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Characterization of a recombinant immunodiagnostic antigen (NIE) from *Strongyloides stercoralis* L3-stage larvae☆

Varatharajalu Ravi, Srinivasan Ramachandran [1], Robert W. Thompson, John F. Andersen, Franklin A. Neva *

Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 4 Center Drive, Room 126, Bethesda, MD 20892, USA

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Abstract

Due to the process of internal autoinfection, even chronic asymptomatic infections with *Strongyloides stercoralis* have the potential to become severe disseminated disease with fatal outcome. Intermittent and scanty larval excretion makes parasitologic diagnosis difficult. Serodiagnosis is helpful, but antigen preparation from infective larvae requires access to patients or immunosuppressed experimental animals. For these reasons, attention has turned to recombinant antigens for immunodiagnosis. A 31-kDa candidate antigen (NIE) derived from an L3 cDNA library is described in this report. Multiple alignment of the deduced amino acid sequence of NIE showed approximately 12–18% identity with various other organisms, including 17.9% of Asp1 of *Ancylostoma caninum*, 12.6% of *Hemonchus contortus*, and 17.6% of insect venom allergen 5 of yellow jacket. By ELISA, antibodies to the purified recombinant NIE antigen were demonstrated in 87.5% of 48 sera from strongyloides-infected patients and in only 6.5% of sera from presumed normal controls. Immunoreactivity of purified NIE antigen with parasite-specific IgE from sera of strongyloides-infected patients indicated its potential use as an immediate sensitivity skin test antigen. This application of the NIE antigen was supported by its capacity to trigger release of histamine upon in vitro exposure to blood from strongyloides-infected patients and its failure to produce histamine release from blood of normal controls.

Keywords: Antibodies; Basophils; Histamine; Immunodiagnosis; *Strongyloides stercoralis*

1. Introduction

Although infection with the intestinal nematode *Strongyloides stercoralis* has worldwide distribution, most affected individuals are asymptomatic. Some of the parasite larvae normally excreted in the feces may transform to infective stages prior to excretion and re-infect the same host. This process, known as internal autoinfection, enables the parasite to persist for many years even in immunocompetent individuals; however, in people who are immunosuppressed—especially from administration of steroids for other underlying conditions—the autoinfective process is greatly increased, so that the parasite literally multiplies in the host. The consequent dissemination of larvae to various organs, referred to as hyperinfection syndrome, is often accompanied by Gram-negative bacterial sepsis, leading to a fatal outcome if not recognized and treated appropriately [1,2], yet in the chronically infected immunocompetent individual, excretion of larvae is scanty and intermittent. Thus parasitologic diagnosis of *S. stercoralis* is difficult.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; kDa, kilodalton; PBST, phosphate-buffered saline plus Tween; TBST, Tris buffered saline plus tween-20.

☆ *Note:* GenBank™ accession numbers: NIE protein from *S. stercoralis* (AAB97359); Asp1 from *Ancylostoma caninum* (Q16937); *C. elegans* (T31959); *Hemonchus contortus* (AAC47714); insect venom allergen 5 of Ves5 of *Vespula vulgaris* (AAA30333); Pol5 from *Polistes annularis* (Q05109); plant pathogen-related protein of tobacco (PR-1A protein precursor) (P08299).

* Corresponding author. Tel.: +1-301-496-2486; fax: +1-301-480-1394

E-mail address: fneva@nih.gov (F.A. Neva).

[1] Present address: Functional Genomics Unit, Center for Biochemical Technology, Delhi 110 007, India.

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Immunologic detection of specific strongyloides antibodies by enzyme-linked immunosorbent assay (ELISA), using filariform (L3) larvae of the parasite as antigen, has been found useful for diagnosis [3–6]. Recently, another method of immunodiagnosis by means of an immediate hypersensitivity skin test has been described [7]. An advantage of these immunologic tests for diagnosis, in contrast to direct demonstration of the parasite, is that they are not dependent on certain levels of larval excretion. All immunologic tests require a readily available and, hopefully, convenient source of antigen. For these reasons, attention has turned to use of recombinant antigens that show potential for sensitive and specific immunodiagnosis of *S. stercoralis* infections [8]. In this report, we describe preparation, purification, and properties of another recombinant protein antigen (NIE) derived from the L3 (infective) stage of the parasite. In contrast to earlier recombinant antigens reported [8], the recombinant NIE antigen is capable of releasing histamine from basophils of infected patients, a requirement for an immediate skin test antigen.

2. Materials and methods

2.1. Immunoscreening of L3 cDNA library

Preparation of the library from infective-stage (L3) larvae obtained from an infected human has been described [9], as has the protocol used for immunoscreening [8]. After inducing the phage by adding 10 mM isopropyl-1-β-D-thio-1-galactopyranoside (Gold Biotechnology, St. Louis, MO) for 3.5 h at 37 °C, about 200 000 plaques were screened. The plaque blots were blocked overnight with 2% casein (Casein Hammerstein, ICN, Aurora, OH) in 1 × Tris buffered saline (TBS; 20 mM Tris–HCl, pH 7.5, 150 mM NaCl) and then washed with TBST (1 × TBS plus 0.05% Tween-20).

