

STRONGYLOIDES STERCORALIS RECOMBINANT NIE ANTIGEN SHARES EPITOPE WITH RECOMBINANT Ves v 5 AND Pol a 5 ALLERGENS OF INSECTS

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Abstract. A new recombinant protein (NIE) for immunodiagnosis of human *Strongyloides* infection has 13% to 18% amino acid identity with antigen 5 insect venom allergen, but the C-terminal segment of NIE showed highest identity with Ves v 5 (yellow jacket) and Pol a 5 (paper wasp). A rabbit polyclonal anti-NIE antibody identified a single band of NIE antigen as well as bands of Pol a 5 and Ves v 5 antigens, and mouse anti-Pol a 5 and anti-Ves v 5 sera reacted with recombinant NIE antigen by Western blot. A cyanogen bromide-digested C-terminal fragment of NIE was reactive with mouse anti-Ves v 5 and Pol a 5 antibodies as well as with rabbit anti-NIE serum. Although IgE and IgG antibodies from pooled sera from *Strongyloides*-infected patients reacted with Pol a 5 and Ves v 5 recombinant antigens on immunoblots, neither antigen inhibited human IgG reaction with NIE antigen in a competitive enzyme-linked immunosorbent assay.

INTRODUCTION

In the process of characterizing a new recombinant protein (NIE) for use in immunodiagnosis of strongyloidiasis,¹ the recombinant protein was found to exhibit 13–18% amino acid homology with a venom allergen, antigen 5 (Ag 5), of hornets, wasps, and yellow jackets.² NIE has variable degrees of homology with proteins from diverse sources, including mammalian testis protein,³ human brain,⁴ plants,⁵ and hookworm.⁶

A recombinant NIE (rNIE) protein was found to be capable of stimulating release of histamine from basophils containing parasite specific IgE of patients infected with *Strongyloides stercoralis*. This property of the NIE antigen suggested that it could also be used as a skin test antigen for immediate hypersensitivity responses, as has been reported with crude larval antigen from the parasite.⁷ In view of the frequent multiple sensitivity to different vespids seen in patients,^{2,8} it was considered important to examine further the extent of antigenic relationships of the insect venoms with the rNIE antigen from *S. stercoralis* parasites. Such considerations are particularly relevant if the antigens are used to evaluate immediate hypersensitivity.

In this report, we present evidence for considerable cross-reactivity among recombinant Pol a 5, Ves v 5, and the NIE antigens by their respective polyclonal antibodies. Cross-reactivity of the recombinant antigens extended to serum IgE and IgG from *Strongyloides*-infected patient sera; however, the recombinant vespid venom antigens were unable to inhibit NIE reactivity with pooled serum from *Strongyloides* patients by competitive enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Expression and purification of antigens. Recombinant venoms Ves v 5 of yellow jacket (*Vespa vulgaris*) and Pol a 5 of paper wasp (*Polistes annularis*) were expressed in a yeast expression system.⁹ These proteins were purified and their respective antibodies raised in mice by one of the authors.⁹ NIE protein was expressed in bacteria *Escherichia coli* and purified by 2-step purification using as the first step an immobilized metal affinity purification column and then a size-exclusion column.¹

Western blotting. Recombinant purified proteins Pol a 5,

Ves v 5, and NIE antigens, 0.5 µg/lane, were loaded onto either 10% or 4–12% gradient gels.¹⁰ Protein was transferred to nitrocellulose membranes and blocked in 5% skim milk overnight at 4°C. Membranes were washed in phosphate-buffered saline containing 0.1% Tween 20 (PBST) and incubated separately with 25,000-times diluted rabbit anti-NIE serum in PBST. Mouse anti-Pol a 5 and Ves v 5 sera were diluted 1:2,000 in PBST and incubated 1 hour at room temperature. Blots were separately washed in PBST and incubated with goat anti-mouse or goat anti-rabbit sera conjugated with horseradish peroxidase (HRPO; Jackson ImmunoResearch, West Grove, PA) diluted to 1:50,000 in PBST for 1 hour. For IgE Western blots, membranes were first incubated with IgG-depleted sera (2 volumes Gamma Bind G Sepharose [Pharmacia, Piscataway, NJ] to 1 volume serum) for 2 hours at a 1:50 dilution in PBST. The membranes were then exposed to affinity-purified goat anti-human IgE (Kirkegaard and Perry, Gaithersburg, MD) conjugated with peroxidase at a 1:5,000 dilution. Bound antibodies were detected by chemiluminescence on addition of luminol substrate (Super Signal West Pico stable peroxidase; Pierce, Rockford, IL).

