[Jpn. J. Parasitol., Vol. 39, No. 3, 258–266, June 1990]

Immunoblot Analysis of Three Antigen Preparations from *Strongyloides stercoralis* Larvae in Human Strongyloidiasis

YOSHIYA SATO[1,2], FUMIHIDE INOUE[3], SUSUMU KIYUNA[4] AND YOSHIYUKI SHIROMA[4]

(Accepted for publication; May 11, 1990)

Abstract

The antigenic polypeptide composition in three isolates extracted with PBS and detergents (DOC and SDS) from *Strongyloides stercoralis* larvae was analyzed by the immunoblotting technique with sera from strongyloidiasis patients. It was found that the immune responses of the patients were mainly directed toward the polypeptides in the relatively low molecular weight region of the detergent-extractive preparations. Three polypeptides with 41, 26 and 22 kDa, predominant in the SDS-extractive preparation, were also demonstrated to be specific components for *S. stercoralis* larvae. Of the three polypeptides, two were considered to correspond to the antigenic components which had been demonstrated in a previous study to be a significant antigen commonly recognized in a great majority of the patients. The SDS-extractive preparation revealed a satisfactory antigenic activity when used as a diagnostic antigen for the enzyme-linked immunosorbent assay.

Key words: strongyloidiasis, *Strongyloides stercoralis,* immunoblotting, antigenic analysis

Introduction

Strongyloidiasis is one of the major human parasitic diseases. Although the great majority of patients have no symptoms clearly attributable to the presence of the parasite, the infection often progresses to a fatal hyperinfected state under immunosuppressed conditions. The diagnosis of the disease depends upon the detection of *S. stercoralis* larvae from stool or other specimens. The parasitological confirmation, however, is sometimes difficult to achieve from larvae in these specimens, because the larvae are present only in very small numbers or are frequently absent in a chronic infection.

On the other hand, as is well documented, several immunological tests have proven effective in providing a sensitive diagnostic method to detect the *Strongyloides* infection (Dafalla, 1972; Tribouley-Duret *et al.*, 1978; Carroll *et al.*, 1981; Grove & Blair, 1981; Neva *et al.*, 1981; Genta & Weil, 1982; Sato *et al.*, 1985, 1986). There has been little investigation, however, of the nature of antigens used for such immunological tests. Although the protein composition and the reactivity of serum antibodies to the proteins were recently investigated in a murine infection model with *S. ratti* (Northern & Grove, 1987; 1988), few attempts have been made to investigate this in human strongyloidiasis (Genta *et al.*, 1987). In a previous study, the authors examined the composition of antigenic polypeptides and demonstrated that four antigen polypeptides commonly recognized by the patients' sera may be reliable antigens for immunological testing of the infection (Sato *et al.*, 1990).

In the present study, the authors further analyzed the antigenic compositions in three preparations which were isolated from *S. ster-*

[1]*Department of Parasitology,* [2]*Research Center of Comprehensive Medicine and* [3]*Department of Biochemistry, School of Medicine, University of the Ryukyus, Nishihara, Okinawa 903-01, Japan*
[4]*Izumizaki Hospital, Naha, Okinawa 900, Japan*

This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (Nos. 61570196 and 61304037).

佐藤良也（琉球大学医学部寄生虫学教室，同附属地域医療研究センター）
井上文英（同生化学教室）
喜友名　進　城間祥行（泉崎病院）

coralis larvae, using an immunoblotting technique.

Materials and Methods

Patients

Fifty patients were diagnosed as cases of chronic strongyloidiasis in the Izumizaki Hospital, Okinawa, Japan, by one or more fecal examinations. There were 38 male and 12 female subjects. The serum samples of 37 uninfected persons also served as controls. No concurrent infection with other gastrointestinal helminth was observed in any of the patients nor in the uninfected controls.

