Hookworm (*Necator americanus*) Larval Enzymes Disrupt Human Vascular Endothelium

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Abstract. Knowledge of the molecular mechanisms used by *Necator americanus* larvae to penetrate the human skin and the vasculature would aid the development of effective vaccines against this important pathogen. In this work, the impact of *N. americanus* exsheathing fluid (EF) and excretory/secretory products (ES) on the endothelial barrier was examined using human umbilical vein endothelial cells (HUVEC). Cellular responses were assessed by investigating molecular changes at cell–cell junctions and by determining levels of secreted IL-6, IL-8, and vascular endothelial growth factor (VEGF) in the culture medium. It would appear that a repertoire of larval proteases caused a dose-related increase in endothelial permeability as characterized by a decrease in monolayer resistance with increased permeation of tracer-albumin. These barrier changes were associated with disruption of junctional vascular endothelial cadherin (VE-cadherin) and F-actin and an increase in endothelial secretion of IL-6 and IL-8. Our data suggest that larval proteases play an important role in negotiating the endothelium.

INTRODUCTION

Necator americanus needs to penetrate the human skin to facilitate its life cycle, and is thought to use a repertoire of secreted and exsheathment enzymes to do so.^{1–3} As part of the infection process, larvae are required to enter the vasculature, presumably by disrupting the vascular endothelium, as has been shown for other parasites such as *Dirofilaria immitis*⁴ and *Schistosoma mansoni*.⁵

The vascular endothelium forms the intact, innermost layer of the vessel wall, which acts as a physical barrier to maintain vascular homeostasis and regulate vascular permeability.^{6,7} Several studies have shown vascular permeability to change in response to physiological and pathological factors including permeability increasing agents such as vascular endothelial growth factor (VEGF)⁸ and inflammatory mediators including histamine and thrombin.^{9,10} Parasite proteases¹¹ also have the potential to enhance vascular permeability,^{4,5,12,13} as do cytokines such as interleukin-6 (IL-6) and IL-8.^{14,15} On a morphological and a functional basis, one of the major structures regulating vascular permeability is the inter-endothelial cleft. Junctional adhesion molecules located in this cleft are essential for establishing strong homotypic and heterotypic connections and anchoring junctional molecules to the cellular actin cytoskeleton, promoting stabilization of junctions.^{16,17} *Necator americanus* larval enzymes may target these structures to breach the endothelium and enter the blood circulation.

To investigate this possibility, we have used an *in vitro* model of human endothelium to study the interaction between hookworm larval enzymes and vascular endothelium. The interaction was studied using human umbilical vein endothelial cells (HUVEC), exposed to well-defined hookworm larval exsheathing fluid (EF) and secretions (ES), with cell monolayer integrity examined using transendothelial electrical resistance (TEER) and tracer permeability. Furthermore, the response of the model to larval enzymes was assessed through the release of IL-6, IL-8, and VEGF and changes in the molecular structure.

MATERIALS AND METHODS

Materials. Ethical approval for the use of human term umbilical cords was obtained (from Nottingham Local Research Ethics Committee; Reference OG010101). Reagents were purchased from Sigma-Aldrich Ltd. (UK) unless indicated otherwise.

Preparation of *N. americanus* larval exsheathing fluid (EF) and excretory/secretory (ES) products. Infective *N. americanus* larvae were cultured as described by Kumar and Pritchard.¹⁸ Fecal material was collected from a *N. americanus*-infected individual, mixed with charcoal, 1% fungizone, and water to form a smooth paste, which was applied as a thin layer on the upper half of 0.5 × 8 cm strips of Whatman chromatography paper (Sigma, UK). Each strip was then placed in a 15 mL centrifuge tube (containing ~2 mL of distilled water) and incubated at room temperature for 8 to 10 days. The strips were carefully removed and the water containing the larvae transferred to a larger measuring cylinder. The larvae were allowed to settle for 1 to 2 hr, excess water aspirated off, and the concentrated larvae washed extensively in RPMI 1640 medium containing penicillin (100 IU/mL), streptomycin (100 µg/mL), and 1% fungizone. Larval EF was prepared by bubbling 100% CO₂ through the larval suspension in media for 2 hr at room temperature. The media containing the EF was collected after centrifugation at 52 × *g* for 45 min, and stored at –20°C. The exsheathed larvae were then cultured for an additional 72 hr at 37°C and the ES products were collected every 24 hr after centrifugation at 52 × *g* for 45 min. The ES products were pooled, dialyzed against water for 48 hr at 4°C, and stored at –20°C until needed. The protein content of larval EF and ES products was estimated using the BioRad protein assay (UK) and ranged between 15 and 30 µg/mL. The presence of lipopolysaccharide (LPS) in larval EF and ES products was estimated using an E-TOXATE kit (Sigma) and was not detected or else present at a level below the detection limits of this assay (0.015 EU/mL). Positive controls were chosen following the manufacturer's recommendations.

Larval proteins were concentrated by trichloroacetic acid (5% TCA) precipitation, resuspended in 20 µL of 2 × reducing sample buffer, loaded at 20 µg per lane, and separated on 10% acrylamide resolving gels at 20 mA constant current for 60 to 90 min. Sodium dodecyl sulfate-polyacrylamide gel

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(SDS-PAGE) gels were silver stained using a modified method by Yan and others.¹⁹ Briefly, gels were fixed in destain solution (25% methanol, 10% glacial acetic acid, and distilled water) for 1 hr before being sensitized with 0.02% sodium thiosulphate for 1 min. The 0.2% silver nitrate was added for 20 to 40 min and gels were washed twice (20 seconds each) and developed with a solution of 3% sodium carbonate, 0.05% formaldehyde, and 0.0004% sodium thiosulphate on an orbital shaker.