Inhibition ELISA. Purified rNIE antigen, in volumes of 50 µL in coating buffer and containing 0.125 µg/mL of protein (optimum concentration by box titration), was added to all 96 wells of an ELISA plate (Immulon 2B) and left overnight at 4°C. The next day, the plate was blocked with 5% skim milk for 1 hour. Meanwhile, three series of tubes were prepared, each containing 50 µL of a 1:64 dilution of pooled sera from *S. stercoralis*-infected patients. Serial 10-fold dilutions of the recombinant venom antigens, starting with 100 µg/mL for Pol a 5 and Ves v 5 and 1.25 µg/mL protein for the NIE antigen in 4-fold dilutions, were made in the series of tubes containing the 1:64 dilution of *Strongyloides* patients' serum. These antigen-antibody mixtures were allowed to absorb for 1 hour at 37°C. The antigen-antibody mixtures were then transferred in duplicate to the ELISA plate containing the fixed concentration of NIE antigen. The optical density (OD) readings on the serial dilutions of the NIE antigen serves as the control against which possible competing action of the Pol a 5 and Ves v 5 antigens will become apparent.

Rabbit and mouse antibodies to rNIE and insect venom proteins. Purified rNIE antigen was used for immunization as

described.¹ A primary dose of 200 µg protein in complete Freund's adjuvant was given intradermally to a rabbit (Spring Valley Laboratories, 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 against NIE antigen and 1:25,000 against Ves v 5 and Pol a 5 of insect venom. Groups of BALB/c mice were immunized intraperitoneally each with 0.2 mL of a mixture of 10 µg/mL Ag 5 and 5 mg/mL alum as adjuvant.⁹ These sera were diluted to 1:2,000 for immunoblot assays.

Purification of NIE-specific antibody. Purification of rabbit anti-NIE-specific antibody was performed using activated cyanogen bromide (CnBr) coupled to Sepharose 4B as explained by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ). One milliliter of matrix was added to 5 mg of purified NIE antigen and incubated rocking at 4°C overnight. The coupled matrix was washed with PBS, and 1 mL of immunized rabbit serum was added and left overnight at 4°C. The column was then packed with the sample and washed with 15 column volumes of PBS and then eluted with two column volumes of glycine buffer, pH 2.4. The eluted antibody sample was immediately neutralized with 3 M Tris HCl (pH 9.0), and glycerol was added to a final concentration of 50% and stored at -20°C.

Chemical digestion of NIE antigen. One milligram of purified rNIE antigen was suspended in 1 mL of 70% formic acid containing 1 mL of 70 mg/mL CnBr stock solution. The sample was incubated in the dark for 24 hours at room temperature. The sample was dried with a speed vac (HetoVac, Columbia, MD), and 100 µL of water was added and re-dried under the same conditions. The digested NIE sample was dissolved in sample solubilizing buffer and analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels.

RESULTS

Amino acid sequence analysis of NIE with amino acid sequences of Ves v 5 and Pol a 5. A sequence search of the conserved domain database using Pfam showed that NIE protein had sequence similarity to a protein from insect venom allergens 5 of Ves v 5⁸ from the yellow jacket and Pol a 5⁸

from the paper wasp, human testes protein,³ pathogen-related protein,¹¹ and human hookworm.⁶ Further, Ves v 5 and Pol a 5 were selected to identify their cross-reactive epitopes with NIE antigen. The NIE protein showed amino acid similarity (Figure 1) that was equivalent between it and both the Ves v 5 and Pol a 5 (46%), whereas the degree of amino acid identity varied between Ves v 5 and Pol a 5 (34% and 30%, respectively).

Cross-reacting epitope of NIE with Ves v 5 and Pol a 5. A 38-kDa purified rNIE protein was probed separately with rabbit anti-NIE antibody, mouse anti-Ves v 5, and mouse anti-Pol a 5 antisera. All these antibodies recognized the rNIE antigen at 38 kDa (Figure 2C). A similar experiment with rabbit anti-NIE showed reactions with recombinant Ves v 5 and Pol a 5 antigen at 26 kDa and 25.5 kDa, respectively (Figure 2B). The intensity of anti-NIE antibody reactivity with Ves v 5 was more prominent than with the Pol a 5 antigen (Figure 2B), possibly reflective of the differences in amino acid identity between the vespid allergens. Preimmunized rabbit and mouse sera were not reacted to their respective recombinant antigens (data not shown). Recombinant proteins NIE, Ves v 5, and Pol a 5 were run on nondenatured gels, and their respective antibodies were not reacted in criss-cross experiments (data not shown).

