

Possible Approach for Serodiagnosis of Ascariasis by Evaluation of Immunoglobulin G4 Response Using *Ascaris lumbricoides* Somatic Antigen

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Somatic antigen of *Ascaris lumbricoides* was purified to homogeneity (molecular mass, 34 kDa) by ammonium sulfate fractionation and successive chromatographic procedures, namely, gel permeation, ion exchange, and high-performance gel permeation liquid chromatographies. The antigen showed strong binding with immunoglobulin G (IgG) in *Ascaris*-infested patients and was cross-reactive with IgE and IgG in patients infested with other nematodes. It reacted specifically with IgG4 ($P < 0.001$) in 63 *Ascaris*-infested patients, which represented 65% of the total IgG response, though cross-reactivity with IgG1, IgG2, and IgG3 subclasses was observed, indicating the unique specificity of this test system and its potential utility in the serodiagnosis of ascariasis.

Ascaris lumbricoides infects about one-fourth of the world's population (21) and is considered to be one of the causes of various other ailments, namely, intestinal obstruction, acute pancreatitis, acute appendicitis (12), and malnutrition in children (9). Despite the high prevalence of *Ascaris* infection, no good method for diagnosing ascariasis in the context of an epidemiological investigation has yet been devised except for parasitological screening for the presence of eggs in stool. However, this method poses logistical and social problems. The use of an alternative method, such as serodiagnosis, is limited by the extensive cross-reactivity between the antigenic epitopes of different nematodes infective to humans. Ascariasis is associated with elevated immunoglobulin E (IgE) and IgG responses in humans and animals (11, 24). Serological tests based on IgG detection may overestimate the prevalence of infection, due to the persistence of antibodies for a long time after the deworming of patients. Although a novel, specific, and sensitive technique for the serodiagnosis of ascariasis that involves the assessment of *Ascaris* excretory-secretory (ES) antigen-specific IgG4 has been developed (3), procurement of ES antigen from living worms is limited due to the small number and death of the worms after deworming.

As a substance released from living worms, ES antigen possesses a significant antibody response; however, the source of the ES antigen is unknown. The possibility of its derivation from the worm's somatic cell component could not be ruled out. If ES antigen responsible for the IgG4 response in the infected host persists in the worm's somatic cell component(s), this could be a chief alternative source of IgG4-specific antigen for the diagnosis of ascariasis, due to its easy availability from the whole worm rather than from the ES antigen.

The present study describes the purification of the *A. lumbricoides* somatic antigen and its reactivity with serum IgE and IgG, especially with subclasses of IgG by enzyme-linked immunosorbent assay (ELISA), which may be useful markers for diagnosis of *Ascaris* infection in an epidemiological study.

Sixty-three patients (29 males and 34 females, 8 to 65 years of age) from urban and rural areas of West Bengal, India, infested with *Ascaris* were treated with albendazole (400-mg single dose), and during the first 72 h of their posttreatment period, stool samples were collected for three consecutive days from each subject and examined under a microscope. The number of worms expelled (range, 1 to 50) was counted to provide an estimate of the worm burden for each patient. Sera separated from pretreatment peripheral blood were stored in aliquots at -50°C for analysis. For a comparison of the parasitological screening with the serological evidence of ascariasis, stool samples from 126 dyspeptic patients were collected for three consecutive days and examined for the presence of eggs and/or larvae of helminths as before. Ten subjects (six males and four females, 20 to 50 years of age) with no known history of worm infection and with an absence of intestinal nematodes, confirmed by stool examination, served as controls. Groups of 10 subjects (five males and five females, 5 to 40 years of age) infested with hookworm, *Strongyloides stercoralis*, and *Trichuris trichura*, as confirmed by stool tests, were studied. Sera from control subjects and helminth-infested patients were stored at -50°C , and those with mixed infections (i.e., those infested with more than one type of nematode) were excluded from this study.

A. lumbricoides worms were collected from stool from each patient, washed thoroughly with saline, and dissected longitudinally. The body wall of each worm was again washed, homogenized in Tris-buffered saline (TBS; 50 mM, pH 8.0), centrifuged (10,000 rpm; Sorvall RC5B refrigerated centrifuge) for 1 h at 4°C , and concentrated (PM10 membrane). After protein estimation (16), the solution was stored in aliquots at -50°C .