A high titre anti-*S. stercoralis* serum from a single patient was pre-absorbed with 10 vol of saturated *Escherichia coli* lysate and subsequently diluted to 1:250 in 2% casein in 1 × TBS. Blots were incubated with the serum at a final dilution of 1:250 for 1 h at room temperature. The filters were washed several times with TBST and incubated with goat anti-human IgG-conjugated alkaline phosphatase (Jackson Immuno-Research, West Grove, PA) at a 1:50 000 dilution in TBST for 1 h at room temperature. The filters were washed again with TBST and the blots developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate kit (Kirkegaard & Perry, Gaithersburg, MD).

2.2. Sequencing and cloning of NIE gene

Positive plaques were re-screened using the same procedure until a purified plaque was obtained. Bluescript SK^- plasmid containing the recombinant inserts was excised using an excision kit (Stratagene, La Jolla, CA), and the plasmid DNA was purified using the Qiagen kit (Chatsworth, CA). As the recombinant inserts were generally small, they were sequenced on both strands using a combination of several primers (reverse, T3, SK^-, and universal) with an ABI Prism automated sequencer (Perkin–Elmer, Foster City, CA). Additional analysis of deduced amino acid sequence of NIE was performed with the on-line tools at http://www.expasy.org, a BLAST search was done against *S. Stercoralis* dbEST database available at http://www.nematode.net, and protein multiple alignment was done with DNAstar program (DNAstar Inc., Madison, WI).

The cDNA insert was excised from pBluescript SK^- with *Eco*RI and *Xho*I (New England Biolabs, Beverly, MA), and the fragments were cloned into pET30b (Novagen Inc., Madison, WI), which has the same reading frame as the Bluescript plasmid at the *Eco*RI site. Recombinant plasmid was isolated and inserts were sequenced to ensure authenticity of the reading frame [10]. Recombinant plasmid containing the NIE insert was transformed into *E. coli* strain BL21 (DE3), and protein was produced with an N-terminal fusion protein containing plasmid encoded 52 amino acids including six his tag in addition to NIE protein.

2.3. Expression and purification of NIE protein

The NIE recombinant protein was isolated from insoluble inclusion bodies using the following protocol. Luria broth (6 l, with 100 μg ml^{-1} ampicillin in six bacterial culture flasks; Beckman, Fullerton, CA) was inoculated with an overnight culture of *E. coli* BL21 (DE3) containing the plasmid NIE cDNA gene in pET30b. The flasks were shaken at 37 °C and 150 rpm until the optical density $(OD)_{600}$ of the culture reached 0.6. Isopropyl-1-β-D-thio-1-galactopyranoside was added to a concentration of 1 mM, and the cultures were shaken at 37 °C for 4 h. The cells were harvested by centrifugation, washed with 20 mM Tris–HCl (pH 7.5), and stored at −70 °C. Inclusion bodies were purified as described by Palmer and Wingfield [11]. After centrifugation at 100 000 × *g* for 1 h at 4 °C, the protein was purified using the His Bind kit (Novagen, Inc.). Six milliliter of 50% saturated resin was added to the 20-ml protein sample containing 20 mM imidazole and shaken for 15 min. The mixture was packed under gravity flow on a polypropylene column and washed with 40 column volumes of wash buffer (20 mM Tris–HCl [pH 7.5], 300 mM NaCl, 20 mM imidazole, and 6 M urea). Protein was eluted with 10 column volumes of

eluting buffer (20 mM Tris–HCl [pH 7.5], 300 mM NaCl, and 250 mM imidazole). Recombinant protein samples were concentrated to 10 ml by ultrafiltration through Amicon filters (Amicon, Beverly, MA) with 10-kDa cutoff. Recombinant protein samples were then run on a size-exclusion column (Superdex 75 HiLoad^{TM} 16/60; Amersham Pharmacia Biotech, Piscataway, NJ) to remove high molecular weight contaminating proteins. The eluates were diluted 10 fold and dialyzed in stepwise decreasing concentrations of urea (5–0.25 M in 20 mM Tris–HCl [pH 7.5] with 300 mM NaCl, 5 mM EDTA, and 2 mM DTT) to allow slow renaturation and subsequently against PBS at room temperature. Dialysis was done at each step for at least 2 h. The protein sample was concentrated with Centricon 10 (Amicon) in such a way that the concentration of protein was between 70 and 100 μg ml^{-1}. The concentration of protein was estimated using the micro-bicinchoninic acid method [12] (Pierce, Rockford, IL).