NIE, Ves v 5, and Pol a 5 reactions with human sera. Ves v 5 and Pol a 5 are components of insect venom and have been identified as allergenic, but their functions are as yet undefined. An experiment was carried out to determine the reactivity of Ves v 5 and Pol a 5 antigens with IgE in pooled serum from *S. stercoralis*-infected patients. The status of individuals contributing to the serum pool with respect to insect sensitization was not known.

Purified recombinant antigens of Ves v 5, Pol a 5, and NIE were reacted with human sera from patients with *S. stercoralis* and IgE and IgG reactivity (Figure 3); the reactivity was weaker with Pol a 5 compared with NIE and Ves v 5 antigens (Figure 3). There was, in general, reactivity to each of the proteins by both IgE and IgG isotypes.

Localization of epitope on rNIE antigen. Purified NIE antigen was chemically digested with CnBr to identify the location of the cross-reactive epitopes on the digested fragments. The rNIE, containing two methionines, generated three frag-

NIE	102	AHGAKKLIKSKDLEKKAQAYAEVIARLCRLERHDPKNRIEG--TGENLAY-GTTFIGH---	155
Ves5	71	AKNMKNLVWDELAYVAQVWANQ-CQYQ---HDTCRDI AKYQVGVNVALTGSTA AKYDDP	126
Pol5	73	ASNMNNLVWDELAKIAQVWA---SQCIILVHDKCRNTEKYQVGONTIAYAGSS-NHFPSV	128
NIE	156	-LAVKGVYDEIALYNFKKPGFSP----ATGHFTQLVWKGTHAGFCVV---EKGDRVYV	206
Ves5	127	VKLVKMTEDEVKDYNPKKK-FSGNNFLKTGHYTMVWVANTKEVCCGSLKFIQEKWHKHLYL	185
Pol5	129	TKL IQLWENEVKDFNYNTGITNKNFGKV-GHYTMVWVGNTEKVCGLSKYVEKNMQIHLYL	187
NIE	207	VCKYSPPGNYPRQ	219
Ves5	186	VCNYGPSGNFQNE	198
Pol5	188	ICNYGPAGNY	197

FIGURE 1. Comparison of the C-terminal segment of the recombinant NIE (AAD46493) amino acid sequence with the C-terminal segments of homologous insect venom allergens from Ves v 5 (P35783) and Pol a 5 (P81656). Best fitting segment-to-segment pairwise alignment was done using nonredundant protein sequence database WxAl1 with similarity matrix 62 (available at www.ebi.ac.uk). Identical residues are darkly shaded, and conserved substitute residues are shaded gray.