Parasites and preparation of antigens

Filariform larvae of *S. stercoralis* were obtained by culturing feces from relatively severe cases of strongyloidiasis, as described previously (Sato *et al.*, 1983). Two species of rodent *Strongyloides, S. ratti* and *S. venezuelensis,* were also used as antigen sources. The parasites have been maintained in our laboratory by biweekly passage in Wistar rats, since they were isolated from a wild rat in 1986 (Hasegawa *et al.*, 1988). The filariform larvae were collected from the feces of the infected rats by the same method used for collecting *S. stercoralis* larvae. The larvae were washed 6 times in PBS and were homogenized with a Teflon homogenizer. The homogenates were stirred to extract antigenic components in PBS containing 15 mM ethylenediamine-tetraacetic acid (EDTA; Wako Pure Chem, Osaka), 1.0 mM phenylmethylsulfonyl fluoride (PMSF; Wako) and 0.01% NaN_3, for 24 hr at 4°C. The supernatant fluid was collected by centrifugation at 8,000 *g* for 1 hr and then filtered through a millipore membrane (pore size 0.45 μm; Sartorius, Göttingen, FRG) before use as an antigen. Subsequently, the residues after extraction of the PBS-soluble components were further solubilised with detergents. Sequential two-step extractions with 1% sodium deoxycholate (DOC; Sigma, St. Louis, MO) and 1% sodium dodecylsulfate (SDS; Wako) were performed according to the method of Northern and Grove (1987). The antigen preparations are referred to as PBS-, DOC- and SDS-fractions, respectively. The protein concentration was determined by a dye-binding assay with a protein assay kit (Bio-Rad Lab., Richmond, CA).

Polyacrylamide gel electrophoresis and Western blot analysis

The polyacrylamide gel electrophoresis in SDS (SDS-PAGE) was carried out with 10% polyacrylamide slab gels according to the method of Laemmli (1970). The samples for electrophoresis were prepared at a protein concentration of 1.0 mg/ml in sample buffer; 62.5 mM Tris-HCl, pH 6.8, 4% SDS, 5% 2-mercaptoethanol (2-ME: Nacalai Tesque, Kyoto), 10% glycerol and 0.001% bromophenol blue (Merck, Darmstadt, FRG). Electrophoresis was performed at a constant voltage of 120 V for 4 hr. After electrophoresis, gels were silver-stained with a staining kit (Bio-Rad Lab.). A mixture of standard proteins (Bio-Rad Lab.) was co-electrophoresed to estimate the molecular weight of antigen polypeptides separated by the electrophoresis.

An immunoelectrotransfer blot (Western blotting) technique (Towbin *et al.*, 1979) was carried out to detect antigen components reactive with patients' sera. The polypeptides separated by the SDS-PAGE were transferred electrophoretically to a nitrocellulose membrane (Bio-Rad Lab.). After blocking the non-specific protein-binding sites with a 1% bovine serum albumin (BSA; Sigma), the blotted membrane was treated with a 1/10 dilution of the patient's serum in PBS containing 1% BSA for 2 hr at 37°C and then overnight at 4°C. After washing, the membrane was further incubated with ^{125}I-labeled protein A (NEN Research Products, Boston, MA) to detect IgGs which had reacted to antigens on the membrane (Burnette, 1981). The membrane was finally dried after 4 washings and the distribution of ^{125}I-radioactivity on the blotted membrane was examined by autoradiography using Fuji X-ray film (Fuji Photo Film, Tokyo).

Enzyme-linked immunosorbent assay

The reactivities of the patients' sera to the ex-

tracts of *S. stercoralis* were measured by enzyme-linked immunosorbent assay with a polystylene microtiter plate (micro-ELISA). The method has been described previously in detail (Sato *et al.*, 1985). Briefly, antigen was coated onto the wells of the microplate at a concentration of 10 μg/ml (0.2 ml/well). Each serum was tested at the single dilution of 1:50. After reaction of serum samples for 1 hr at 37°C and then overnight at 4°C, horseradish peroxidase-conjugated goat anti-human IgG (Miles-Yeda Ltd, Rehocot, Israel) was added to each well and the plate was incubated for 1 hr at 37°C. After washing, substrate (0.01% orthophenylenediamine; Eastman Kodak, Rochester, NY) was added and the reaction mixture was further incubated for 40 min at room temperature in darkness. The enzyme reaction was stopped by the addition of 25 μl of 8 N H_2SO_4 and the absorbance was measured at 500 nm using a spectrophotometer (Corona MTP12; Nissei Sangyo Co., Tokyo). Values were given as net absorbance (subtracting the absorbance value obtained in wells where only patient's serum was omitted).

Absorption of serum

The antigens of *S. ratti* and *S. venezuelensis* (10 mg/ml in 0.1 M carbonate buffer, pH 8.0, containing 0.5 M NaCl) were coupled with an equal volume of Affi-Gel 10 (Bio-Rad Lab.) by incubation at 4°C for 4 hr. Incubation was continued for 1 hr at 4°C after adding an equal volume of 1.0 M monoethanolamine-HCl, pH 8.0. The resin was washed with a 0.1 M carbonate buffer containing 0.5 M NaCl. The antigen-coupled gels were equilibrated by further washing with 0.01 M Tris-HCl, pH 7.6 containing 0.15 M NaCl, and were made up to a 30% suspension. The serum was mixed with an equal volume of the antigen-conjugated gel suspension. The mixture was incubated at 37°C for 1 hr and shaken gently every 15 min. The mixture was further allowed to remain overnight at 4°C. The gel suspension was then centrifuged at 300 *g* and the supernatants were collected. Antigen-uncoupled gels, which were treated only by 1.0 M ethanolamine-HCl, were used for control absorption.