Following a method by Kumar and Pritchard,¹⁸ larval proteolytic activity was investigated using 10% substrate SDS-PAGE gels containing 0.1% of gelatin or hemoglobin in the resolving gel. Protein samples were used at 5 µg per lane and run, under non-reducing conditions, at a constant current of 20 mA per gel. Hyaluronidase activity was also assessed by incorporating 0.1% hyaluronic acid into a 12% SDS-PAGE gel and run under non-reducing conditions, in a method modified from Hotez and others.²⁰ Hyaluronidase from bovine testes was used as a positive control. Protease activity was visualized by fixing substrate gels for 30 min in destain solution before being stained with Coomassie Brilliant Blue R250 overnight, whereas hyaluronidase activity was stained with 0.1% Stain-All and dissolved in 50% formamide. Activity was detected by destaining the gels in destain solution or water (hyaluronidase activity) and is observed as clear bands against a blue background.

Protease activity assay using casein conjugated to fluorescein isothiocyanate (FITC-casein). *pH profile of EF and ES products.* The effect of pH on protease activity present in larval EF and ES products was studied, as described previously.²¹ Briefly, larval EF/ES products (1 µg, 50 µL) were mixed with 10 µL of fluorescein isothiocyanate-casein (FITC-casein, 250 µg/mL) and 140 µL of buffer (pH 3–5.5, 0.1 M citric acid/sodium citrate buffer; pH 6–8, 0.1 M phosphate buffer; pH 8–10, 0.05 M 2-amino-2-methyl-1,3-propanediol-HCL buffer) containing 5 mM cysteine and incubated at 37°C for 2 hr. The reaction was stopped by adding 120 µL of 5% trichloroacetic acid and the undigested protein was removed by centrifugation at 13,000 × g for 10 min. Twenty microliters of the supernatant were then added to 80 µL of 0.5 M Tris-Cl, pH 8.5 and the fluorescence was measured in triplicates (excitation = 490 nm, emission detection = 525 nm).

Characterization of larval proteases. Protease inhibitors were quality controlled by assaying them against their class-specific proteases (Table 1). To characterize the proteases present in larval secretions, EF/ES products (1 µg, 50 µL) were pre-incubated with protease inhibitors E64 (1 µM), 1, 10-phenanthroline (1 mM), pepstatin A (1 µM), and phenyl-methane sulfonyl fluoride (PMSF, 1 mM), individually or in a mixture for 30 min before the addition of FITC-casein, as described previously. In this assay, only phosphate buffer (0.1 M, at pH 6.5 and 8) was used.

TABLE 1
Quality control of protease inhibitors*

Protease	Protease inhibitor
Trypsin (serine proteases)	PMSF (1 mM), pH 8
Papain (cysteine proteases)	E64 (1 µM), pH 6
Leucine aminopeptidase (metalloproteases)	1, 10-Phenanthroline (1 mM), pH 7.2
Pepsin (aspartyl proteases)	Pepstatin A (1 µM), pH 5

* Protease inhibitors were incubated with their specific proteases (5 µg) at 37°C for 1 hr.

Endothelial cell culture. *Cultivation of HUVECs.* The HUVECs were harvested from fresh umbilical cords, obtained at elective caesarean sections from normal pregnancies, using the method of Jaffe and others.²² Cells were cultured on 1% gelatinized T25 cm² flasks in M20; Medium 199 (Gibco, UK) supplemented with 20% heat-inactivated fetal bovine serum (FBS, Perbio, USA), 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 µg/mL fungizone, 150 µg/mL endothelial cell growth supplement (ECGS), and 50 µg/mL heparin, at 37°C and 5% CO₂ in air. At 90–100% confluence, HUVEC were detached with 0.05% trypsin/0.02% EDTA and seeded on 1% gelatin-coated transwells (12 mm, 0.4 µm, Costar, UK) at a density of 10⁵ cells per well. Growth medium was refreshed every 24 hr (200 µL and 700 µL in the apical and basal chamber, respectively).

Treatment of HUVEC monolayers. For studies on endothelial barrier function, HUVEC cells were used up to the third passage and were incubated with EF/ES products at 37°C for 2 hr. The EF/ES products were used at different concentrations (0.05–1.5 µg/mL), added to the apical, basal, or both chambers (at 1.5 µg/mL), and pre-incubated with protease inhibitors individually or in a mixture before being added to confluent HUVEC monolayers. Alpha-thrombin, a serine protease, was used as a positive control at 0.15 U/mL and EF/ES products were diluted in M0 medium (M20 with no added FBS). Protease inhibitors showed no ability to disturb the integrity of HUVEC monolayers when used in the absence of larval products.

Estimation of cell viability. The HUVEC, grown to confluence on 1% gelatin-coated 96-well plates, were treated with EF/ES products (at 1.5 µg/mL) as described previously, exposed to resazurin solution (10 µg/mL in pre-warmed Hank's balanced salt solution, 100 µL per well),²³ and incubated at 37°C and 5% CO₂ in air for 1 hr. Fluorescence was measured (excitation = 530 nm, emission detection = 590 nm) and values were expressed as the percentage of the fluorescence compared with untreated HUVEC controls.

Measuring the integrity of the endothelial monolayer. *TEER.* After HUVEC were seeded on transwells, the TEER of the monolayers were monitored closely using an EVOM resistance meter (World Precision Instruments, USA) until they reached a steady level (~72–74 Ω.cm²). During treatment with EF/ES products, the resistance across HUVEC monolayers was measured in triplicate at 20 min intervals. Specific TEER values were then calculated by subtracting the resistance of a cell-free transwell insert from the resistance measured across individual monolayers.

