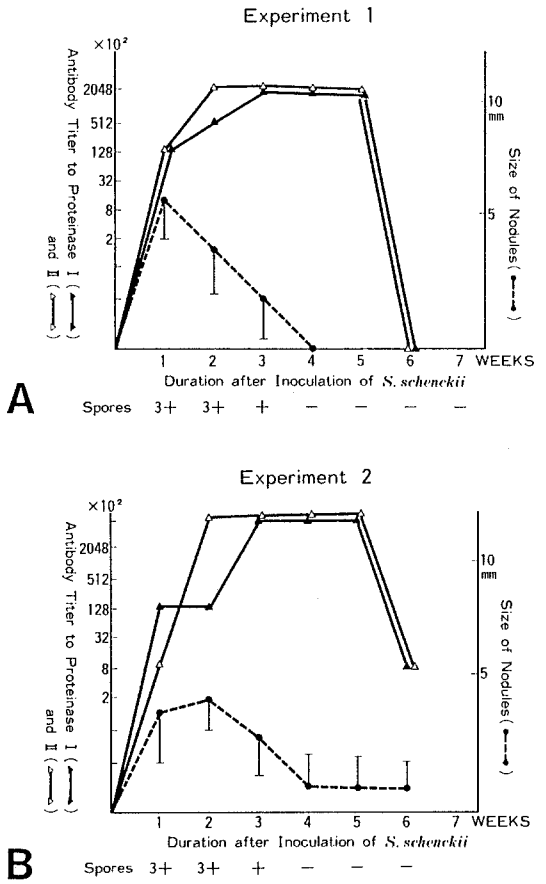

Fig. 1. Time course with respect to the macroscopic and histopathological observations from the cutaneous lesions of hairless mice and the anti-proteinase I and II antibody titers after inoculation of *S. schenckii*. Panel A: Experiment 1; Panel B: Experiment 2. Symbols: ●, mean diameter size of nodules (bar: standard deviation); ▲, antibody titer to proteinase I; △, antibody titer to proteinase II. Spores: PAS-positive organisms, 3+ = many; 2+ = moderate; 1+ = little; − = none.

week after healing, antibody titers were sharply reduced to negative control levels or very low values. There were no significant differences with respect to the time courses between the anti-proteinase I and anti-proteinase II antibody titers.

Discussion

Serologic tests for sporotrichosis are of great diagnostic and prognostic value particularly with regard to extracutaneous forms as *S. schenckii* has an exogenous and protean nature [1–8]. In this study, using an *S. schenckii* inoculated mouse model, serum antibody titers against purified proteinases I and II were measured by EIA. As a result, high titers of antibodies were successfully raised, suggesting that both proteinases were produced in vivo.

In addition, the time course of the antibody titers to both proteinases I and II was parallel to that of the macroscopic observations in an experimental mouse sporotrichosis model. It is of great interest that the antibody titers rose and dropped very rapidly in accordance with fungal activity. In general, antibody responses differ following primary and secondary antigenic challenges [20]. The phenomenon observed in this study is not simply explained by either primary or secondary antibody responses. The steep rise of IgG antibody is consistent with secondary response in a sensitized host. However, the rapid drop in antibody titers is a characteristic of a primary response. Following primary antigenic challenge, the antibodies are naturally catabolized or bind to the antigen and are cleared from circulation. Our results may reflect a primary and local antibody production [21]. Even though this is a preliminary study using a spontaneously healing mouse model, anti-proteinase antibody production was unique and the antibody measurement would not only be of diagnostic value but also be of assistance when evaluating prognosis and disease activity. In terms of the phenomenon that the antibody titers were parallel to the disease activity, antibody measurement can be of a similar value to antigen-oriented serodiagnosis. We believe that the antibody titers of the study herein reported closely reflected antigen amounts. From this viewpoint, it is likely that two proteinases are produced in the same manner during the course of infection.

Detection of the proteinase is proof of viability of the fungus so that an assay makes it possible to determine when antimycotic therapy should cease. Meanwhile, the antigen-oriented serodiagnosis would be more helpful for the evaluation of the disease state and therapeutic effectiveness not

only in sporotrichosis but also for other deep mycoses such as chromomycosis.

As this is a preliminary study, a larger experimental sample size and appropriate controls are required.

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