2.4. Assay for antigen-induced release of histamine

Quantitative determination of histamine released from blood of Strongyloides-infected patients by in vitro exposure to *S. stercoralis* antigens was performed with a histamine immunoassay kit (Immunotech, Beckman Coulter, France). The assay is based on the reaction of parasite-specific IgE bound to specialized receptors on the surface of peripheral blood basophils (and tissue mast cells), which trigger the release of histamine and other mediators from intracellular granules on exposure to parasite-specific antigens that crosslink the IgE receptors. Two hundred microliters of 1:7 diluted blood (must be collected in heparin as anticoagulant) was added to 300 μl of histamine-releasing buffer containing serial concentrations of either NIE or *S. stercoralis* somatic antigens under test. A sample for total histamine release was prepared by adding 50 μl of undiluted heparinised blood to 950 μl of distilled water, freeze-thawing three times, and storing at −20 °C until tested. For measuring spontaneous histamine release, 100 μl of histamine-release buffer was added to 200 μl of a 1:7 diluted suspension of heparinised blood. Quantification of the released histamine was performed by acylating samples and standards, which were then added to antibody-coated plates provided in the kit, along with histamine-alkaline phosphatase conjugate (also provided in the kit). Plates were developed with *p*-nitrophenol phosphate substrate (Kirkegaard & Perry) and read at 410 nm (Dynatech-MR5000; Springfield, VA). The lowest concentration of acylated histamine detected by the assay (sensitivity) was 0.5 nM. Histamine release was calculated using the following formula:

$$\%\ \text{of histamine release} = \frac{\text{Sample histamine release} - \text{spontaneous release}}{\text{Total histamine release} - \text{spontaneous release}} \times 100.$$

2.5. Preparation of somatic S. stercoralis L3-stage antigen

Somatic *S. stercoralis* antigen was prepared as described previously [4,8] from fecal samples of humans and monkeys infected with a Southeast Asian strain of the parasite. The method involved culture of a fecal suspension mixed with charcoal at room temperature for 7–14 days to permit development of free-living adult worms and production of infective (L3) larvae. The larvae were separated from fecal debris and charcoal by the Baermann procedure [13] and washed extensively. The antigen was stored at −70 °C for later use.

2.6. Western blotting and ELISA

Purified recombinant protein (1 μg per lane) or *S. stercoralis* somatic antigen (10 μg per lane) was separated by either 10 or 4 to 12% NuPAGE bis-Tris polyacrylamide gels (Invitrogen, San Diego, CA) at 70 mA for 40 min (200 V, constant) and transferred to nitrocellulose membranes under 25 V (constant) for 90 min. Blots were blocked in 5% skim milk in phosphate-buffered saline containing 0.1% Tween (PBST) overnight, washed with PBST, and subsequently incubated with either 1:100 human sera or 1:50 000 rabbit anti-NIE serum (dilutions made in PBST containing 2% skim milk) for 1 h at room temperature. After washing with PBST, membranes were incubated with either 1:50 000 diluted goat anti-human IgG or goat anti-rabbit IgG (both Fc-fragment specific) conjugated with peroxidase. For IgE Western blots, the membranes were incubated at room temperature for 2 h in a 1:50 dilution of IgG-depleted serum in PBST (by overnight exposure at 4 °C–2 vol of 50% Gamma Bind G Sepharose [Pharmacia] to 1 vol of pooled undiluted serum). The membranes were then exposed to affinity-purified goat anti-human IgE (Kirkegaard & Perry) conjugated with peroxidase at a 1:5000 dilution. Bound antibodies were detected by chemiluminescence on addition of luminol substrate (Super Signal West Pico stable peroxidase; Pierce).

For ELISA, the recombinant NIE antigen was coated on 96-well polystyrene plates (Costar, Cambridge, MA) at a protein concentration of 0.125 μg ml^{-1} in coating buffer and held at 4 °C overnight. The working antigen concentration was determined by prior 'box titration' of 2-fold dilutions of a standard positive pool of human serum versus serial antigen concentrations from 2.0 to

0.062 μg protein per ml. After overnight incubation of antigen, the wells were rinsed and remaining sites blocked by incubation at 37 °C for 1 h with 150 μl PBST (Tween 0.05%) containing 5% skim milk. The standard positive serum pool was always tested in duplicate at 2-fold dilutions ranging from 1:16 to 1:1024 to establish a curve of reactivity. A standard antibody-negative pool of serum was also always tested at dilutions of 1:16, 1:32, and 1:64. After 1 h at 37 °C, plates were washed with PBST and incubated with goat anti-human IgG-conjugated alkaline phosphatase at a 1:5000 dilution in PBST. Plates were washed in PBST and measured at 410 nm (Dynatech–MR5000) after developing with *p*-nitrophenol phosphate substrate.