Statistics

The statistical significance of the difference in the ELISA values was analyzed by Student's *t*-test. A *P* value of more than 0.05 was considered to be insignificant.

Results

Polypeptide composition of the three preparations isolated from *S. stercoralis* larvae were tested by the SDS-PAGE (Fig. 1) and many polypeptide bands with a wide range of molecular weights were shown in each preparation. The PAGE patterns of the PBS- and DOC-fractions were more complex than that of the SDS-

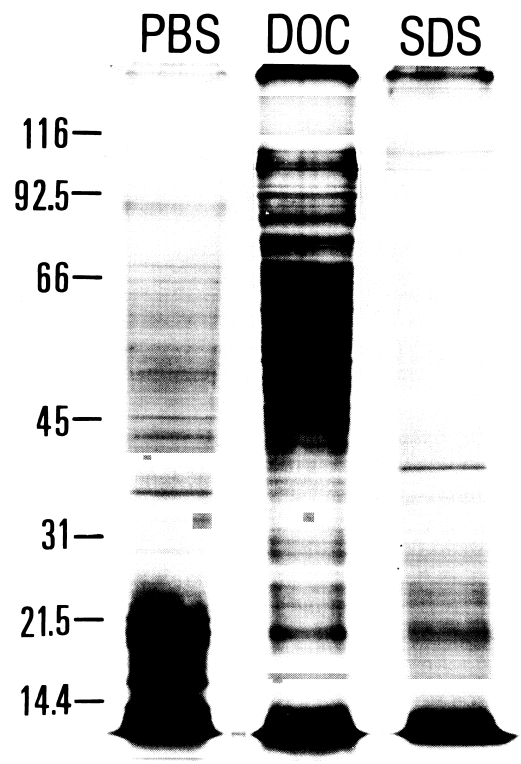

Fig. 1 PAGE patterns of three antigen preparations from *S. stercoralis* larvae. The left-hand lane indicates the PBS-fraction, the central lane indicates the DOC-fraction and the right-hand lane indicates the SDS-fraction. The polypeptide bands were detected by silver staining. The position and molecular weights (kDa) of marker proteins are represented at the far left.