Permeability assay. The leakage of tetramethylrhodamine isothiocyanate TRITC-albumin (~68 kDa) across pre-treated HUVEC monolayers was assessed. The TRITC-albumin (50 mM in RPMI 1640 phenol red-free medium with 20% FBS) was added in the apical chamber (200 µL) and cells were incubated and alternated between 37°C and room temperature (on an orbital shaker to ensure homogenous distribution of tracer) over a total period of 2 hr. Triplicate, 20 µL aliquots were collected from the basal chamber at 20–30 min intervals and made up to 100 µL with RPMI 1640 with 20% FBS. Retrieved aliquots from the basal chamber were always replaced. The amount of fluorescence crossing individual monolayers was then measured using a microtiter fluorescence plate reader (excitation = 530 nm, emission = 590 nm) and values were converted to concentrations of TRITC-albumin.

Immunocytochemistry. The HUVEC monolayers were gently washed and fixed for 10 min in 1% paraformaldehyde, permeabilized for 10 min with 0.15% Triton X-100, and blocked for 30 min in phosphate buffered saline (PBS) containing 5% normal human serum, all at room temperature. Cells were incubated overnight at 4°C with a monoclonal anti-CD 144 (anti-VE-cadherin, at 10 µg/mL) antibody or bovine serum albumin (0.1% BSA/PBS). After washing with 0.1% BSA/PBS, cells were incubated for 2 hr at room temperature with FITC conjugated goat anti-mouse IgG (10 µg/mL), or FITC conjugated phalloidin to stain F-actin (50 µg/mL in PBS). The cells were washed again and finally mounted in Vectorshield with propidium iodide to visualize the nuclei. Cells were then observed using a Leica TCS SP2 confocal laser scanning microscope (UK).

Cytokine quantification by enzyme-linked immunosorbent assays (ELISA). Following treatment with EF/ES products, cell supernatants were collected, centrifuged for 10 min at 14,000 × g to remove cell debris, and stored at -20°C pending analysis. Human VEGF, IL-6 DuoSet ELISA Development kit (R&D systems, UK), IL-8 ELISA kit (BD Biosciences, UK) were used to quantify VEGF, IL-6, and IL-8 in the supernatants according to the manufacturers' recommendations. The detection limits for VEGF, IL-6, and IL-8 were 1 and 0.1 pg/mL, respectively. Samples were assessed in duplicate and results are expressed as means ± SEM.

Statistical analyses. Data were analyzed with GraphPad Prism version 5.01. Values are presented as means ± SEM and

statistical significance was determined by two-way analysis of variance (ANOVA) tests unless otherwise stated. Statistical significance was defined as $P < 0.05$.

RESULTS

Protease activity in *N. americanus* larval EF and ES products. The pH optima for larval EF and ES were broad, ranging between 6.5 and 8.5 (Figure 1A). Protease activity was significantly higher in ES (at pH 8, $P < 0.01$) and EF products (at pH 6.5, $P < 0.01$), as shown in Figure 1B. Divergence among larval products was also observed in silver stained gels. The EF proteins were mainly observed at molecular weights of 55 to 170 kDa, whereas ES protein bands were concentrated at molecular weights from 11 to 60 kDa (Figure 1C). Substrates including gelatin and hemoglobin were degraded by both EF and ES products, as shown in Figure 1D. A single clear band with an apparent molecular weight of 25 kDa was characteristic of gelatin degradation, whereas two clear bands (~20 and ~27 kDa) were observed on hemoglobin gels. The 27 kDa protease in ES products showed a greater ability to degrade both gelatin and hemoglobin than the corresponding enzyme in EF products. Hyaluronidase activity was absent from ES and EF products (Figure 1D).

Characterization of protease activities in *N. americanus* larval EF and ES products. The contribution of different protease classes to larval EF and ES proteolytic activities was examined at their optimal pH (6.5 and 8). Figure 2A shows

FIGURE 1. (A) Effects of pH on protease activities present in *Necator americanus* larval preparations. (B) Comparison of protease activities present in exsheathing fluid (EF) and excretory/secretory (ES) products at optimal pH. Larval products were incubated with fluorescein isothiocyanate (FITC-casein) at 37°C for 2 hr. The specific activity, in (A) and (B), was obtained by subtracting values for a blank buffer control and presented as the mean ± SEM ($N = 3$) of fluorescence units per microgram of products/hr. The SEM bars are not visible in (A) because of the reproducibility of the assay. Protease activity was optimal between pH 6 and pH 8.5 and a significant difference in the proteolytic activities in EF (pH 6.5, ** = $P < 0.01$) and ES (pH 8, ** = $P < 0.01$) products was observed, compared with their activities at different pH (6–8.5). (C) Larval products separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver stained as described in the Methods with arrow heads illustrating the main protein bands. (D) Substrate gels showing degradation of gelatin, hemoglobin, and hyaluronic acid by *N. americanus* larval products. Protease activity appeared as clear bands against a blue background (arrow heads). Hyaluronidase activity was not detectable in EF and ES products.