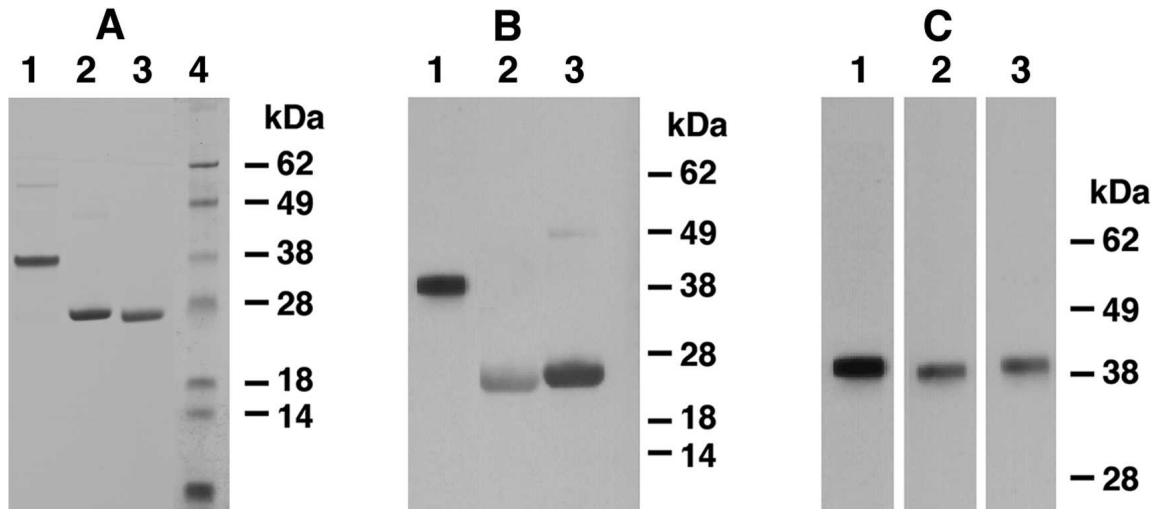


FIGURE 2. SDS-PAGE gel and immunoblot analysis of purified recombinant antigens of NIE and its homologs, Ves v 5 and Pol a 5. **A**, Purified recombinant antigens were resolved on a 10% SDS-PAGE gel and stained with Coomassie blue. Lane 1, NIE antigen (38 kDa); Lane 2, purified rVes v 5 (26 kDa); Lane 3, purified rPol a 5 (25.5 kDa); Lane 4, molecular weight protein markers. **B**, Immunoreactivity of purified rNIE antigen and its homologs reacted against rabbit anti-NIE antibody. Lane 1, NIE reacted at 38 kDa; Lane 2, Pol a 5 reacted at 25.5 kDa; Lane 3, Ves v 5 antigen reacted at 26 kDa. **C**, Immunoreactivity of NIE antigen with mouse anti-Ves v 5 and Pol a 5 sera. Lane 1, NIE reacted with rabbit anti-NIE antibody; Lane 2, NIE reacted with mouse anti-Pol a 5 antibody; Lane 3, NIE recognized by mouse anti-Ves v 5 antibody.

ments: C-terminal of 17.5 kDa, a middle portion of 7 kDa, and an N-terminal of 3.5 kDa (Figure 4A). Appearance of an extra band at 24 kDa was likely due to modification of methionine at position 204.¹ The 24-kDa band is formed by the middle portion and the C-terminal fragment of NIE, as determined by N-terminal amino acid sequencing by the Edman degradation method.

Mouse anti-Pol a 5, mouse anti-Ves v 5, and rabbit anti-NIE sera reacted with both the 24-kDa and 17-kDa fragments of the C-terminal portion of the NIE antigen (Figures 4B–4D). *S. stercoralis*-infected pooled human serum also reacted

with the C-terminal fragment of NIE, but the reactivity was very poor, suggesting the presence of a discontinuous epitope. Immunoreactivity of these sera with synthetic peptides designed to the C-terminal fragment of NIE was identified by ELISA (data not shown). These results further confirm that all these different sera exhibit a crossreacting epitope located at the C-terminal fragment of NIE.

Inhibition ELISA. Although the NIE antigen showed shared epitopes with the recombinant venom antigens, the specificity of NIE as a diagnostic antigen for *Strongyloides* infection was also tested by inhibition ELISA. For this purpose, a wide range of recombinant Ves v 5 and Pol a 5 concentrations (10-fold decreasing dilutions starting at 100 µg/mL protein) was tested for capacity to inhibit the reaction of pooled sera from *Strongyloides*-infected patients. The NIE antigen was also serially diluted in 4-fold steps starting at 1.25 µg/mL and served as the control against which the possible inhibitory effects of Ves v 5 and Pol a 5 antigens could be evaluated. Figure 5 shows the control curve of OD for the rNIE antigen, with a progressive decline in OD that would be expected with the serial 4-fold dilutions of antigen. The presence of either Pol a 5 or Ves v 5 recombinant antigens did not inhibit the binding of NIE to anti-NIE antibody. Detection of cross-reactivity of NIE with Ves v 5 or Pol a 5 on immunoblot but absence of cross-reactivity on ELISA may possibly be because cross-reactivity is observed with denatured proteins on immunoblot and that denaturation occurred during SDS gel electrophoresis.

