Basophil Competence during Hookworm (*Necator americanus*) Infection

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Abstract. A popular hypothesis to explain parasite survival in the presence of a pronounced T helper 2 phenotype in helminth-parasitized populations has been FceRI blockade by parasite-induced polyclonal IgE. To begin to test this hypothesis that FceRI-bearing cells would be refractory to activation in parasitized populations, we investigated basophil function in 43 individuals from a hookworm endemic area. Study individuals had high levels of total IgE and eosinophilia and a mean hookworm burden of 2,257 epg. Basophils from all members of this parasitized population were shown to release histamine to a number of agonists, including anti IgE and a hookworm allergen, calreticulin. These data would indicate that FceRI blockade at the level of the basophil did not occur in this parasitized population despite the presence of possible immunologic blocking agents. This would suggest that this effector arm of the T helper 2 phenotype remains operative in infected populations.

INTRODUCTION

Hookworms survive relatively well in an immunologically hostile environment, although the T helper 2 phenotype is associated with partial protection. The parasite may promote its survival by secreting a molecular screen of immunosuppressive agents and, possibly, by stimulating the appearance of regulatory T-cell populations. However, it is also possible that the parasite induces immunologic incompetence in one of the effector arms of the T helper 2 response, eliciting FceRI blockade in mast cells and basophils. This could be achieved through the upregulation of non-parasite-specific polyclonal IgE and autoantibodies acting in isolation and, possibly, in tandem. Therefore, it is reasonable at this stage to hypothesize that these and other immunologic components downregulate the capacity of FceRI to signal, and for cells of the basophil/mast cell series to respond biochemically to antigenic insult in infected individuals by releasing histamine. Such mast cell/basophil blockade could also partially explain the protective effect of hookworms against the development of respiratory wheeze against environmental allergens and the apparent asymptomatic nature of hookworm infection at mucosal surfaces, although investigating this is not the primary aim of the present study.

To test the hypothesis that FceRI blockade and basophil incompetence could indeed occur in hookworm-infected individuals, blood was collected from a parasitized population in Madang Province, Papua New Guinea, and cells exposed to a range of biologically relevant agonists including a known hookworm allergen and anti-human IgE, to assess the capability of basophils to release histamine. As a result, we have been able to demonstrate clearly the competence of basophils to release histamine in blood from a community infected with the hookworm *Necator americanus*. These data would indicate that blockade at this level is probably not responsible for parasite survival, nor for the protective effects of hookworm infection against the development of allergic disease.

Materials and Methods

**Fieldwork parasitology and blood collection.** The study sample comprised 43 inhabitants from the villages of Haven and Wasab in lowland Madang province, Papua New Guinea. This was an unselected sample taken during a parasitological survey of all individuals aged 4 years or over and formed part of a larger study assessing the immune biology of hookworm infection (Table 1). *Necator americanus* is the only hookworm species in this area. Informed consent was obtained from all subjects and their parents, and the study was approved by the Medical Research Advisory Committee of Papua New Guinea. Fecal samples were taken during September 2001; helminth eggs were counted by a modified McMaster salt-flotation technique and expressed as eggs per gram feces (epg). Blood samples were taken during October 2001; 10–20 mL venous blood was taken into heparin (1000 U/ml). An aliquot of the blood collected was also diluted 1:20 into Kimura’s stain to carry out differential white cell counts using an improved Neubauer chamber. Most inhabitants of the study area, including 39 of 43 of the study sample, had been treated with anthelmintics in 1998. All sampled individuals were offered anthelmintic treatment at the conclusion of the study.

**Parasite maintenance and basophil agonist preparation.** *Necator americanus* was maintained in the laboratory in syngeneic DSN hamsters. Two- to 4-day-old neonate hamsters were infected percutaneously with 100 infective larvae and adult worms harvested from the small intestine 35 days post-infection. All animal work was licensed under the UK Animals (Scientific Procedures) Act 1986. Adult ES products were prepared as described previously. The protein content of ES products was determined using the BioRAD protein assay. ES products were finally dialyzed against distilled water, freeze dried, and stored at −20°C prior to shipping to Papua New Guinea. Some ES products were boiled for 30 min as described by Brown and others to inactivate the proteinase activities present, given that hookworm proteinases themselves can cause basophil activation.