FIGURE 2. (A) Quality control of protease inhibitors, assayed against their cognate enzymes. (B, C) Characterization of protease activities present in *Necator americanus* larval exsheathing fluid (EF) and excretory/secretory (ES) products at their optimal pH, respectively. The specific activity was obtained by subtracting values for a blank buffer control and presented as the mean $\pm$ SEM ($N = 3$) of fluorescence units per microgram of products per hour. ** = $P < 0.001$, * = $P < 0.01$ indicates significant inhibition compared with a non-inhibited control (+ EF [B] or ES [C] at the same pH).

protease inhibitors to cause a reduction of at least 90% in protease activities against their cognate enzymes, shown in Table 1, except for 1, 10-phenanthroline (~59%). Overall, protease activity in both EF and ES products was decreased by individual inhibitors especially PMSF (at pH 6.5 and 8). Reduction was most significant when all inhibitors (PMSF, E64, pepstatin A, and 1, 10-phenanthroline) were combined together, as shown in Figure 2B and C). Table 2 summarizes the inhibition of protease activities (Figure 2) as the percentage of reduction in proteolysis of FITC-casein to the specific activity observed with larval products. Protease activity in ES products was noticeably inhibited by PMSF at pH 6.5 and 8, whereas EF

TABLE 2

Summary of inhibition of protease activities by *Necator americanus* larval products*

Protease inhibitors	EF products		ES products	
	pH 6.5	pH 8	pH 6.5	pH 8
E64 (1 μ M)	-13.6	-1.97	-18.58	-16.69
Pepstatin A (1 μ M)	-9.62	-15.88	-13.31	-16.56
PMSF (1 mM)	-21.11	-33.72	-28.07	-27.83
1, 10-phenanthroline (1 mM)	-10.31	-8.96	-26.5	-11.82
All inhibitors	-31.58	-51.66	-41.87	-40.49

* Inhibition was determined as the % of reduction in protease activity to the total specific activity in larval products at their optimal pH.

proteases were significantly inhibited at pH 8. Results indicated the presence of serine proteases in both EF and ES products, which are primarily active not only at pH 8 (optimal pH for PMSF), but also at pH 6.5. Inhibition of cysteine proteases by E64 was highest at pH 6.5 suggesting that cysteine proteases are markedly active in larval products at this pH. Unlike EF products, cysteine proteases were considerably inhibited by E64 in ES products at pH 8, signifying that cysteine proteolytic activity in ES products was not affected by pH. These findings indicated that cysteine proteases were distinctly present in larval ES products and to a lesser extent in EF products.

At pH 6.5 and 8, a modest inhibition of protease activity by pepstatin A (optimal pH for pepstatin A is 5) was indicative of the presence of aspartyl proteases in both EF and ES products. Inhibition by 1, 10-phenanthroline was higher with ES products and highest at pH 6.5 indicating that although metalloproteases were present in both larval products, these proteases were exceptionally more active in ES products at pH 6.5. In addition, the use of all protease inhibitors in combination, which resulted in the greatest inhibition of proteolytic activity, confirmed the presence of the four main protease classes; serine, cysteine, aspartyl, and metalloproteases in larval EF and ES products.

Effects of larval EF and ES products on HUVEC monolayer permeability. *Effect of concentration of larval EF/ES preparations.* Changes in endothelial permeability as assessed by a decrease in resistance (TEER) and an increase in tracer leakage across endothelial monolayers were related to concentration of EF/ES products in the culture medium. Both EF and ES products caused a statistically significant reduction of TEER (46.8% and 50.9%, respectively) from control values when used at a final concentration of 1.5 μ g/mL ($P < 0.001$, Figure 3A). This correlated with a significant increase in levels of TRITC-albumin crossing treated monolayers (85.1% and 135.6%, respectively) compared with untreated monolayers ($P < 0.001$, Figure 3B). Although similar in pattern, ES products significantly showed greater tracer leakage at any one dose compared with EF products and thrombin ($P < 0.01$, Figure 3B). The dose of larval products at which the monolayer permeability was most significantly disrupted (1.5 μ g/mL) was then used for all following experiments.

Effects of the apical/basal administration. The increase in endothelial permeability was significant ($P < 0.001$) when larval products were administered to the apical chamber and highest when applied to both chambers of transwells. The latter resulted in a reduction in TEER (37.3% versus 46.8% for EF, 40.5% versus 50.9% for ES) compared with control values and an increase in tracer leakage (74.7% versus 85.1% for EF, 130.3% versus 135.6% for ES), respectively (Figure 4A and B). The presence of both larval products individually in the basal chamber caused a significant decrease in monolayer resistance (24.7% for EF, 30.6% for ES) but a minor increase in TRITC-albumin levels (1.4% for EF, 8.6% for ES) over time, compared with untreated monolayers (Figure 4B).

Inhibition of EF/ES protease activities. The use of protease inhibitors singly and in combination before treatment of HUVEC monolayers with larval EF/ES products resulted in a better preservation of endothelial barrier properties as revealed by improved TEER and reduced tracer leakage across treated monolayers. Inhibitors, used together, was the most effective treatment resulting in a minimal reduction in TEER (16.6% for EF, 18.4% for ES) compared with the

FIGURE 3. Effects of concentration of larval product on human umbilical vein endothelial cell permeability (HUVEC). Experiments were conducted on confluent HUVEC monolayers treated with different concentrations of larval products. **(A)** Percentage changes from control transendothelial electrical resistance (TEER) at different concentrations of exsheathing fluid (EF)/excretory/secretory (ES) preparations. The TEER values are presented as a percentage of reduction in TEER $\pm$ SEM ($N = 3$). The TEER was significantly reduced compared with control resistance where $* = P < 0.01$, $** = P < 0.001$ compared with the TEER of untreated controls. **(B)** Leakage of tetramethylrhodamine isothiocyanate TRITC-albumin across treated monolayers. TRITC-albumin concentrations were calculated by subtracting tracer leakage across a blank monolayer and reported as the mean $\pm$ SEM ($N = 3$). Tracer leakage was significantly increased in response to EF and ES preparations, $* = P < 0.01$ and $** = P < 0.001$ compared with untreated controls.

resistance of individually treated (in particular PMSF; 27.9% for EF, 33.3% for ES) and inhibitor-free (46.8% for EF, 50.9% for ES) monolayers (Figure 5A). The measured TEER values agreed with the subsequent, statistically significant reduction in tracer leakage values measured post-treatment with all inhibitors in combination ($P < 0.01$, 30.6% for EF, 43.7% for ES), or individually (Figure 5B). These findings suggest that alterations in monolayer permeability and barrier functions are dependent on the involvement of all four protease classes present in EF or ES products. Preservation of endothelial permeability was greater with inhibition of ES products than EF products ($P < 0.05$).