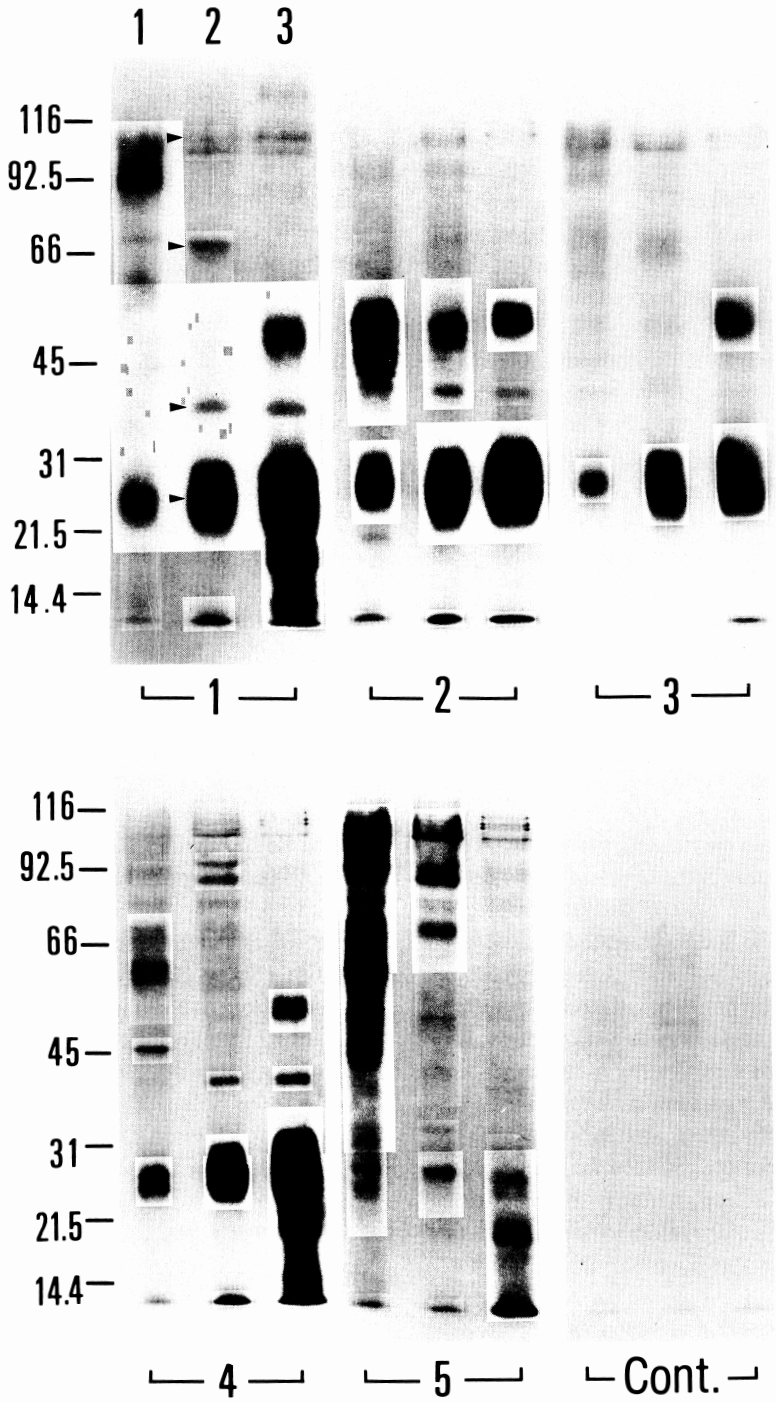

Fig. 2 Immunoblot analysis of antigen components in the three fractions of *S. stercoralis* larvae. In each case, *lane 1* indicates the PBS-fraction, *lane 2* indicates the DOC-fraction and *lane 3* indicates the SDS-fraction. The position and molecular weights (kDa) of marker proteins are represented at the far left. The arrowheads indicate the sero-reactive bands at 97, 66, 41 and 26 kDa.

fraction. Especially in the DOC-fraction, more than 50 tiny bands with a wide range of molecular sizes were demonstrated by silver staining. Intensity of the bands in the region of high molecular masses over 45 killodaltons (kDa) were very tiny in the PBS-fraction or the bands were almost absent in the SDS-fraction, whereas the polypeptide bands were much more abundant in number and were detectable as intensified silver-stained bands in the high molecular weight region of the DOC-fraction.

In order to determine the components reactive with the patients' sera, the Western blot analysis was performed using the serum samples from fifteen patients and the three antigenic preparations. The several patterns are illustrated in Fig. 2. The immunoblotting analysis showed strong reactivities of the patients' sera to the polypeptide bands in the molecular weight regions from about 20 to 30 kDa. Strong bindings of antibodies to the bands in the 20–30 kDa region were observed in the SDS-fractions but the reactions in the region were only faintly detectable in the PBS-fraction. In several subjects (only 4 out of the 15 patients examined), however, the sera displayed a very weak reactivity in the molecular region (Fig. 2, Cases 5). In such cases, the sera markedly recognized the bands in the high molecular region of the PBS-fraction. Several distinct differences of the reactivity to the polypeptide bands other than the 20–30 kDa bands were also noted among the three preparations; the reactions to the bands in about the 41 and 50 kDa regions were also demonstrated significantly in the DOC- and SDS-fractions but were almost absent in the PBS-fraction. In general, the reactivities in the higher molecular region of over 50 kDa were characteristic in the PBS-fraction, whereas the reactivities in the lower molecular region were significant in the SDS-fraction. On the other hand, the DOC-fraction contained the common reactive components both in the higher and lower molecular weight regions.

Only a few bands were visualized non-specifically by use of the uninfected control sera in the molecular weight region from about 43–47 kDa. The reactions to these bands, however, were extremely faint.