**Proteolytic activity of ES products.** The proteolytic activity of normal and heat-inactivated ES products was determined using fluorescein isothiocyanate labeled casein (FITC-casein) as described by Brown and others; 12 μg of ES (20 μL) products were mixed with 10 μL of FITC-casein (stock 0.5
mg/ml) and 170 μL of 50 mM phosphate buffer, pH 6.5 containing 5 mM cysteine and incubated at 37°C for 2 h. The reaction was stopped with 120 μL of 5% w/v trichloroacetic acid and the tubes allowed to stand at room temperature for 1 h. Precipitated protein was removed by centrifugation at 13,000 g for 10 minutes. Triplicate, 20 μL aliquots of the supernatant were added to 80 μL of 0.5 M Tris pH 8.5 and the fluorescence measured (excitation 490 nm, emission detection 525 nm) using a Dynex MFX microplate fluorimeter. In this assay, untreated ES products gave a specific activity of 3387 ± 91.6 fluorescence units/min/mg. No activity was detected in heat-treated ES products.

**Recombinant** *N. americanus* calreticulin. Recombinant *N. americanus* calreticulin was expressed in culture as described previously and purified using a combination of 15 °C - 861 r 1 h and incubation overnight at 4 °C. The authenticity of the purified calreticulin was confirmed by MALDI-TOF mass spectrometry peptide sequencing.

**Assessment of LPS in agonists.** To eliminate any possible stimulatory effects of LPS in agonist preparations, *N. americanus* ES products and recombinant calreticulin were assessed for LPS content using the Sigma E-TOXATE assay kit in accordance with the manufacturer’s instructions. Fractions containing calreticulin as determined by SDS-PAGE were pooled, dialyzed against PBS, and stored at −20°C until required. The authenticity of the purified calreticulin was confirmed by MALDI-TOF mass spectrometry peptide sequencing.

**Western blotting.** Fifty μg of adult somatic extract, 120 μg of ES products (untreated and heat inactivated), and 10 μg of recombinant calreticulin were resolved by 12% SDS-PAGE under reducing conditions and transferred onto nitrocellulose. Western blots were blocked with 5% non-fat dried milk powder in Tris buffered saline (TBS) for 1 h and incubated overnight at 4°C with either rabbit anti *N. americanus* calreticulin (1:600 in blocking agent) or pooled post-infection human sera (1:20 in blocking agent) obtained during this study and a previous study in 1996. After washing with TBS/0.05% Tween 20, the blots were incubated with either alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma, 1: 10000 in blocking agent) or alkaline phosphatase conjugated goat anti-human IgE (Sigma, 1:500 in blocking agent). After further washing with TBS/0.05% Tween 20, antibody binding was visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium in 0.75 M Tris pH 9.6.

**Human whole blood challenge for histamine release.** To assess histamine release from challenged basophils 100 μL of whole blood (collected into heparinized tubes as described previously) was challenged with untreated ES products (10–0.001 μg/ml), heat-inactivated ES products (10–0.001 μg/ml), recombinant calreticulin (40–0.001 μg/ml), goat anti-human IgE (50–0.001 μg/ml), goat anti-human IgG (isotype control, 50 μg/ml), and ovalbumin (10–0.01 μg/ml) in a total volume of 500 μL made up with Pipes buffer pH 7.4 (0.01 M Piperazine-N‘N'-bis[2-ethanecarbonyl acid], 0.14 M sodium acetate, 5 mM potassium acetate, 0.1% glucose, 1 mM CaCl2, and 0.03% human serum albumin). All challenges and spontaneous histamine release (100 μL of whole blood in Pipes buffer) were assessed after incubation for 1 h at 37°C. Total histamine release was assessed by mixing 50 μL of whole blood in 450 μL dH2O and freeze-thawing three times. Standard histamine calibrators of 0, 10, 25, 50, 100, and 250 ng/ml (Hycor Biomedical Ltd., Penicuik, UK) were included with the whole blood challenges.

Histamine released in each whole blood challenge was determined using a Histamine Assay Kit (Hycor Biomedical Ltd.); 50 μL of challenged whole blood was added to histamine-coated 96 well plates followed by 50 μL of mouse anti-histamine monoclonal antibody conjugated to alkaline phosphatase. The plates were incubated for 1 h at room temperature followed by three washes with a provided EIA wash solution. Antibody binding was visualized by the addition of 100 μL of 1 mg/ml p-nitrophenyl phosphate (pNPP) substrate. The plates were developed for 1 h at 37°C. Absorbance was measured at 405 nm using a Dynex MRX absorbance microplate reader. A standard curve was constructed from each histamine calibrator and the levels of histamine released in each challenge determined from this curve. Histamine levels were analyzed after subtraction of background histamine production release. The data presented are combined data obtained from all 43 individuals; not all individuals were examined at every agonist concentration indicated.