Endothelial cell responses to larval products. *Quantification of secreted cytokines and VEGF.* To assess the effects of larval products on the release of VEGF and cytokines from endothelial cells, levels of VEGF, IL-6, and IL-8 were

FIGURE 4. Effects of administration of larval products on monolayer permeability. **(A)** Percentage changes in transendothelial electrical resistance (TEER) from original human umbilical vein endothelial cell (HUVEC) monolayers, during treatment with larval products in the apical, basal, and both chambers. TEER values are presented as a percentage of reduction in TEER $\pm$ SEM ($N = 3$), $** = P < 0.001$ and $*** = P < 0.0001$ compared with original TEER of untreated controls. **(B)** Leakage of tetramethylrhodamine isothiocyanate TRITC-albumin over time with exsheathing fluid (EF)/excretory/secretory (ES) products at the apical, basal, or both chambers. TRITC-albumin concentrations are reported as the mean $\pm$ SEM ($N = 3$), $** = P < 0.001$ compared with untreated HUVEC controls at the same time-point.

determined in culture supernatants from cells pre-treated with EF/ES products, using ELISA. Although larval products (both EF and ES) caused almost no change in VEGF release from HUVEC cultures (Figure 6A), post-treatment with larval ES products at a final concentration of 1.5 $\mu\text{g/mL}$ significantly increased endothelial secretion of IL-6 (~2-fold) and IL-8 (~9-fold), respectively (Figure 6B and C).

Treatment of HUVEC monolayers with larval ES products from the apical, basal, and both chambers induced a significant increase in the levels of secreted IL-6 ($P < 0.01$) and IL-8 ($P < 0.001$), as shown in Figure 7. Alterations in IL-6 and IL-8 secretions were highest following treatment in both chambers (~2-fold and ~13-fold, respectively) compared with cytokine levels observed with the apical treatment only (~1.6-fold and ~8-fold, respectively). These results signify a direct correlation between IL-6 and IL-8 release and changes in endothelial permeability, mainly caused by larval ES products.

Measuring VEGF and cytokines release from HUVEC monolayers, pre-treated with four protease inhibitors individually or in a mixture, showed that both EF and ES products failed to induce any significant changes in the levels of VEGF, whereas significant alterations in IL-6 and IL-8 production were observed (Figure 8A and B). The use of inhibitors separately to block protease activities in EF and ES products caused either low or no decrease in IL-6 and IL-8 release by endothelial cells (ranging from 0% to 25%), whereas the use of all inhibitors together demonstrated a greater reduction in the levels of secreted IL-6 (40.7%, $P < 0.001$) and IL-8

FIGURE 5. Effects of protease inhibition of larval products on monolayer permeability. (A) Percentage changes in transendothelial electrical resistance (TEER) from control resistance during treatment with larval products. TEER values are presented as a percentage of reduction in resistance from original TEER $\pm$ SEM ($N = 3$). * = $P < 0.01$ and ** = $P < 0.001$, describing a significant effect of protease inhibitors compared with % reduction in TEER of exsheathing fluid (EF)/ excretory/secretory (ES) treated controls. (B) TRITC-albumin leakage across human umbilical vein endothelial cell (HUVEC) monolayers post-treatment with protease inhibition. Tracer concentrations are reported as the mean $\pm$ SEM ($N = 3$). A significant decrease in the concentration of tracer compared with EF/ES treated controls (* = $P < 0.01$).

(52.0%, $P < 0.0001$) in response to larval ES products only. Hence, alterations in the endothelial permeability, which are likely mediated by the cellular production of IL-6 and IL-8, are largely dependent on differential contributions of all studied protease classes.

Viability and molecular changes at cell-cell junctions. Viability, determined as the ability of viable cells to oxidize resazurin to the fluorescent resorufin, was measured in HUVEC monolayers post-treatment with EF/ES products and compared with untreated controls (data not shown). The optical reading of fluorescent resorufin in untreated HUVEC controls was 644.13 ± 57.36 compared with 637.93 ± 39.14 (EF) and 649.33 ± 25.25 (ES). Treatment with both EF and ES products showed no statistically significant changes in cell viability ($P > 0.05$) indicating that both EF and ES products had no cytotoxic effects on HUVEC cells.

Changes in VE-cadherin and F-actin staining were associated with alterations in monolayer permeability. Different concentrations of larval ES products (Figure 9) induced a dose-dependent fragmentation of the continuous, junctional VE-cadherin (Figure 9A–D), ultimately leading to the formation of wide intercellular gaps at higher concentrations (Figure 9D). Additionally, increased concentrations of larval ES products caused changes in F-actin localization, loss of cortical actin, and an increase in stress fibers and perinuclear actin, as seen in Figure 9 (a'–d'). Similar changes of junctional VE-cadherin and F-actin were seen with larval EF products.