2.7. Sources of serum from patients

Parasite-positive sera were obtained from patients in whom *S. stercoralis* larvae had been demonstrated in fecal specimens not more than 1 month before being tested for presence or absence of antibody. Some of these patients had signs or symptoms of clinical disease, but many were asymptomatic. Most of the parasite-positive patients had also been skin tested with immediate hypersensitivity *S. stercoralis* skin test antigens [7]. A pool of nine such serum samples was used as a positive control in ELISA. A negative control serum pool was made from eight patients seen at the National Institutes of Health Clinical Center for other illnesses such as cutaneous leishmaniasis and unexplained eosinophilia. The 48 Strongyloides-negative sera used to screen the NIE antigen for reactivity came from normal blood donors at the National Institutes of Health blood bank. For patients representing filarial infections, the cause was determined by history of appropriate exposure, clinical findings such as recurrent calabar swellings, itching of the skin, finding of microfilariae in the blood or in skin snips, and positive filarial antibody tests. In a few instances, dual infections of *Loa loa* with *Onchocerca volvulus* could not be ruled out. Most of these sera were made available by Dr Thomas B. Nutman (Laboratory of Parasitic Diseases, NIAID, NIH).

2.8. Rabbit antibody to recombinant NIE protein

The band of purified recombinant NIE protein was excised from a SDS polyacrylamide gel after electrophoresis to provide antigen for immunization. A primary dose of 200 μg protein in complete Freund's adjuvant was given intradermally to a rabbit (Spring Valley, Woodbine, MD), followed by three booster injections each containing 100 μg protein in incomplete Freund's adjuvant. For use as a probe, the polyclonal rabbit antiserum was used at a dilution of 1:50 000.

2.9. Statistical analysis

The significance of variance between control and experimental groups was determined by a Mann–Whitney *U*-test using a STATVIEW program (SAS Institute, Inc., Cary, NC).

3. Results

3.1. Sequence analysis of NIE clone

The *S. stercoralis* cDNA library was screened with a single high-titre human serum to identify an immunodiagnostic antigen. One clone, referred to as NIE, was selected for further characterization. Lack of a start codon for methionine indicated a partial cDNA clone, and examination of the sequence showed the presence of 792 nucleotides. The 3′ end of the clone contained a translation stop codon (TAA) and a polyadenylation site (AATAAA) located 11 base pairs upstream of the polyA tail. A partial cDNA coding sequence contained 687 base pair codes for 229 amino acids. The deduced amino acid sequence of NIE scanned against PROSITE [14,15] suggested that the clone was a highly basic protein with an isoelectric point of 9.32. Possible N-linked glycosylation site residues NNTF and tyrosine phosphorylation site residues KLFNEQNKY are shown in Fig. 1. NIE protein containing an extracellular domain from 180 to 190 (-GHFTQLKWKGT-) amino acid residues agreed 100% with the signature sequence of extracellular proteins ([G, D, E, R]) H [F, Y, W, H] TQ [L, I, V, M] 2W × 2 [S, T, N]) as with sperm-coating glycoprotein, plant pathogenic response protein (PR-1), insect venom allergen 5, mammalian glioma pathogenesis-related protein, and Ancylostoma secreted protein 1 (Asp1) from the dog hookworm.

The multiple alignment of amino acid sequence of NIE antigen showed approximately 12–18% identity with other organisms, such as 17.9% with Asp1 (Ancylostoma secreted protein1) and PR-1A (plant pathogenesis related protein 1A precursor) of *Ancylostoma caninum* [16] and tobacco [17], respectively; 12.6% with *Hemonchus contortus* [18]; and 14% with *C. elegans*. Insect venom allergen 5 of Ves5 of yellow jacket and pol 5 of paper wasp showed 17.6 and 13.4% identity, respectively, [19]. The function of the protein NIE and other homologues is yet to be elucidated.

3.2. Western blot analysis

The NIE antigen appeared as a dominant band at 38 kDa when a lysate of *E. coli* transformed by the NIE clone was electrophoresed on a 4–12% NuPAGE gradient gel. This is shown in lane 2 of panel A in Fig. 2. Lane 1 of the same panel shows the NIE antigen after

