

# Allergy Controls the Population Density of *Necator americanus* in the Small Intestine

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**Background & Aims:** Nearly 700 million people remain infected with hookworms. Although allergy is intuitively linked to immunity against helminths, few positive examples have been characterized. Larval migration through the lungs has been considered the likely interface at which hookworm attrition occurs. As part of a study evaluating a potential role for hookworms in the modulation of human autoimmunity, we examined parasite migration and intestinal colonization. **Methods:** Capsule and conventional endoscopies supplemented the evaluation of healthy volunteers and Crohn's disease patients recently inoculated with larvae of the human hookworm *Necator americanus*. Two healthy volunteers with a previously established and stable hookworm infection were inoculated with 50 larvae and had serial capsule endoscopies performed. **Results:** Eosinophilic enteritis developed in all subjects after the initial inoculation. Newly inoculated larvae in the 2 subjects with an established infection reliably reached the intestine within 4 weeks. Thereafter, the colony diminished to the host's constitutive status quo because mostly immature worms failed to attach. The intensity of the eosinophilic response correlated negatively with the time available for hookworms to feed and positively with hookworm attrition. **Conclusions:** *Necator* larval migration to the intestine is uncontested. We propose that allergic inflammation purposefully degrades the hookworm's bite, causing premature detachment, restricted feeding, and expulsion. This novel biological dynamic suggests a new paradigm of hookworm resistance.

For nearly 100 years, hookworms have been targeted for eradication. That nearly 700 million people in the undeveloped world remain infected is testament to our incomplete understanding of this complex dynamic. In the case of *Necator americanus*, it has been suggested that a mutually symbiotic relationship has evolved.<sup>1</sup>

A theme consistent in parasitology is uneven dispersion.<sup>2</sup> Older humans infected with *N americanus* sustain a larger population or colony compared with children, which is contrary to what might be expected based on exposure-prone behavior.<sup>3</sup> However, even among demographic equals, colony size varies. Most members sustain

an easily tolerated burden, but, at the extremes, some appear relatively resistant, and others are heavily infected, sufficient to cause anemia.<sup>4</sup> The size of the colony a host will sustain is genetically predestined.<sup>5,6</sup> Pre- and posttreatment studies on people living in endemic areas have shown that participants, when reinfected, sustained a hookworm burden that was inversely correlated with the pretreatment level of interleukin 5, the cytokine most closely linked to eosinophil induction.<sup>7</sup> Immunoglobulin E (IgE) though, the antibody intuitively linked to resistance against parasites, is positively correlated with the hookworm burden and, paradoxically, negatively correlated with the weight of individual hookworms and fecundity.<sup>7,8</sup>

In nonpermissive hosts, hookworm third-stage larvae (L3i) sometime appear vulnerable. Human cutaneous *larva migrans* identifies the cat-adapted *Ancylostoma braziliense* L3i trapped in the superficial dermis, and migration fails.<sup>9</sup> A lesser but still obvious reaction occurs after contact with *N americanus* L3i.<sup>4</sup> Passage through the lung brings L3i into contact with immune cells, potentially providing the host a larvicidal opportunity. Loeffler's syndrome, an eosinophilic pneumonitis, is provoked by migrating ascarid larvae.<sup>4</sup> A similar, more subtle response to hookworm migration has been proffered, but infections with *N americanus* and the dog-adapted *Ancylostoma caninum* in volunteers cause little if any pulmonary reaction.<sup>10,11</sup> For hookworms, the intestine has never been seriously considered as an interface at which host-mediated attrition might occur.

The emergence of autoimmune diseases in the developed world has been linked to hygiene, some believing that the disappearance of endoparasites has been an important immune modifier.<sup>12,13</sup> *Trichuris suis*, the pig whipworm, was reported as benefiting patients with

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Abbreviations used in this paper: CE, capsule endoscopy; EMS, excretory-secretory molecules; L3i, third-stage larvae; RD, reservoir donors.