DISCUSSION

The moderate degree of homology found in the database between *Strongyloides* recombinant NIE protein and several nematode proteins, such as *Caenorhabditis* and hookworm, might be expected, but the relationship to insect venom proteins was unexpected. Except for the fact that both groups of

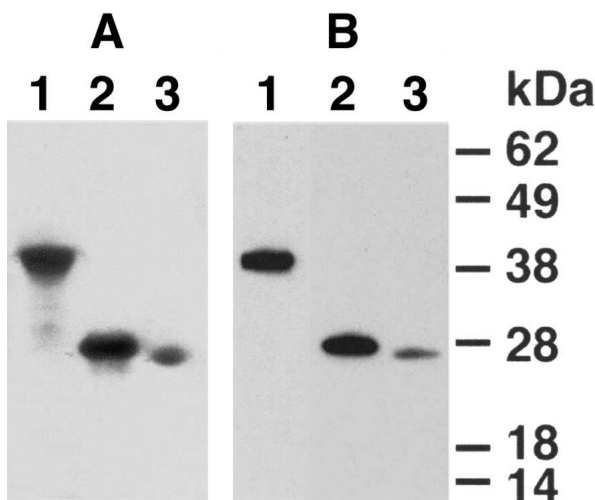


FIGURE 3. **A**, Immunoblot of purified antigens of NIE, Ves v 5, and Pol a 5 reacted with IgG of pooled sera from humans infected with *S. stercoralis*. Lane 1, NIE antigen; Lane 2, Ves v 5; Lane 3, Pol a 5. **B**, Immunoblot of purified recombinant antigens of NIE and its homologs Ves v 5 and Pol a 5 reacted with IgE antibody of pooled *S. stercoralis*-infected human sera (IgG depleted). Lane 1, purified rNIE antigen; Lane 2, purified rVes v 5; Lane 3, purified rPol a 5.

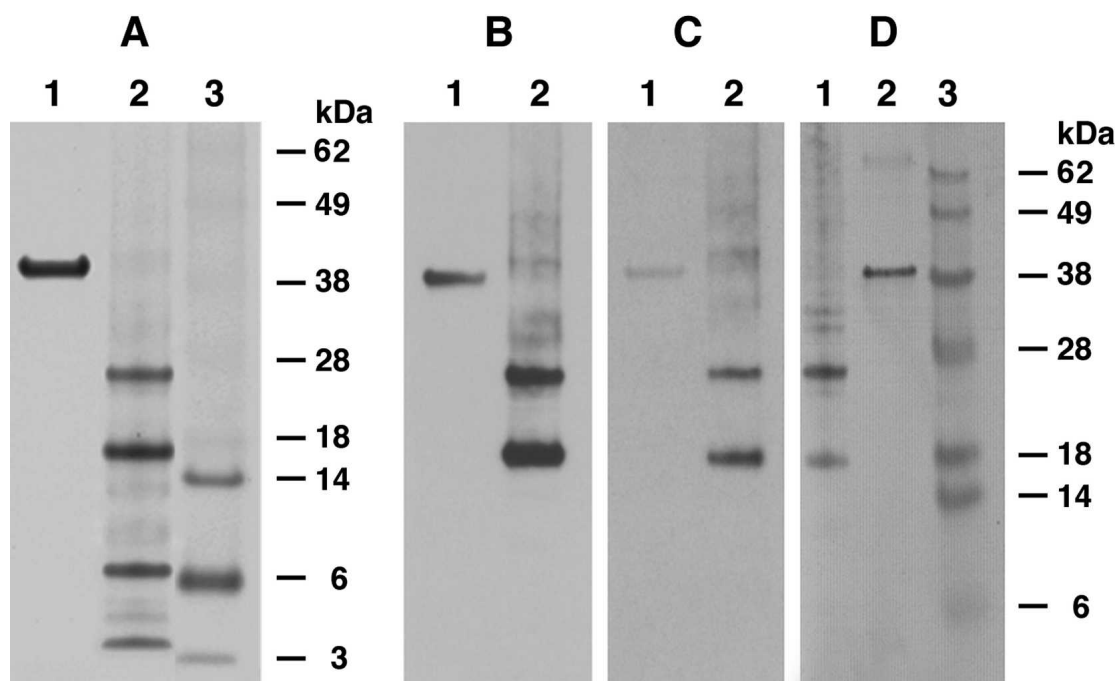


FIGURE 4. CnBr-digested rNIE protein on SDS-PAGE and immunoblot analysis. **A**, CnBr-digested NIE antigen was separated on 12% SDS-PAGE gel with running electrophoresis buffer containing 2-[N-morpholino]ethanesulfonic acid. Lane 1, 38-kDa undigested NIE protein; Lane 2, fragments of NIE digested with CnBr; Lane 3, molecular weight marker. **B**, Immunoblot of undigested and CnBr-digested fragments of NIE with mouse anti-Ves v 5 sera. Lane 1, undigested NIE reacted at 38 kDa; Lane 2, CnBr-digested fragments of NIE protein. **C**, Immunoblot of undigested and CnBr-digested fragments of NIE with mouse anti-Pol a 5 sera. Lane 1, undigested NIE reacted at 38 kDa; Lane 2, CnBr-digested fragments. **D**, Immunoblot of undigested and CnBr-digested fragments of NIE with rabbit anti-NIE sera. Lane 1, CnBr-digested fragments; Lane 2, undigested NIE reacted at 38 kDa; Lane 3, molecular weight markers.