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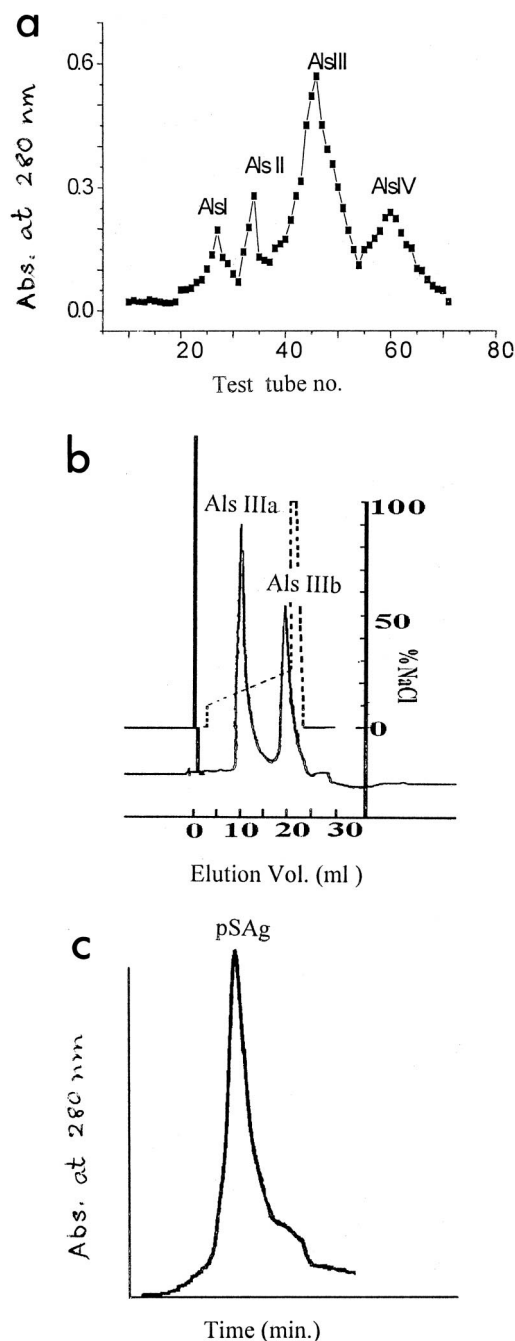


FIG. 1. (a) Gel permeation chromatography of the 30 to 70% ammonium sulfate fraction of *A. lumbricoides* SAg on a Sephacryl S-300 column. (b) Separation of Als III on a Resource-Q anion exchanger by FPLC. (c) Purification of Als IIIb by HPGPLC on a protein PAC 300 SW column. Abs., absorbance.

The extract was precipitated with ammonium sulfate to give products of 30, 70, and finally 100% saturation; these were centrifuged as before, dissolved separately in TBS, dialyzed against the same buffer, and tested for antigenicity by ELISA using sera from *Ascaris*-infested patients. The most immunogenic fraction (30 to 70%, 1 ml, 20 mg of protein) was resolved into four fractions on a Sephacryl S-300 column (1.6 by 90 cm) with TBS as the eluent; these fractions were concentrated and

tested for their binding activities with specific IgE and IgG. Fraction III (0.5 ml, 0.5 mg of protein/ml), which showed high binding activity with specific IgE and IgG, was further passed through a Resource-Q anion exchanger (6.4 by 30 mm) in a fast protein liquid chromatography (FPLC) system with a continuous gradient set up with TBS. The eluted fractions were concentrated and assayed for antigenic activity as before. The more active fraction was further purified by high-performance gel permeation liquid chromatography (HPGPLC) on a protein PAC 300 SW column (7.5 by 75 mm) with 10 mM phosphate-buffered saline (pH 6.8) as the eluent.

Homogeneity of the purified somatic antigen (pSAg) was tested by 7.5% alkaline polyacrylamide gel electrophoresis (4). The molecular size of pSAg was determined by sodium dodecyl sulfate (SDS)-7.5% polyacrylamide gel electrophoresis (14) with the following protein markers: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), lactalbumin (14 kDa), and aprotinin (6.5 kDa).