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Crohn's disease (CD) and ulcerative colitis, but concern has been raised over the possibility of adverse migration.<sup>14–16</sup> Our experience of *N americanus* in humans is extensive. Apart from anemia, chronic infection is subclinical. Neither ocular nor visceral *larva migrans* has been described.<sup>4</sup> After commencing a proof-of-concept study to evaluate *N americanus* as a means to modulate human immunity in Crohn's disease patients, wireless capsule endoscopy (CE) became available.<sup>17,18</sup> This technology provides a clear view of the small intestine, and it is minimally invasive and able to be repeated.

Using capsule endoscopy to monitor *N americanus* larval migration to and development in the intestine, we have shown that migration appears uncontested, but, once intestinal feeding starts, an allergic response sets in train a strategy to protect the host against over colonization.

## Materials and Methods

### Participants

Conventional and capsule endoscopies, hematology, fecal testing, and total IgE were performed on 5 subjects recently inoculated with *N americanus*: Three were healthy researchers, intended as future reservoir donors (RD1–3), and 2 were long-standing, immune suppressed Crohn's disease (CD1–2) patients. Fifteen capsule endoscopy examinations were performed on 4 subjects (RD1, RD2, CD1, and CD2 had 7, 5, 2, and 1 endoscopies, respectively). Ethics approval for all levels of this study was provided independently by the appropriately constituted ethics committees of the regional health service (Townsville Health Service District Institutional Ethics Committee; protocol number: 32/03) and of James Cook University.

### Parasite

The donor strain of *N americanus* was originally obtained from a naturally infected person in Madang, Papua New Guinea, and maintained in a researcher at the University of Nottingham, United Kingdom. The identity of the L3i as *N americanus* was verified morphologically.<sup>19</sup> Of 2000 L3i transported to Australia, 306 were motile after 6 weeks. Subsequent L3i were obtained from RD1-feces cultured using a Harada–Mori technique.<sup>20</sup>

### Inoculations

For inoculation, carefully counted L3i in deionized water were placed on an absorbent-paper dressing that was applied to the forearm or base of the thumb for 30 minutes. Initial inoculation: In May 2004 (winter), using the L3i imported from the United Kingdom, CD1 and CD2 each received 25 L3i, RD1 received 100 L3i, RD2 received 31 L3i, and RD3 received 50 L3i. In November 2004 (summer), CD1 and RD2 and, in October 2005, RD1 were inoculated a second time with 50, 1-week-old L3i obtained from RD1.

### Capsule Endoscopy

Capsule endoscopy was commenced after an overnight fast. Subjects then undertook normal duties with food permitted after 4 hours. No adverse incident occurred. All analyzed data were as reported by an experienced endoscopist (J.C.). The examination time averaged 3 hours. On RD1, capsule endoscopy was performed twice 13 weeks after an inoculation with 100 L3i, separated by a day, when a stable hookworm population was expected; 36 and 17 hookworms were counted, the difference reflecting technologic limitations. Bowel preparations varied, and hookworms were easily obscured. To test observer bias, 3 studies were independently examined by a second investigator with limited endoscopic experience (R.S.). The hookworm counts per investigator were 39 and 36, 3 and 2, and 4 and 8, respectively ( $P = .77$ ). A close correlation between other findings also occurred.

### Feeding Time

Hookworms, red spots (interpreted as recent feeding sites), and white spots (interpreted as aphthous ulcers or degraded feeding spots) were counted (Figure 1). A hemorrhagic zone surrounded the attachment site of hookworms. When seen independently of hookworms, these were interpreted as recently abandoned feeding sites. Capsule endoscopy does not allow for an extended static observation, which means that it is not possible to directly measure feeding time. However, the time spent feeding and the number of red spots per hookworm correlate inversely. Consider a hypothetical study with a single hookworm present and all red spots recorded. The most recently formed red spot (RS) is  $nRS$ , with the hookworm (HW) just detached, and the first red spot produced and now almost faded is  $1RS$ . The sequence appears as  $1RS, 2RS, \dots, nRS$ . Provided that red spots disappear after a bite at a rate independent of the time attached, the higher the  $n$  value, the shorter the attachment, the shorter the time available to feed. The restricted feeding index has been measured as  $nRS:nHW$ .