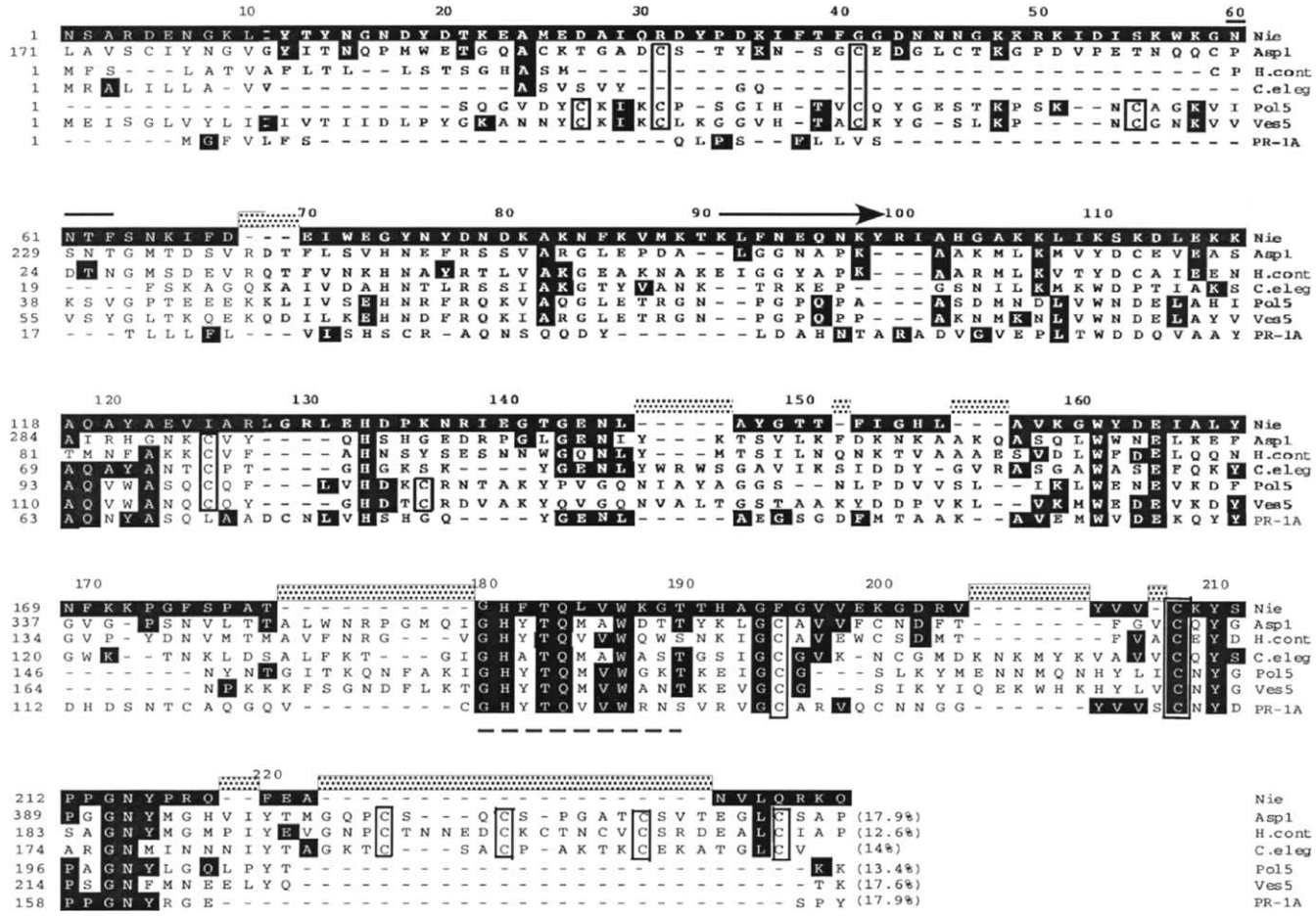

Fig. 1. Comparison of recombinant NIE protein-deduced amino acid sequences with those of selected homologous proteins. Shaded residues are identical to those in NIE; dashed lines from residues 180 to 190 represent an extracellular motif. Conserved cysteine residues are shown in boxes. Possible N-linked glycosylation is indicated by a line from amino acids 60–63. Tyrosine phosphorylation site residues from 91 to 99 are indicated by the arrow. NIE protein from *S. stercoralis* (AAB97359); Asp1 from *A. caninum* (Q16937, residues 171–424); *H. contortus* (AAC47714); *C. elegans* (T31959); Pol5 from paper wasp (Q05109); Ves5 from yellow jacket (AAA30333); plant pathogenesis related protein 1A precursor (PR-1A) of tobacco (P08299).

purification on an ion-exchange and size exclusion column under the same conditions of electrophoresis.

Lanes 1–3 of panel B (Fig. 2) contained purified NIE antigen that was first electrophoresed and then blotted onto nitrocellulose membranes. Lane 1 was probed with mouse anti-His tag antibody. Lane 2 was probed with pooled serum from *S. stercoralis*-infected patients and secondarily with phosphatase-labelled goat anti-human IgG. Lane 3 was probed with the same pooled human serum from infected patients that had been depleted of IgG and secondarily exposed to peroxidase-labelled goat anti-human IgE. This indicated that the NIE antigen had epitopes for both IgG and IgE isotypes.

Panel C of Fig. 2 shows blots of recombinant NIE antigen in Lane 1 and larval somatic antigen in Lane 2 after electrophoresis and staining of both preparations with polyclonal rabbit anti-NIE serum. The appearance of two bands, one weakly reactive of 42 kDa and the other strongly reactive of 31 kDa, reveals that 31 kDa might be a NIE protein in the parasite. The 42-kDa protein might be an indication of one additional NIE-like protein in the parasite. Cluster analysis of the NIE amino acid sequence against the *S. stercoralis* genome database available at http://www.nematode.net revealed the presence of three clusters showing homology of 97, 41, and 40% with clusters SS00064, SS01580, and SS01574, respectively. None of these clusters contain ATG as the first ORF, indicating the submitted EST sequences are missing the 5′ end. Clusters SS01580 and SS01574 shared identity of 77% at the amino acid level. It is difficult to conclude which of these genes codes for the 42-kDa protein.