For ELISA, each fraction of SAg (10 µg/well) in carbonate buffer (pH 9.6), coated on a 96-well assay plate (Flow Laboratories) and blocked with phosphate-buffered saline-Tween 20 (0.05%) containing bovine serum albumin (1%), was incubated with sera from patients (100 µl), which were diluted to 1:64. Biotinylated goat anti-human IgE, IgG, IgG1, IgG2, IgG3, and IgG4 (Sigma), diluted to 1:1,000, were added, following antibiotin-coupled peroxidase. Color developed by *o*-phenylenediamine and H₂O₂ was measured at 492 nm in an ELISA reader to quantify results. For the ELISA inhibition assay, sera from patients, diluted to 1:100, were incubated with SAg and its fractions prior to ELISA. Antigenic proteins needed for inhibition were calculated from a graph of percentage inhibition determined from ELISA reference values.

A nonparametric Mann-Whitney U test was used for testing the significance of differences in worm load between sexes, and Spearman's rank correlation coefficient was used to assess the relation between age, isotype levels, and worm load.

The ammonium sulfate fraction of *A. lumbricoides* somatic extract was separated into four fractions by Sephacryl S-300 column chromatography (Fig. 1a). Als III, being the most im-

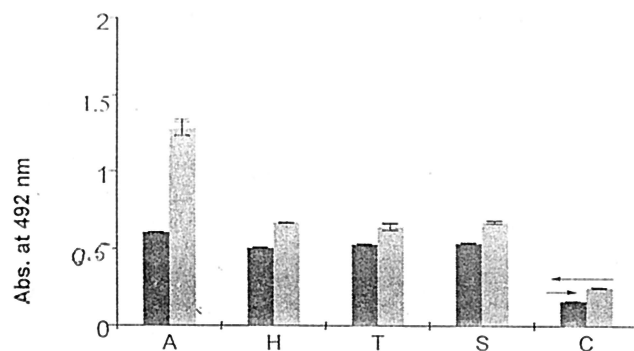


FIG. 2. pSAg-specific IgE and IgG response in patients with different nematode infections. ←, control cut-off value for IgG; →, control cut-off value for IgE. Dark gray bars, specific IgE response; light gray bars, specific IgG response. A, *A. lumbricoides*; H, hookworm; T, *T. trichura*; S, *S. stercoralis*; C, control. Abs., absorbance.

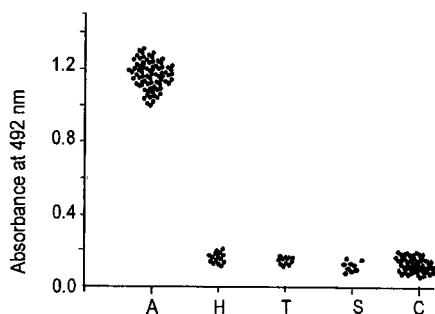


FIG. 3. pSag-specific IgG4 response by ELISA in sera of different groups of individuals: *Ascaris*-infested ($n = 63$), hookworm-infested ($n = 10$), *T. trichura*-infested ($n = 10$), *S. stercoralis*-infested ($n = 10$), and control ($n = 10$) subjects.

munogenic fraction, was separated into two fractions (Fig. 1b), of which Als IIIb, being the more immunogenic of the two fractions as tested by an ELISA inhibition study, resulted in pSag by HPGPLC (Fig. 1c). pSag-specific IgG and IgE were present in the sera of other nematode-infected patients (Fig. 2), suggesting the nonspecificity of this test system; however, specific IgG in *Ascaris*-infested patients was significantly elevated ($P < 0.05$). pSag was homogenous, having a molecular size of ≈ 34 kDa (data not shown).