**Total IgE assay.** Total IgE concentrations in plasma samples were measured in an enzyme-linked immunosorbent assay (ELISA). Ninety-six well polystyrene plates were coated with 50 μL of a monoclonal mouse anti-human IgE (clone GE-1, ascites fluid, Sigma, Poole, UK, 5 μg/ml diluted in 0.05 M carbonate/bicarbonate buffer, pH 9.6) overnight at 4°C. The plates were washed with phosphate buffered saline/0.05% Tween 20, pH 7.2 (PBS/Tween) and blocked with 100 μL of 1% human serum albumin in PBS (HSA/PBS) for 1 h at room temperature. After blocking, the plates were washed again and 50 μL of human serum (diluted in the range 1:50 to 1:5000 in HSA/PBS) was added to each well and incubated overnight at 4°C. In addition, 50 μL of human IgE standards (doubling dilutions from 31.25 to 1.953 IU/ml, National Institute of Biologic Standards and Control, UK) were included on each plate. All assays were carried out in duplicate. After overnight incubation the plates were washed again and 50 μL of alkaline phosphatase conjugated goat anti-human IgE (ε-chain specific, diluted 1:1000 HSA/PBS) was added to each well and the plate incubated at room temperature for 2 h. Antibody binding was visualized by the addition of 100 μL of 1 μg/ml pNPP substrate. Absorbance was measured at 405 nm using a Dynex MRX microplate reader.

**Assay of IgG anti-IgE autoantibodies.** Human IgG anti-IgE autoantibodies were measured in an ELISA with an immobilized (2 μg/ml) myeloma IgE (kind gift from Binding Site, Birmingham) bound to a 96-well plate. After blocking with 1% human serum albumin in PBS (HSA/PBS), plasma samples were diluted 1:5 in HSA/PBS with 0.05% Tween 20 and 50 μL added to the IgE-coated wells. (All samples were assayed in duplicate.) After overnight incubation at 4°C, the
plates were washed and bound anti-IgE autoantibodies were detected with a peroxidase conjugated sheep anti-human IgG (Binding Site, Birmingham) diluted 1:000, 50 μL per well and the plate incubated at room temperature for 2 h. After further washing the plate was developed with 100 μL per well of 2.5 M H₂SO₄ and the absorbance read at 450 nm using a Dynex MRX microplate plate reader. The mean of duplicate absorbance readings was taken after background correction (serum wells with IgE coating omitted) and values above 0.05 were taken as positive.

RESULTS

Immunoepidemiological parameters. The study sample consisted of 43 individuals—27 males and 16 females (Table 1). All sampled individuals in the study were infected with hookworms, with a mean egg count of 2257 epg. Study individuals did not differ significantly by age, sex, or egg count from the total sampled population of the study villages (data not shown). Study individuals had elevated IgE levels, ranging from 677 to 26467 IU/ml compared with baseline values obtained in the UK, which ranged from 12 to 308 IU/ml.

Ten of 43 individuals (23.2%) produced detectable IgG anti-IgE. The number of eosinophils was also elevated, varying from 0.1–1.6 × 10⁹ cells/l (normal range 0.04–0.4 × 10⁹ cells/l). However, basophil numbers did not appear to be elevated in the study individuals (range 0–0.1 × 10⁹ cells/l) when compared with uninfected individuals (normal range 0.01–0.1 × 10⁹ cells/l).

Authentication of basophil agonists. To demonstrate the reactivity of N. americanus ES products and recombinant calreticulin for IgE prior to the study commencing in Papua New Guinea, Western blots of adult somatic extract (SE), ES products (ES), and recombinant calreticulin (rCal) were probed with either rabbit anti N. americanus calreticulin or pooled post-infection IgE obtained during a previous study in Madang Province in 1996 (Figure 1). Panel A illustrates the purity of the recombinant calreticulin used for the study and panel B shows myeloma IgE probed with the secondary antibody used for these studies. On return from Papua New Guinea to Nottingham the agonists were also tested against post-infection sera obtained during the current study. Recombinant calreticulin (rCal) reacted with both rabbit anti-calreticulin and human post-infection IgE from both studies (Figure 1C–1E). Human post-infection IgE from both studies also recognized a band of approximately 33 kDa in N. americanus ES products (Figure 1D and 1E). The reactivity and specificity of the anti-human IgE (Sigma) used in this study has been authenticated in previous studies. Therefore, all agonists were biologically intact and capable of recognizing or being recognized by IgE, an important consideration if blockade is a distinct possibility.