### Inflammatory Indices

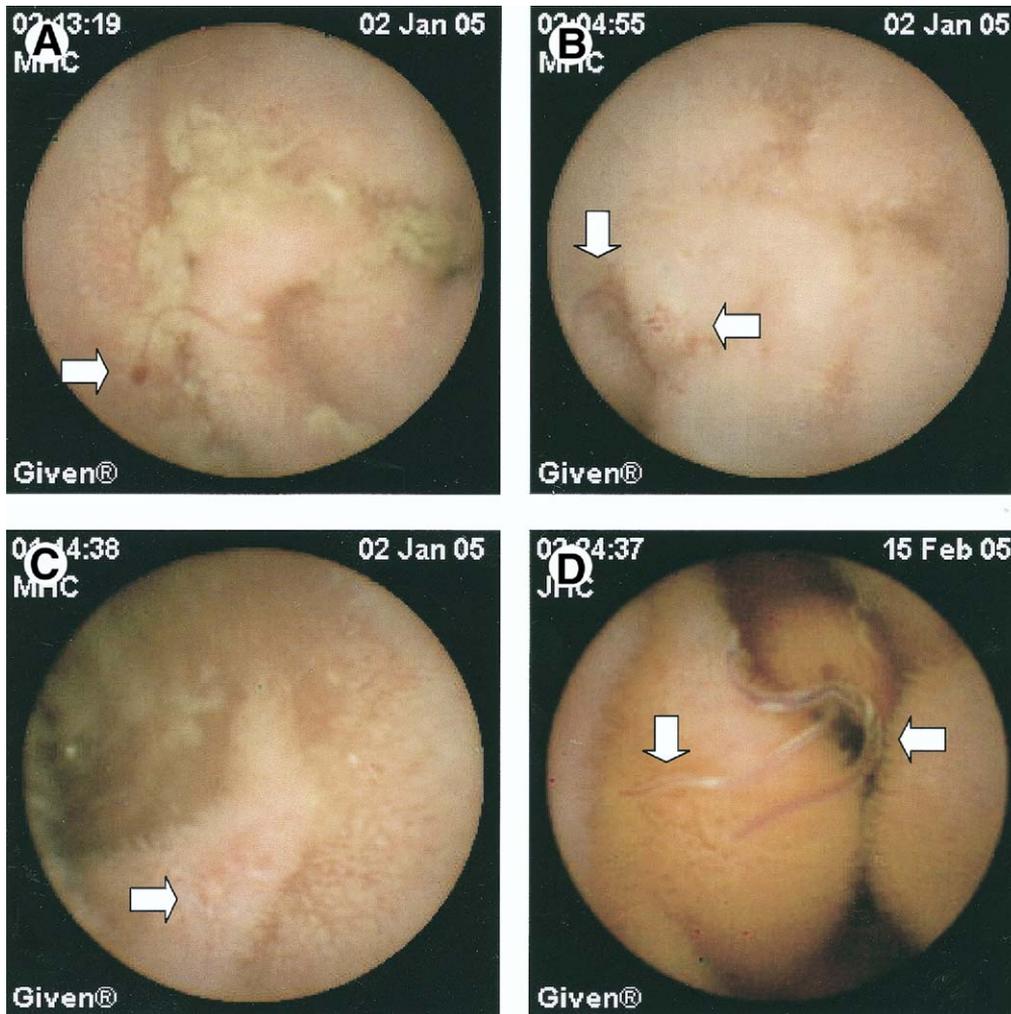
Aphthous ulcers were recognized as ill-defined white spots (WS). These were frequently found evolving in the center of a feeding spot. The propensity for feeding spots to ulcerate, measured as  $nWS:nRS$ , was interpreted as an index of the intensity of the mucosal inflammation caused by a bite. The peripheral blood eosinophil count ( $n \times 10^9/L$ ) was also interpreted as an indirect index of mucosal inflammation.

### Hookworm Maturity and Location

The maturity of a hookworm was adjudged from the size and the visceral detail evident (Figure 1). Localization in the small bowel was represented as a proportion to the capsule's transit time. The first duodenal image was at point 0.0, and the last small intestinal image was at 1.0.