The immunoblotting patterns in ten patients were compared before and after the absorption of sera with the PBS-fractions from *S. venezuelensis* and *S. ratti.* As shown in Fig. 3, the immunoblotting reactivities to the antigen preparations of *S. venezuelensis* and *S. ratti* were almost completely abolished after absorption either with *S. venezuelensis* or *S. ratti* antigens. On the other hand, the reactions to the two bands of *S. stercoralis,* estimated as 26 and 41 kDa, clearly remained after the absorption, suggesting that the two polypeptides may be specific for *S. stercoralis.*

Fig. 4 represents the immunoblotting patterns to the three fractions from *S. stercoralis* before and after the absorption. In the reaction to the PBS-fraction, the reactivities diminished significantly after the absorption by either *S. venezuelensis* or *S. ratti* antigens, although a band, estimated as 22 kDa, remained as a consistently strong band. On the other hand, the antibodies to the two polypeptides corresponding to the above 26 and 41 kDa bands in the DOC- and SDS-fractions remained without absorption by the heterologous antigen preparations (Fig. 4, left figure). In the other immunoblotting patterns in which strong reactions in the 20–30 kDa region were absent, the positive bands diminished almost completely after the absorption, except for a band which corresponds to 22 kDa in the SDS-fraction (Fig. 4, right figure). Similarly when the patients' sera were absorbed by the detergent-extractive fraction (the DOC-fraction) of either *S. venezuelensis* or *S. ratti,* the responses to the above three polypeptide bands (22, 26 and 41 kDa) also remained after the absorption.

Finally, in order to evaluate the use of the three fractions for detection of *S. stercoralis* infection, the ELISA values were measured on 50 patients and 37 uninfected controls (Fig. 5). The mean ELISA value was highest in response to the SDS-fraction, whereas it was lowest in response to the DOC-fraction. Also, non-specific reactions in the uninfected controls were higher in the SDS-fractions than in the PBS and DOC-fractions. Presuming that the mean value plus 3SD in the controls is regarded as the cut off value of positive reaction, false-negative results were

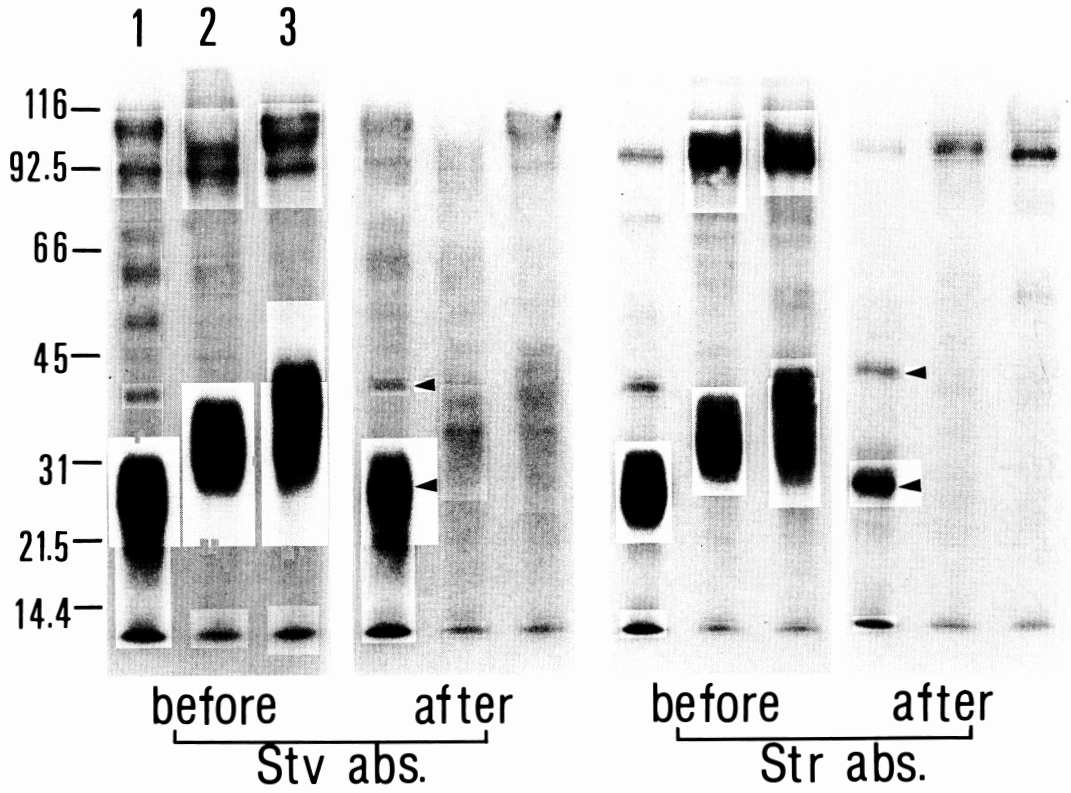

Fig. 3 Immunoblot analysis of antibody responses to the PBS-fractions from three *Strongyloides* species before and after absorption of serum samples with the *S. venezuelensis* or *S. ratti* antigen. To each case, the trans-blotted membranes (*lane 1*: *S. stercoralis* antigen, *lane 2*: *S. venezuelensis* antigens, and *lane 3*: *S. ratti* antigens, respectively) were treated with serum samples before and after the absorption with the PBS-fraction of either *S. venezuelenesis* (Stv) or *S. ratti* (Str) as indicated. The positions of marker proteins are represented at the far left and the arrowheads represent the bands at 41 and 26 kDa.

observed in 24% (12/50) of patients with known infection both in the responses to PBS- and DOC-fractions, but in only 8% (4/50) in the response to the SDS-fraction.