Basophil histamine release. Anti-human IgE induced histamine release from human basophils in a dose-dependent manner between 1 and 50 μg/ml (Figure 2A). Basophils challenged with anti-human IgG at a concentration of 50 μg/ml failed to release histamine (data not shown). Recombinant calreticulin, a hookworm allergen, induced basophil degranulation in a similar dose-dependent manner to anti-human IgE (Figure 2A). N. americanus ES products induced histamine release in a dose-dependent manner between 0.01 and 10 μg/ml (Figure 2B). Heat-inactivated ES products also induced similar levels of histamine release from five individuals tested, within the same dose range, indicating that the factor responsible for inducing basophil degranulation is not proteolytic in nature. Ovalbumin, a ‘bystander’ antigen control, failed to induce significant levels of histamine release over the range 0.01–10 μg/ml (N = 5).

These results are compared statistically to those obtained from UK naive individuals under the same conditions (Figure 2C). Basophils from non-parasitized donors released significantly less histamine after exposure to anti IgE, and to recombinant calreticulin, confirming the competence of basophils from a parasitized population to release histamine on challenge.

![Figure 1](image_url)
The higher levels of histamine release seen from basophils from parasitized individuals probably reflects the higher degree of FcεRI loading by IgE, given the differences seen in levels of total and specific IgE between the parasitized (mean 7594 I.U./ml) and non-parasitized (mean 136 I.U./ml). The higher background level seen with calreticulin possibly reflects the fact that it is both an allergen per se, and lectin.

Ironically the eventuality that we are witnessing a combination of IgE-specific and non-specific events, where agonists can possibly engage IgE via Fab and Fc regions does not detract from our main argument that substances released from hookworms retain the capability to release histamine from basophils under conditions deemed by others to be prohibitive of the event.

**DISCUSSION**

The present study has demonstrated clear indications of basophil and FcεRI competence in an immunologically well-studied hookworm-infected population from Papua New Guinea. Histamine release was recorded to anti-IgE (but not to anti-IgG) and to the hookworm allergen calreticulin (Figure 2A), indicating an intact IgE - FcεRI axis in basophils from this population. No release was seen to the ‘bystander’ antigen ovalbumin (Figure 2B). This is a potentially significant finding, and indicates that polyclonal IgE of the levels recorded in this population were insufficient to block or saturate FcεRI, at least on basophils.

The histamine release data in Figure 2C indicate significance over background for all agonists, proving that basophils from parasitized individuals retain competence whether triggering is through polyclonal IgE, allergen-specific IgE, or a combination of both. The lower release values for anti-IgE probably reflect the fact that, in a whole blood assay, the presence of high levels of polyclonal IgE act to sequester agonist. Whatever the scenario, mast cell blockade is unlikely to be a realistic event in the parasitized, given that some parasite secretions may be able to act through IgE per se.

It has been reported previously that only 6% to 17% of the polyclonal IgE produced is parasite specific, allowing ample opportunity for non–parasite-specific IgE to interfere with histamine release. This was clearly not seen in the present study. In addition, the presence of autoantibodies to IgE had no deleterious effect on basophil function despite the ability of anti-IgE antibodies to interfere with signalling and to potentially downregulate FcεRI expression.

From a parasitological standpoint, these data show that potentially protective FcεRI-dependent immune responses are not blocked in human necatoriasis. This is consistent with observations that the Th2 phenotype is associated with both reduced worm size and fecundity and reduced re-infection with hookworms, and with post-vaccination immunity in murine models.

There are a number of other potential mechanisms for helminth-mediated regulation of immunologic responses if FcεRI blockade is not operating to the extent previously thought. For example, the production of regulatory cytokines, such as IL-10, has been suggested to be responsible for the downregulation of allergic responses in schistosomiasis, and unregulated IL-10 responses have both also been re-
ported in hookworm infection, though the functional relationship between them has not been investigated.

It is also possible that FceRI blockade can indeed occur differentially at the mast cell level, although we consider this to be unlikely given the molecular conservation of this receptor across these cell types.

Whatever the mechanisms involved, we can categorically report at this stage that the IgE- FceRI basophil histamine release axis is fully operational in a tropical population infected with hookworm exhibiting high levels of polyclonal IgE, and in the presence of autoantibodies to IgE. This is perhaps not too surprising given that the membrane frequency of FceRI is in fact upregulated in the presence of high levels of IgE, which undoubtedly exist in hookworm infection. This effect presumably outweighs any downregulatory effect of anti IgE, leaving the original system intact. Therefore, we now suggest that hookworm-mediated downregulation of immune responses is instead mediated by secreted parasite immune suppressants and the induction of regulatory leukocyte populations rather than at the level of FceRI blockade.

Acknowledgments: Fieldwork was supported by the Wellcome Trust and RJQ was supported by a MRC Career Development Award. We thank Moses Lagog (Papua New Guinea Institute of Medical Research) for technical support in the field, and the inhabitants of Haven and Wasab for their kind participation.

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