The cross-reactivities in regard to IgE and IgG turned our attention towards IgG subclass response in the study population, which showed that pSag predominantly reacted with IgG4 in the sera of 63 *Ascaris*-infested patients, comprising 65% of the total IgG response; no such reactivity was observed with hookworm-, *Trichuris*-, and *Strongyloides*-infested patients ($P < 0.001$) (Fig. 3). Cross-reactivity was observed with pSag-specific IgG1 (20% of hookworm, 10% each of *T. trichura* and *S. stercoralis*), IgG2 (60% of hookworm, 80% of *T. trichura*, and 60% of *S. stercoralis*), and IgG3 (20% of hookworm, 10% of *T. trichura*, and 30% of *S. stercoralis*).

A comparison of the results of stool examination and of serological evidence of ascariasis in 126 individuals (Table 1) shows that these results are compatible and that such a comparison can be regarded a sensitive assay. Figure 4 shows the relationship between absorbance levels of IgG4 response and worm loads of patients.

The Mann-Whitney U test showed no significant difference ($P > 0.05$) between males ($n = 29$) and females ($n = 34$) regarding the worm load. Spearman's rank correlation coefficient analyses also revealed no significant association between isotypes, age, and worm load between sexes ($P > 0.05$).

TABLE 1. Comparison of stool examination and serological evidence of ascariasis

Subjects	Ascaris/IgG4 ^a			
	+/+	+/-	-/+	-/-
Men ($n = 33$)	23/23	0/0	0/0	10/10
Women ($n = 28$)	16/16	0/0	0/0	12/12
Boys ($n = 34$)	24/24	0/0	0/0	10/10
Girls ($n = 31$)	20/20	0/0	0/0	11/11
Total ($n = 126$)	83/83	0/0	0/0	43/43

^a Values are the number of samples with the indicated results.

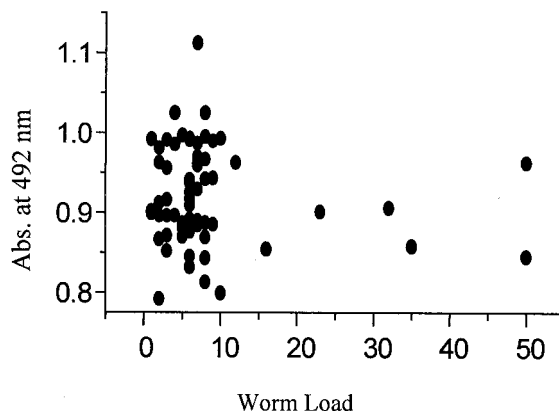


FIG. 4. Relationship between absorbance levels of IgG4 response and *A. lumbricoides* worm load. Abs., absorbance.

Adult *A. lumbricoides* worms produce pSag-specific IgE and IgG in infested humans. We observed significant cross-reactivity of *A. lumbricoides* ES antigen, fraction Als IIIb, with IgE and IgG of different nematode-infected patients (3), which was not specific for serodiagnosis. The present study shows that, in ascariasis, distribution of specific antibodies occurs among the IgG subclasses and IgG4 response is highly elevated. IgG4 response was studied in lymphatic filarial infections (13), onchocerciasis (5, 17), hookworm infection (19), strongyloidiasis (22), and ascariasis (3), and this subclass appears to be a marker of active infection that is useful for serodiagnosis. In our *Ascaris*-infested study population, IgG4 level was found to be independent of the worm load. IgG4 antibody is prominent in total antigen-specific IgG response when antigenic exposure is chronic (1), and this finding is supported by results of several clinical studies showing high IgG or IgG4 levels in subjects with various atopic disorders (2, 6–8, 20, 23, 25) and chronic schistosomiasis (10), as well as those with chronic filariasis (18). The fact that this immunoglobulin cannot be induced in humans by the epitope phosphorylcholine, which is shared by all helminthic parasites (15), and consequently that the possibilities of false-positive results are omitted, enables the assay system to be more predictive about *Ascaris* invasion.

Recombinant proteins, though they have been used for diagnostic purposes, still show limitations in clinical tests and are high in cost and not readily available in less developed regions, where the disease is an important public health issue. In this regard, somatic antigen, which contains epitope against specific IgG4 and is widely available, may be considered an alternative diagnostic method of nominal expense and no cross-reactivity. This increased specificity of an IgG4-based assay may be used for serodiagnosis of ascariasis in large populations.

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