Discussion

Although there have been many reports on quantitative studies on the humoral immune responses to *S. stercoralis,* the qualitative investigation of antibody responses is very scanty in human strongyloidiasis. The improvement of diagnostic assays for strongyloidiasis will largely depend upon the understanding of host responses to defined antigens of the parasite. The authors have examined for the immunoblotting patterns of many patients with chronic strongyloidiasis (Sato *et al.*, 1990) and have indicated that there were considerable differences in the antibodies produced among the individual patients. Although it is reasonable to assume that individual patients recognize the different antigens of the parasite, due to the different reactivity in each patient it appears to be of essential importance for reliable serodiagnostic assay to identify the antigenic components which are parasite-specific and highly immunogenic for a large majority of patients. As shown in the previous study, four antigenic polypeptides with molecular sizes of about 97, 66, 41 and 26 kDa were found by the authors to be the significant antigens for the immunological testing of

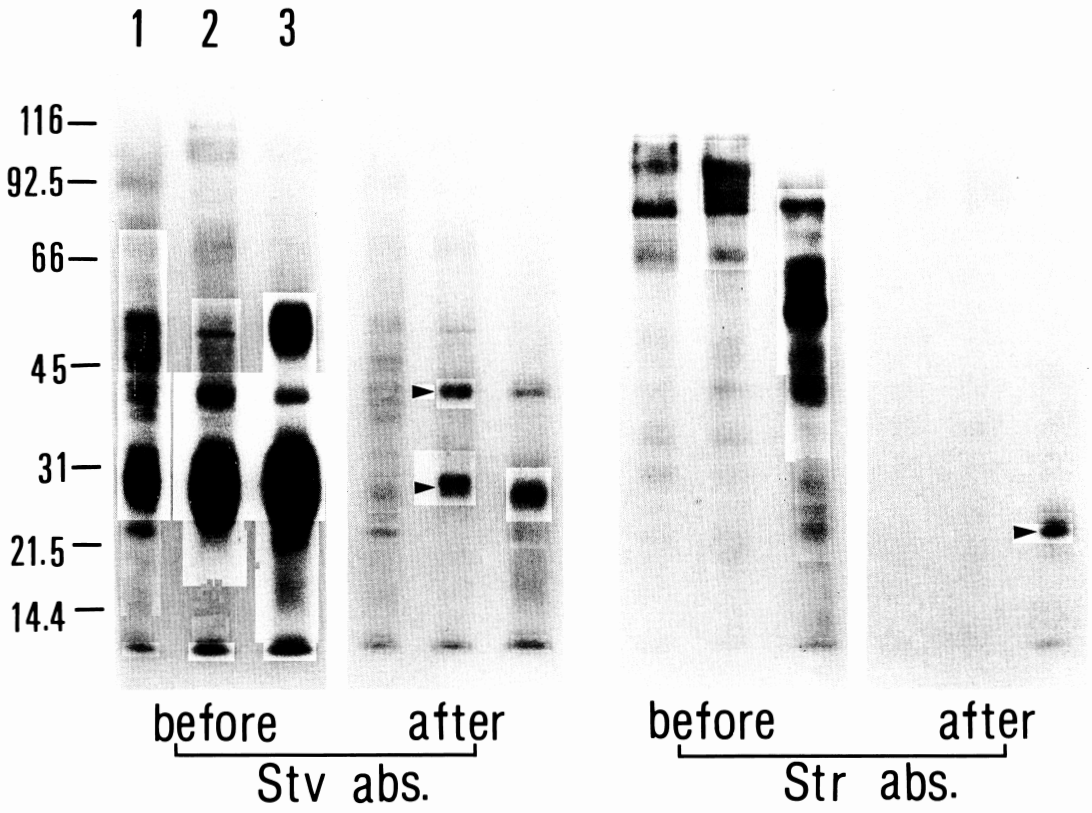

Fig. 4 Immunoblot analysis of antibody responses to the three antigen preparations from *S. stercoralis* before and after absorption of serum samples with heterologous *S. venezuelensis* or *S. ratti* antigen.