3.3. Recombinant NIE antigen for immunodiagnosis

Serum samples from 48 individuals with parasitologically confirmed *S. stercoralis* infections were tested at dilutions of 1:16, 1:32, and 1:64 for IgG antibodies against the recombinant NIE antigen by ELISA. All of these sera had previously been found to have signifi-

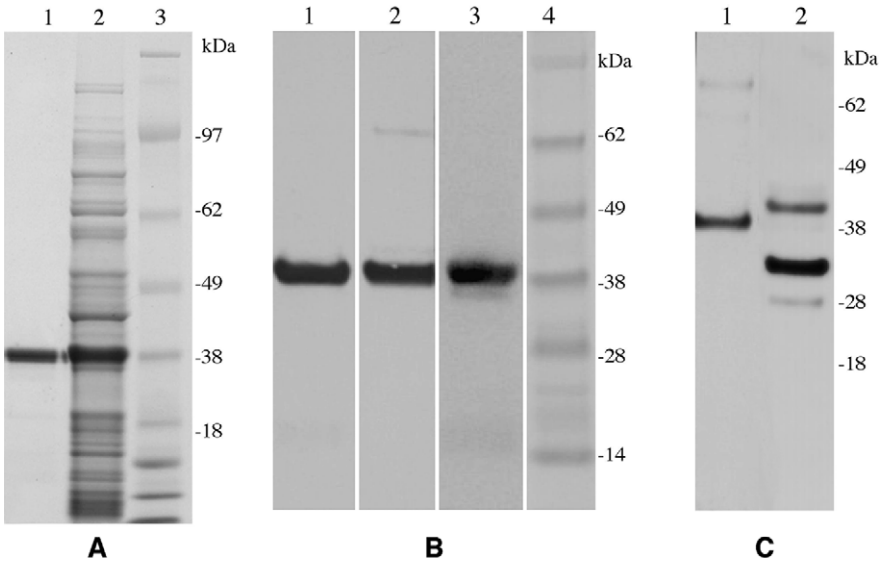

Fig. 2. SDS-PAGE and immunoblot analysis of recombinant NIE antigen. Panel A. Lane 1 shows the NIE antigen after purification on an ion-exchange column. Lane 2 is a lysate of *E. coli* BL21 (DE3) transformed with NIE plasmid. Lane 3 of the same panel shows molecular weight markers. The proteins were fractionated on a 4–12% SDS-PAGE gradient gel. Panel B. Lanes 1–3 contain purified NIE antigen that was first electrophoresed and then blotted onto nitrocellulose membranes. Lane 1 was then probed with mouse anti-His tag antibody. Lane 2 was probed with pooled serum from *S. stercoralis*-infected patients and secondarily with hydrogen peroxidase-conjugated goat anti-human IgG. Lane 3 was probed with the same pooled human serum from infected patients that had been depleted of IgG and secondarily exposed to affinity-purified goat anti-human IgE conjugated with hydrogen peroxidase. Lane 4 has molecular weight markers. Panel C. Lane 1, recombinant NIE antigen; Lane 2, larval somatic antigen. Both lanes were electrophoresed on a 4–12% SDS-PAGE gel, blotted, and probed with polyclonal anti-NIE serum raised in rabbit. The appearance of two bands in lane 2, one weakly reactive at 42 kDa and the other strongly reactive at 31 kDa, suggests that the 31-kDa band represents the NIE protein in the parasite.

cantly elevated IgG levels by ELISA with L3 *S. stercoralis* somatic antigen. A similar number of sera to represent presumably strongyloides-negative people were obtained from normal blood donors from the National Institutes of Health blood bank; however, their negative infection status was not confirmed by fecal examinations. The antibody level, or 'cutoff' (35 U ml^{-1}), separating positive from negative was based on the results obtained with a mean value plus three times the standard deviation of 48 negative control sera. Sera from 42 of 48 infected patients, or 87.5%, were reactive with the recombinant NIE antigen. The frequency of positive reactions among the group of blood bank normal donors was only 3 of 48, or 6%. These results are shown in Fig. 3; Mann–Whitney *U*-test analysis of variance ranks, $P < 0.0001$. The recombinant NIE antigen was 87.5% sensitive and 94% specific in detecting antibodies from *S. stercoralis*-infected people.