Consistent with changes in monolayer permeability, junctional VE-cadherin and F-actin filaments were also observed to be disrupted subsequent to treatment of HUVEC monolay-

FIGURE 6. Graphs showing post-treatment secretions of (A) vascular endothelial growth factor (VEGF), (B) IL-6, and (C) IL-8 in human umbilical vein endothelial cell (HUVEC) culture supernatants. Larval excretory/secretory (ES) products caused a significant increase in secreted IL-6 (** = $P < 0.01$) and IL-8 (***) = $P < 0.001$) from treated monolayers compared with untreated controls, whereas VEGF levels were not affected. VEGF, IL-6, and IL-8 values are expressed as the mean concentration (pg/mL) $\pm$ SEM ($N = 3$).

FIGURE 7. Graphs showing effects of the apical/basal administration of larval products on human umbilical vein endothelial cell (HUVEC) secretions of (A) IL-6 and (B) IL-8. Treatment of HUVEC monolayers with excretory/secretory (ES) products, applied to the apical and the basal chamber, caused a significant increase in levels of IL-6 (** = $P < 0.01$) and IL-8 (** = $P < 0.001$), which were measured in culture supernatants from treated monolayers, compared with untreated HUVEC controls. Values are expressed as the mean concentration (pg/mL) ± SEM ($N = 3$).

ers from the apical or both chambers (data not shown). Basal treatment with ES products appeared to have a lesser effect and caused less discontinuous staining of VE-cadherin. There were more continuous stain pattern of VE-cadherin at any one time.

Protease inhibition of ES products reduced loss of monolayer integrity when inhibitors were used in combination, here minimal disruption of both VE-cadherin (Figure 10F) and F-actin at cell-cell junctions was observed. Again, pre-treatment of larval ES products with individual inhibitors showed less improvement in monolayer integrity (Figure 10B–E), indicating that the proteases studied are jointly responsible for the VE-cadherin and F-actin disruption and eventually alterations in endothelial integrity and permeability.

DISCUSSION

Necator americanus larvae secrete a repertoire of enzymatic secretions, presumably with roles to play in the infection process.^{18,24,25} In this work, we have studied the effects of these enzymes in the context of their interaction with the vascular endothelium.

FIGURE 8. Graphs showing effects of protease inhibition of larval products on endothelial secretions of (A) IL-6 and (B) IL-8. Protease inhibition caused a significant decrease in IL-6 (** = $P < 0.001$) and IL-8 (** = $P < 0.001$) secretions from monolayers in the presence of all inhibitors, compared with an excretory/secretory (ES)-treated control. Significant reduction in secreted IL-8 was also observed with PMSF (** = $P < 0.001$) and 1, 10 phenanthroline (* = $P < 0.01$), compared with ES-treated monolayers. Values are expressed as the mean concentration (pg/mL) ± SEM ($N = 3$).

Necator americanus larval products showed pH-related protease activity, which was optimal between pH 6.5 and 8.5 for both the EF and the ES products. Knowing that “normal” skin pH ranges between 4.5 and 6.5,²⁶ these results suggest that protease activity was present over a broad range of pH possibly to cater for pH changes in or on the skin and subsequently in the deeper tissues. Protease activity in the EF is more likely to be involved in the penetration process at the skin surface, whereas the ES products are believed to help the larvae move deeper into the skin, finally arriving to the blood microcirculation.

Substrate gel analysis indicated the presence of active proteases in larval products at apparent molecular weights of 20, 25, and 27 kDa. Variation in the number, size, and intensity of clear bands suggested the involvement of a number of proteases in the degradation of substrates, which was more evident with ES products. Using protease inhibition assays,²¹ we were able to confirm the presence of serine, cysteinyl, aspartyl, and metalloproteases in both EF and ES products, as described before.^{1,18} Consequently, the use of inhibitors in combination resulted in the most significant reduction in protease activity in both EF and ES products, although complete inhibition was not achieved with inhibitor cocktails even though each inhibitor was quality controlled against its cognate enzyme class,

FIGURE 9. Confocal microscopy of human umbilical vein endothelial cell (HUVEC) cell-cell junctions pre-treated with different concentrations of larval excretory/secretory (ES) products. (a–d) Staining of junctional VE-cadherin (green), (a'–d') staining of F-actin (green), and nuclei were stained with propidium iodide (red). The HUVEC monolayers were grown on 1% gelatin-coated transwells to confluence, incubated with excretory/secretory (ES) products, and stained as described previously; control (a, a'), at 0.05 µg/mL (b, b'), at 0.5 µg/mL (c, c'), and at 1.5 µg/mL (d, d') of ES products. Bar = 20 µm, arrows point to gaps in the monolayer, and * shows regions of discontinuous VE-cadherin. This figure appears in color at www.ajtmh.org.

suggesting that no protease is dominant and that the penetration process may be mediated by the contribution of all proteolytic activities in the larval products. The data of Williamson and others²⁴ and the combinational gelatinolytic activity of larval products, previously described by Kumar and Pritchard,¹⁸ support the involvement of a multi-protease cascade in the degradation of host tissue substrates. The involvement of more than one protease in the infection process is likely caused by the complexity of and the need to negotiate the different layers of skin (i.e., keratinocytes, epidermis, and dermis) to reach the vasculature.

Having established the proteolytic profiles of EF and ES, this investigation focused on the manner by which enzymes mediated alterations in endothelial permeability, possibly as a mechanism to reach the microcirculation. Our findings showed a dose-related increase in HUVEC monolayer permeability following treatment with larval products, as shown by reduced TEER and increased leakage of TRITC-albumin (Figure 3). Disruption of endothelial barrier functions (i.e., permeability) was most significant on treatment with larval ES products, emphasizing the potential involvement of ES products in the interaction between *N. americanus* larvae and the endothelium.