The duplicated membranes trans-blotted the three antigen preparations (*lane 1*: the PBS-fraction, *lane 2*: the DOC-fraction, and *lane 3*: the SDS-fraction, respectively) were treated with serum samples before and after the absorption with the PBS-fraction of either *S. venezuelensis* or *S. ratti*. The positions of marker proteins are represented at the far left and the arrowheads indicate the bands at 42, 26 and 22 kDa.

strongyloidiasis. In the present study, the polypeptide composition and reactivity with the patients' sera were further investigated in three fractions from *S. stercoralis* larvae.

The PAGE profiles and reactivity to the polypeptide bands varied considerably among the three fractions from *S. stercoralis* larvae. The polypeptide composition was most complex in the DOC-fraction showing the presence of at least 50 tiny bands with a wide molecular range, whereas it was much simpler in the SDS-fraction. The immunoblot analysis showed that the sero-reactive components are abundant in the relatively high molecular weight region of the PBS-fraction and in the lower molecular weight region of the SDS-fraction. In particular, many patients showed strong sero-reactivities in the 20–30 kDa region of the two preparations extracted by the detergents. Among the four components detected frequently in the previous examinations, the 97 and 66 kDa bands were mainly detected in the PBS-fraction, whereas the 41 and 26 kDa bands were observed predominantly in the DOC- and SDS-fractions. It is well known that the parasite can propagate in its host by internal autoinfection and that the majority of the larvae invaded are trapped by the host's immune system under an immunocompetent condition. The strong responses to the somatic components solubilizable with the detergent may be induced during

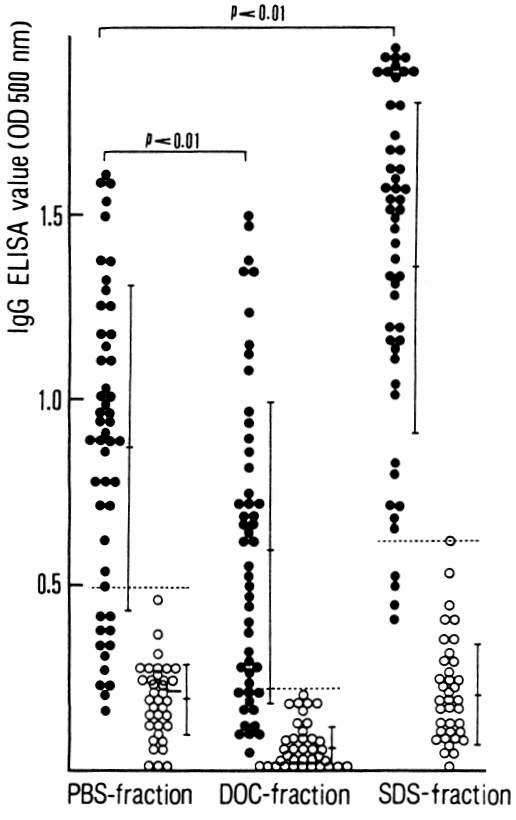

Fig. 5 ELISA results of sera from fifty patients with chronic strongyloidiasis (●) and from 37 uninfected controls (○) using the three antigen preparations of *S. stercoralis* larvae. The ELISA were performed using the PBS-, DOC- and SDS-fractions, respectively. Dashed lines indicate statistical limitation for positive samples (mean value of negative sera + 3SD). Vertical lines represent the mean ± SD.

the effective killing of such larvae in the autoinfection.

When the patients' sera absorbed previously with the heterologous rodent *Strongyloides* antigens, the reactivities to the majority of the bands were abolished almost completely and only the several bands corresponding to the molecular sizes of 41, 26 and 22 kDa remained as specific bands for *S. stercoralis*. These three bands were detected mainly in the detergent-extractive fractions.

In their investigation of the IgG and IgA responses in human strongyloidiasis, Genta *et al.* (1987) have detected 19 bands recognized by IgG antibodies in a patient. Because of the different experimental conditions used in each study, an exact comparison could not be done of the results. However, several bands at approximately the same location as the 41, 26 and 22 kDa bands were also observed in their studies.