3.4. Lack of crossreactivity of recombinant NIE antigen with filarial sera

Serum samples from 23 individuals with different filarial infections caused mainly by *O. volvulus* and *L. loa* (Table 1) were selected for study of immunologic crossreactions with somatic *S. stercoralis* and recombi-

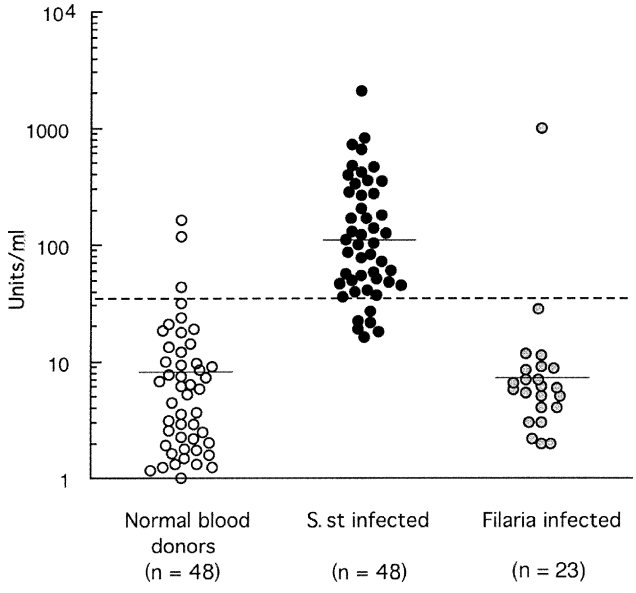

Fig. 3. Distribution of antibody levels to NIE antigen as determined by ELISA in 48 normal blood donors, 48 *S. stercoralis*-infected individuals, and 23 filarial parasite-infected individuals. A level of 35 U ml^{-1} separated positive from negative reactions. NIE-specific antibody was elevated significantly in *S. stercoralis*-infected patients vs. normal donors and filaria-infected patients ($P < 0.0001$, Mann–Whitney *U*-test). There is no statistical difference between normal blood donors and filaria-infected patients.

Table 1
Lack of crossreactivity of NIE antigen with sera of patients with various filarial infections

Filarial infection	Number antibody positive	
	Somatic *S. stercoralis* antigen	Recombinant NIE antigen
O. volvulus	7/8	0/8
L. loa	4/7	0/7
O. volvulus+*Loa*	1/1	0/1
M. perstans	0/1	0/1
M. perstans+*Loa*	1/1	0/1
Tropical pulmonary eosinophilia[a]	5/5	1/5
Total	18/23	1/23

[a] Tropical pulmonary eosinophilia presumably caused by *W. bancrofti* [20].

nant NIE antigens. Both antigens were tested against sera at 1:16, 1:32, and 1:64 dilutions for IgG antibodies by ELISA. Patients with tropical pulmonary eosinophilia were from India and were considered to have occult infections with *Wuchereria bancrofti*. Eighteen of 23 sera gave positive reactions with the somatic *S. stercoralis* antigen, which included all except one *O. volvulus* case, four of seven *L. loa* patients, and all tropical pulmonary eosinophilia cases; however, the recombinant NIE antigen was positive with only one of the 23 filarial sera (Table 1). There was no significant difference in mean antibody levels to NIE antigen between 48 normal blood donors and 23 filarial patients ($P > 0.8018$). In contrast, there was a clear difference in mean antibody levels in the 23 filaria-infected individuals as compared with the 48 *S. stercoralis*-infected patients ($P < 0.001$) as shown in Fig. 3.

3.5. Histamine release by NIE antigen from blood of strongyloides-infected patients

The capacity of allergens to induce release of histamine from peripheral blood basophils or cutaneous mast cells results from crosslinking of antigen with specific IgE on the surface of the sensitized cells and consequent discharge of histamine and other mediators from intracellular granules [21]. This reaction was used to assess the capacity of antigens to be used in an immediate hypersensitivity skin test. All patients whose blood was used to test the NIE antigen for histamine release had *S. stercoralis* larvae in fecal specimens and also had positive immediate skin tests with both the somatic and E/S skin test antigens [7]. Heparinised blood from nine of ten parasite-positive individuals showed positive histamine release tests with NIE antigen concentrations ranging from 10 to 0.61 μg of protein per ml. All ten of the parasite-positive cases showed positive histamine release tests with L3 somatic antigen at protein concentrations ranging from 10 to 0.61 μg ml^{-1}. The percentage of total histamine released by the NIE antigen ranged from 20 to 40%. The somatic larval antigen showed greater effectiveness of histamine release, with percentages as high as 58%. The mean percent histamine release obtained with the recombinant as compared with the somatic larval antigen is shown in Fig. 4. All of six blood samples collected from presumably normal blood bank donors were negative, showing less than 5% histamine release with a full range of antigen concentrations. Total histamine release on each specimen was determined by freeze-thawing the specimen several times and then processing the freeze-thawed specimen in the same way as the test specimens. The percent of histamine released spontaneously was also assayed on each specimen and subtracted from the total value.