FIGURE 10. Immunostaining of human umbilical vein endothelial cell (HUVEC) monolayers for junctional VE-cadherin after being treated with protease inhibitors; (A) control, treated with excretory/secretory (ES) products only, (B) 1 µM E64, (C) 1 mM 1, 10-phenanthroline, (D) 1 µM pepstatin A, (E) 1 mM PMSF, and (F) all inhibitors in combination. Bar = 20 µm, arrows point to gaps in the monolayer, and * shows regions of discontinuous VE-cadherin. This figure appears in color at www.ajtmh.org.

Permeability was increased following treatment of cell monolayers in the apical and apical/basal chambers with larval products (Figure 4). The changes observed suggested that a more rapid increase in endothelial permeability is caused by direct bathing of endothelial cells with larval products on the apical side, whereas application from the basal side resulted in a delay of effects. However, this may be primarily because of technical reasons, such as dilution of larval products in the basal chamber and the need to navigate multiple layers (i.e., plastic membrane and gelatin coating) before reaching the cells.

The use of enzyme inhibitors showed the importance of the combined effect of serine, cysteinyl, aspartyl, and metalloproteases in modulating endothelial permeability. Although the use of all inhibitors in combination exhibited the highest protection of endothelial integrity, a total protection of endothelial barrier functions was not seen, which suggests that other larval enzymes may also be involved, therefore requiring further characterization of larval EF and ES products, or that enzyme inhibitors are not wholly effective, even as cocktails, against mixed enzyme populations. In addition, treatment of HUVEC with larval products might also trigger the activation of matrix metalloproteinases (MMPs) from endothelial cells, which in turn, can be partially responsible for inducing an increase in vascular permeability.^{27–29}

In all experiments, the increase in endothelial permeability was associated with a moderate, but significant, increase in IL-6 and a large, significant increase in IL-8 secretions (Figures 6–8), with no increase in VEGF release. These results agree with previous work, in which endothelial permeability was shown to be mediated in part by IL-6¹⁴ and most significantly by IL-8, in a VEGF independent manner.¹⁵ Although IL-6 was reported to increase permeability through the generation of reactive oxygen species (ROS),¹⁴ IL-8 seems to induce permeability by stimulating transactivation of the IL-8 receptors CXCR1 and CXCR2 and VEGFR2 which in turn, is required for endothelial gap formation.¹⁵

The increase in cytokine secretions also mirrored intercellular gap formation in endothelial monolayers at VE-cadherin-rich junctions. Additionally, disruption of the F-actin filaments, which is thought to be triggered by secreted IL-6 and IL-8,³⁰ eventually led to rearrangement into actin stress fibers and emergence of noticeable gaps. Because no cytotoxicity was associated with larval enzymatic activity, cytokine-induced tyrosine phosphorylation of junctional molecules³¹ and modulation of the actin-myosin contractile system^{30,32} have been suggested as potential mechanisms for regulating adherens junctions, thereby altering endothelial barrier functions and paracellular permeability.^{33,34}

In conclusion, we have showed that *N. americanus* larval products are capable of interacting with the endothelial monolayer causing increased endothelial permeability in parallel with elevated IL-6 and IL-8 release. At the cellular mechanistic level, larval products also caused junctional disruption and gap formation, which in reality would permit *N. americanus* larvae to pass through the endothelium and enter the microcirculation. The contribution of a repertoire of larval enzymes to this process suggests that multiple enzymes may have to be targeted to prevent the infection process.

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REFERENCES

1. Kumar S, Pritchard DI, 1992. Secretion of metalloproteases by living infective larvae of *Necator americanus*. *J Parasitol* 78: 917–919.
2. Loukas A, Prociv P, 2001. Immune responses in hookworm infections. *Clin Microbiol Rev* 14: 689–703.
3. Quinnell RJ, Bethony J, Pritchard DI, 2004. The immunoepidemiology of human hookworm infection. *Parasite Immunol* 26: 443–454.
4. Boreham PF, 1984. Activation *in vitro* of some biological systems by extracts of adult worms and microfilariae of *Dirofilaria immitis*. *J Helminthol* 58: 207–212.
5. Fallon PG, Teixeira MM, Neice CM, Williams TJ, Hellewell PG, Doenhoff MJ, 1996. Enhancement of *Schistosoma mansoni* infectivity by intradermal injections of larval extracts: a putative role for larval proteases. *J Infect Dis* 173: 1460–1466.
6. Ruffer C, Strey A, Janning A, Kim KS, Gerke V, 2004. Cell-cell junctions of dermal microvascular endothelial cells contain tight and adherens junction proteins in spatial proximity. *Biochemistry* 43: 5360–5369.
7. Stevens T, Garcia JG, Shasby DM, Bhattacharya J, Malik AB, 2000. Mechanisms regulating endothelial cell barrier function. *Am J Physiol Lung Cell Mol Physiol* 279: L419–L422.
8. Esser S, Lampugnani MG, Corada M, Dejana E, Risau W, 1998. Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. *J Cell Sci* 111: 1853–1865.
9. Vogel SM, Easington CR, Minshall RD, Niles WD, Tiruppathi C, Hollenberg SM, Parrillo JE, Malik AB, 2001. Evidence of transcellular permeability pathway in microvessels. *Microvasc Res* 61: 87–101.
10. Lampugnani MG, Corada M, Caveda L, Breviaro F, Ayalon O, Geiger B, Dejana E, 1995. The molecular organization of endothelial cell to cell junctions: differential association of plakoglobin, beta-catenin, and alpha-catenin with vascular endothelial cadherin (VE-cadherin). *J Cell Biol* 129: 203–217.
11. Imamura T, Potempa J, Pike RN, Travis J, 1995. Dependence of vascular permeability enhancement on cysteine proteinases in vesicles of *Porphyromonas gingivalis*. *Infect Immun* 63: 1999–2003.
12. Han ED, MacFarlane RC, Mulligan AN, Scafidi J, Davis AE 3rd, 2002. Increased vascular permeability in C1 inhibitor-deficient mice mediated by the bradykinin type 2 receptor. *J Clin Invest* 109: 1057–1063.
13. Hansell E, Braschi S, Medzihradsky KF, Sajid M, Debnath M, Ingram J, Lim KC, McKerrow JH, 2008. Proteomic analysis of skin invasion by blood fluke larvae. *PLoS Negl Trop Dis* 2: e262.
14. Ali MH, Schlidt SA, Chandel NS, Hynes KL, Schumacker PT, Gewertz BL, 1999. Endothelial permeability and IL-6 production during hypoxia: role of ROS in signal transduction. *Am J Physiol* 277: L1057–L1065.
15. Petreaca ML, Yao M, Liu Y, Defea K, Martins-Green M, 2007. Transactivation of vascular endothelial growth factor receptor-2 by interleukin-8 (IL-8/CXCL8) is required for IL-8/CXCL8-induced endothelial permeability. *Mol Biol Cell* 18: 5014–5023.
16. Dejana E, Lampugnani MG, Martinez-Estrada O, Bazzoni G, 2000. The molecular organization of endothelial junctions and their functional role in vascular morphogenesis and permeability. *Int J Dev Biol* 44: 743–748.