proteins can act as allergens, they share no obvious biological properties. The Pol a 5 antigen displayed a weaker degree of cross-reactivity than Ves v 5 when tested by Western blot against the polyclonal rabbit anti-NIE antibody (Figure 2B)

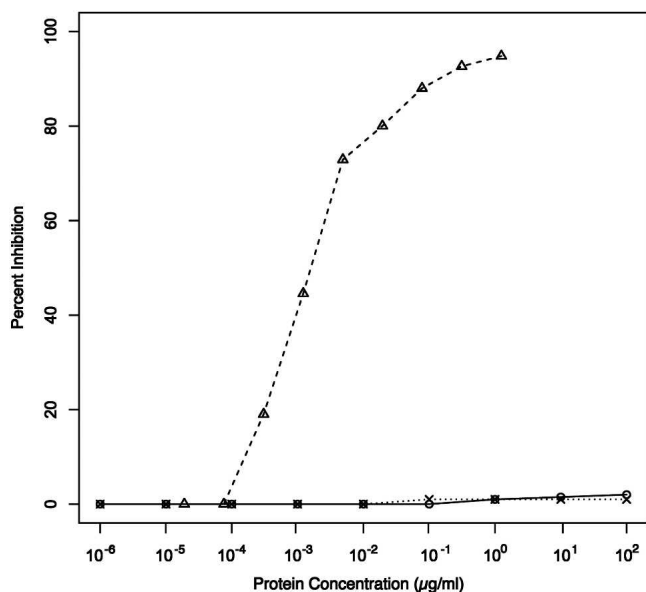


FIGURE 5. Inhibition ELISA of NIE antigen with *S. stercoralis*-infected human patient sera. Patient sera were diluted to 1:128 and depleted with purified recombinant antigens with initial concentrations of 100 µg/mL for both Ves v 5 (circles) and Pol a 5 (diagonal crosses). NIE was used at 1.25 µg/mL (triangles).

as well as when tested against pooled IgE and IgG from *Strongyloides*-infected patients (Figure 3). The reduced intensity of staining of Pol a 5 antigen in comparison with Ves v 5 is in keeping with the degrees of homology assigned to the respective proteins, namely, 30% and 34%, in comparison with the NIE antigens. A similar result was reported for homologous tpx protein from the mouse and the Dol m 5 protein from the hornet. Antibody raised in mice to the C-terminal fragment of Dol m 5 strongly reacted to the C-terminal fragment of tpx.¹² The extensive cross-reactivity between recombinant nematode proteins and recombinant insect venoms raises the possibility that individuals sensitive to certain insect venoms would show false positive serologic tests for antibodies to *S. stercoralis*; however, neither of the recombinant Pol a 5 or Ves v 5 antigens, nor commercial yellow jacket or wasp venom skin test antigens (Alk Abello, Round Rock, TX), showed significant reactions by ELISA to pooled serum from *S. stercoralis* patients (data not shown).

A low level of antibody responses to heterologous antigens may explain the background of immunologic responses that falls below the cutoff level for a given protein-based ELISA. For example, in a recent report on the use of the NIE antigen in *S. stercoralis* serodiagnosis, a result of 35 units or less (mean value plus 3 times SD of negative controls) defined the cutoff level separating positive from negative results.¹ It was somewhat surprising that in the inhibition ELISA, a wide range of either Pol a 5 or Ves v 5 antigen concentrations failed to significantly alter the curve of OD readings for control NIE antigen.

Neither Pol a 5 nor Ves v 5 inhibited the binding of NIE antigen and patient sera, although patient sera did bind these

antigens on immunoblot. The difference is related to the fact that antigen-antibody interaction occurs in solution for inhibition ELISA and it occurs as a solid-phase antigen in immunoblot. Solid-phase immunoassay is known to be more effective than solution-phase assay for detection of low-affinity interaction. A possible explanation for this difference is that high effective local concentration of antigen on the solid phase suppresses dissociation of antigen-antibody complexes.¹³

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