4. Discussion

Immunodiagnosis of active or recent infection by *S. stercoralis*, primarily by ELISA, has been reported by investigators from different geographic areas [3–5]. All reports indicate approximately 85–95% sensitivity of the ELISA in parasitologically positive cases of infection. Except in instances of obvious crossreactivity to other infections, it is difficult to assign precise specificity to a test for strongyloidiasis because absence of infection cannot readily be established. The main drawback to immunodiagnosis of this infection at present is the inconvenience of preparing larval antigen from feces of heavily infected human or experimentally infected animal hosts. Fecal specimens must be cultured for approximately a week to allow development of L3-stage larvae. Then some variety of Baermann concentration method followed by alternate cycles of high- and low-speed centrifugation is required to obtain sufficient concentrations of clean larvae to process for antigen. Preparation of a suitable recombinant antigen with equivalent sensitivity and specificity to existing crude antigens would greatly facilitate one of the problems of immunodiagnosis of strongyloidiasis. Recombinant antigens from *S. stercoralis* larvae have recently been described, although information on their suitability as antigens for ELISA is still scanty [8,22]. Furthermore, the recombinant antigens described in our previous report [8] were able to react with IgG and IgE antibodies by ELISA and Western blots but were not capable of releasing histamine from basophils of infected patients (unpublished data). Therefore, they would not be suitable candidates for use as antigens for an immediate skin test, in contrast to the NIE recombinant antigen described in this report.

A specific and sensitive recombinant antigen that could be produced in large amounts, be easily purified,

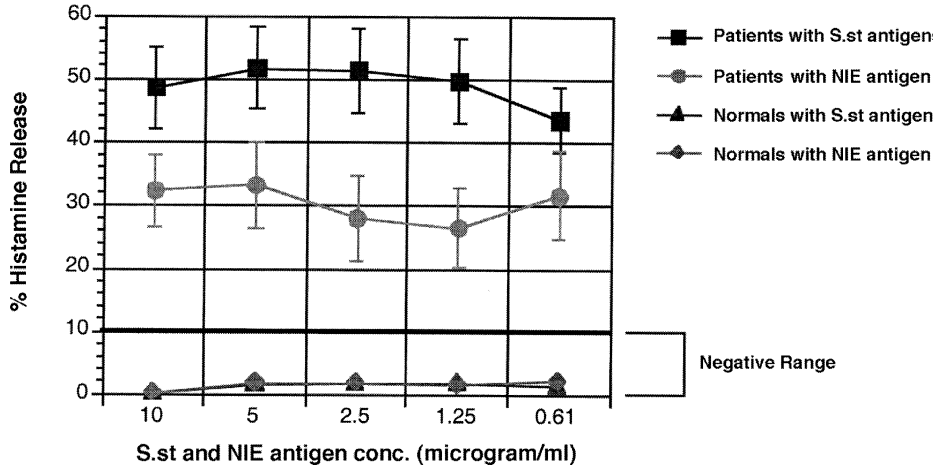

Fig. 4. The means and ranges of percent histamine released by various concentrations of recombinant NIE and strongyloides somatic antigens when exposed to blood of strongyloides-infected patients and normal blood donors.

and be usable for detection of antibody as well as for immediate hypersensitivity skin testing would be ideal. Such an antigen could be used for evaluation of individual patients and for epidemiologic studies. Therefore, the NIE antigen represents a definite improvement over our earlier recombinant antigens.

The homology detected by the amino acid sequences of the NIE antigen was to several secretory proteins of diverse origins that include several nematodes and insect venoms. All of these proteins are known to be rich in cysteine, but the NIE antigen has only one conserved cysteine in the 208th position. In addition to the amino acid sequence homology between the NIE and insect venom antigens, they share properties of immunologic function as indicated by their ability to act as allergens.

Although the NIE protein could be optimally expressed in *E. coli* by the T7 promoter, the final product formed inclusion bodies. Refolding and concentration of the NIE recombinant is still an unresolved problem. For example, one batch of 6-l culture yielded only 300 $\mu g\ ml^{-1}$ of soluble refolded protein. Extensive dilution and dialysis in either Tris–HCl buffer or in PBS with varying concentrations of NaCl, glycerol, SDS, mannitol, sucrose, pH, isopropanol, DTT, EDTA, and Triton X-100 were not useful in increasing solubility. Increasing concentrations of the purified refolded protein greater than 100 $\mu g\ ml^{-1}$ led to aggregation. If the NIE recombinant is to have a future role as a skin test antigen, improvements in purification and refolding of the protein will be necessary.

Various sources cite the specificity of immunodiagnosis of human strongyliodes infection by ELISA as ranging from 85 to 95% [23,24]. Even though only 48 sera were tested with NIE recombinant antigen, it was interesting that the percentage of positive reactions fell within this range. This suggests that recombinant antigens such as NIE may approach or equal the specificity of the crude somatic antigen used in most laboratories for diagnosis. It should also be noted that the recombinant NIE antigen was used at a protein concentration of 0.125 $\mu g\ ml^{-1}$, whereas the somatic antigen protein concentration used in our laboratory is generally 0.50 $\mu g\ ml^{-1}$. A further application of the recombinant antigen for diagnostic use that remains to be explored is the use of synthetic peptides as antigens. Preliminary results indicate that certain synthetic peptides of NIE can detect IgG serum antibodies in strongyloides-infected patients (unpublished data).

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