17. Firth J, 2002. Endothelial barriers: from hypothetical pores to membrane proteins. *J Anat* 200: 524–525.
18. Kumar S, Pritchard DI, 1992. The partial characterization of proteases present in the excretory/secretory products and exsheathing fluid of the infective (L3) larva of *Necator americanus*. *Int J Parasitol* 22: 563–572.
19. Yan JX, Wait R, Berkelman T, Harry RA, Westbrook JA, Wheeler CH, Dunn MJ, 2000. A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. *Electrophoresis* 21: 3666–3672.
20. Hotez PJ, Narasimhan S, Haggerty J, Milstone L, Bhopale V, Schad GA, Richards FF, 1992. Hyaluronidase from infective *Ancylostoma hookworm* larvae and its possible function as a virulence factor in tissue invasion and in cutaneous larva migrans. *Infect Immun* 60: 1018–1023.
21. Brown A, Girod N, Billett EE, Pritchard DI, 1999. *Necator americanus* (human hookworm) aspartyl proteinases and digestion of skin macromolecules during skin penetration. *Am J Trop Med Hyg* 60: 840–847.
22. Jaffe EA, Nachman RL, Becker CG, Minick CR, 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 52: 2745–2756.
23. Kim EA, Kim JA, Park MH, Jung SC, Suh SH, Pang MG, Kim YJ, 2009. Lysophosphatidylcholine induces endothelial cell injury by nitric oxide production through oxidative stress. *J Matern Fetal Neonatal Med* 22: 325–331.
24. Williamson AL, Lecchi P, Turk BE, Choe Y, Hotez PJ, McKerrow JH, Cantley LC, Sajid M, Craik CS, Loukas A, 2004. A multi-enzyme cascade of hemoglobin proteolysis in the intestine of blood-feeding hookworms. *J Biol Chem* 279: 35950–35957.
25. Matthews BE, 1975. Mechanism of skin penetration by *Ancylostoma tubaeforme* larvae. *Parasitology* 70: 25–38.
26. Yosipovitch G, Tur E, Morduchowicz G, Boner G, 1993. Skin surface pH, moisture, and pruritus in haemodialysis patients. *Nephrol Dial Transplant* 8: 1129–1132.
27. Lafleur MA, Hollenberg MD, Atkinson SJ, Knauper V, Murphy G, Edwards DR, 2001. Activation of pro-(matrix metalloproteinase-2) (pro-MMP-2) by thrombin is membrane-type-MMP-dependent in human umbilical vein endothelial cells and generates a distinct 63 kDa active species. *Biochem J* 357: 107–115.
28. Alexander J, 2002. Extracellular matrix, junctional integrity and matrix metalloproteinase interactions. *J Anat* 200: 561–574.
29. Johannsson E, Henriksen T, Iversen PO, 2007. Increase in matrix metalloproteinases from endothelial cells exposed to umbilical cord plasma from high birth weight newborns. *Am J Physiol Regul Integr Comp Physiol* 292: R1563–R1568.
30. Blum MS, Toninelli E, Anderson JM, Balda MS, Zhou J, O'Donnell L, Pardi R, Bender JR, 1997. Cytoskeletal rearrangement mediates human microvascular endothelial tight junction modulation by cytokines. *Am J Physiol* 273: H286–H294.
31. Nwariaku FE, Liu Z, Zhu X, Nahari D, Ingle C, Wu RF, Gu Y, Sarosi G, Terada LS, 2004. NADPH oxidase mediates vascular endothelial cadherin phosphorylation and endothelial dysfunction. *Blood* 104: 3214–3220.
32. Hordijk PL, Anthony E, Mul FP, Rientsma R, Oomen LC, Roos D, 1999. Vascular-endothelial-cadherin modulates endothelial monolayer permeability. *J Cell Sci* 112: 1915–1923.
33. Budworth RA, Anderson M, Clothier RH, Leach L, 1999. Histamine-induced changes in the actin cytoskeleton of the human microvascular endothelial cell line HMEC-1. *Toxicol In Vitro* 13: 789–795.
34. Leach L, Eaton BM, Westcott ED, Firth JA, 1995. Effect of histamine on endothelial permeability and structure and adhesion molecules of the paracellular junctions of perfused human placental microvessels. *Microvasc Res* 50: 323–337.