In order to evaluate the relation between the composition of antigen components and the overall antigenic activity to the patients' sera, the ELISA-based assays were performed on fifty patients using the three fractions. Although all the antigen preparations discriminated between the patients and the controls, the ELISA values estimated by the SDS-fraction were significantly the highest, whereas it was lowest for the DOC-fraction. When the ELISA values were compared among the uninfected controls, the SDS-fraction also gave higher ELISA values than the other two preparations. In general, the preparation extracted with detergent contains many hydrophobic sites. The higher ELISA responses in the SDS-fraction might be due to the non-specific responses to the hydrophobic sites. The control values, however, were consistently lower as compared to those of the patients, and the overall reactivities of the patients and the uninfected controls to the SDS-fraction suggest that the antigen preparation may be useful as a diagnostic antigen for strongyloidiasis. The authors have also successfully utilized the antigen preparation as antigen for the indirect agglutination test of strongyloidiasis (Sato *et al.*, unpublished data). The lowest values in the DOC-fraction may be attributable to the nature of the complex polypeptide mixture, as revealed by SDS-PAGE, in which many components non-reactive with patients' antibodies were demonstrated by the immunoblot assay.

In conclusion, the antigen components specific for *S. stercoralis* and highly immunogenic for the patients were demonstrated in the relatively lower molecular region of the SDS-soluble fraction. The antigen preparation is considered to be of use for the serodiagnosis of human strongyloidiasis. Further studies of how to purify the antigen components for use as diagnostic antigen remain to be done.

References

1) Brunette, W. N. (1981): "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Analy. Biochem., 112, 195–203.
2) Carroll, S. M., Karthigasu, K. T. and Grove, D. I. (1981): Serodiagnosis of human strongyloidiasis by an enzyme-linked immunosorbent assay. Trans. Roy. Soc. Trop. Med. Hyg., 75, 706–709.
3) Dafalla, A. A. (1972): The indirect fluorescent antibody test for the serodiagnosis of strongyloidiasis. J. Trop. Med. Hyg., 75, 109–111.
4) Genta, R. M. and Weil, G. J. (1982): Antibodies to *Strongyloides stercoralis* larval surface antigens in chronic strongyloidiasis. Lab. Invest., 47, 87–90.
5) Genta, R. M., Frei, D. F. and Linke, M. J. (1987): Demonstration and partial characterization of parasite-specific immunoglobulin A responses in human strongyloidiasis. J. Clin. Microbiol., 25, 1505–1510.
6) Grove, D. I. and Blair, A. J. (1981): Diagnosis of human strongyloidiasis by immunofluorescence using *Strongyloides ratti* and *S. stercoralis* larvae. Am. J. Trop. Med. Hyg., 30, 344–349.
7) Hasegawa, H., Orido, Y., Sato, Y. and Otsuru, M. (1988): *Strongyloides venezuelensis* Brumpt, 1934 (Nematoda: Strongyloidae) collected from *Rattus norvegicus* in Naha, Okinawa, Japan. Jpn. J. Parasitol., 37, 429–434.
8) Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680–685.
9) Neva, F. A., Gam, A. A. and Burke, J. (1981): Comparison of larval antigens in an enzyme-linked immunosorbent assay for strongyloidiasis in humans. J. Inf. Dis., 144, 427–432.
10) Northern, C. and Grove, D. I. (1987): Antigenic analysis of *Strongyloides ratti* infective larval and adult worms. Immunol. Cell Biol., 65, 231–239.
11) Northern, C. and Grove, G. I. (1988): Western blot analysis of reactivity to larvae and adult *Strongyloides ratti* antigens in mice. Parasite Immunol., 10, 681–691.
12) Sato, Y., Takai, A., Maeshiro, J., Otsuru, M. and Shiroma, Y. (1983): Studies on the preparation of antigen and application of enzyme-linked immunosorbent assay (ELISA) to immunodiagnosis of strongyloidiasis. Ryukyu Med. J., 6, 35–49.
13) Sato, Y., Takara, M. and Otsuru, M. (1985): Detection of antibodies in strongyloidiasis by enzyme-linked immunosorbent assay (ELISA). Trans. Roy. Soc. Trop. Med. Hyg., 79, 51–55.
14) Sato, Y., Otsuru, M., Takara, M. and Shiroma, Y. (1986): Intradermal reactions in strongyloidiasis. Intern. J. Parasitol., 16, 87–91.
15) Sato, Y., Inoue, F., Matsuyama, R. and Shiroma, Y. (1990): Immunoblot analysis of antibodies in human strongyloidiasis. Trans. Roy. Soc. Trop. Med. Hyg. (in press).
16) Towbin, H., Staehlin, T. and Gordon, J. (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc. Natl. Acad. Sci. U.S.A. 76, 4350–4354.
17) Tribouley-Duret, J., Triboulry, J., Appriou, M. and Megraud, R. N. (1978): Application de test E.L.I.S.A. au diagnostic de la strongyloidose. Ann. Parasitol. Hum. Comp., 53, 641–648.