### Statistical Analysis

Endoscopic interpretation bias was tested for using the paired Student  $t$  test. For comparing the propensity for bites to



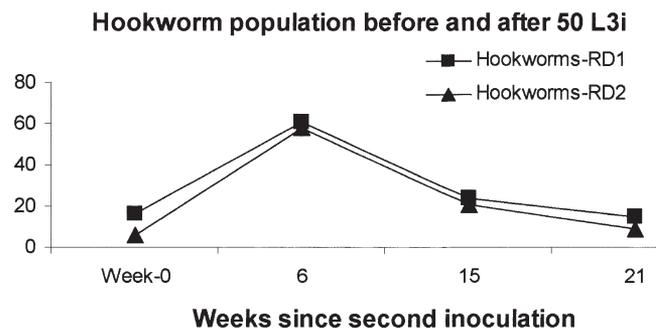
**Figure 1.** (A) Highlights the hemorrhagic spot (*right-pointing arrow*) surrounding an immature adult hookworm. Feeding is evident from the blood that defines the hookworm’s gastrointestinal tract. Red spots occurring independently were interpreted as recent feeding spots. As some red spots age, ulceration occurs. (B) Displays residual hemorrhage in the villous tips surrounding an evolving aphthous ulcer (*left-pointing arrow*). A barely visible hookworm is nearby (*downward-pointing arrow*). Aphthous ulcers were small and mostly difficult to detect as demonstrated in (C) (*right-pointing arrow*). In (D), a mature male (*downward-pointing arrow*) and female (*left-pointing arrow*) hookworms are shown attached to healthy mucosa with prominent villi in an immune suppressed Crohn’s subject. Maturity and gender were gauged from the improved clarity of internal structures in a large worm and the size of the helminths.

ulcerate and the number of bites per mature vs immature hookworm, testing was by  $\chi^2$  analysis with Yates correlation. The  $R^2$  values of trend lines were calculated using the equation:  $y = mx + b$ , where m is the slope and b is the intercept.

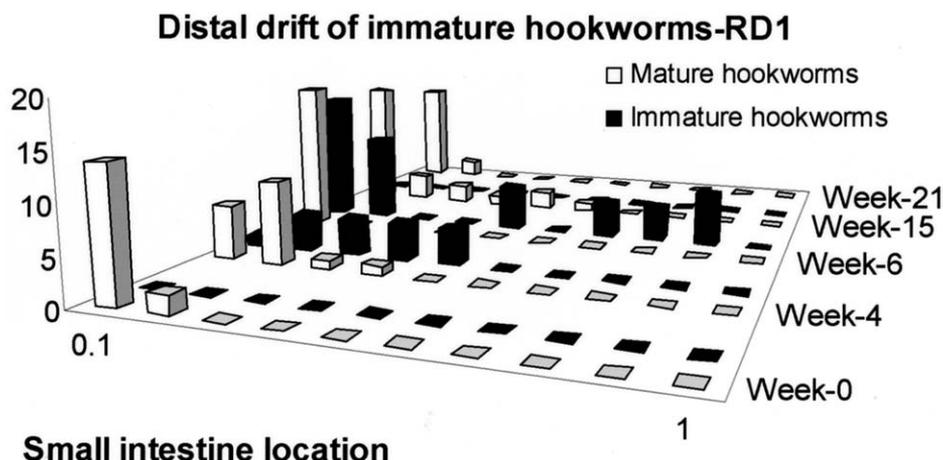
**Results**

***Necator americanus* Migration**

In RD2, inoculations with 31 and 50 L3i, respectively, were applied 27 weeks apart. Capsule endoscopy was performed on 5 occasions (Figure 2). Importantly, capsule endoscopy was performed 2 (CE No. 2) and 6 (CE No. 3) weeks after the second inoculation, estimated to be before and after the newly inoculated larvae might be expected to have completed migration. All inoculated L3i successfully completed migration, after which, the colony diminished.



**Figure 2.** The hookworm count in both RD1 (■) and RD2 (▲) increased 6 weeks after inoculation with 50 L3i, respectively, with most if not all newly inoculated worms being accounted for. By week 21, the size of the hookworm colony had returned to the preinoculation level (week 0), a status quo that appeared to be constitutively set by the host.



**Figure 3.** The location of hookworms was estimated from the time course of the capsule, with 0.0 being the first duodenal image and 1.0 the last ileal image. In RD1, the initial colony of mature hookworms (white bars; week 0) was mostly maintained, but newly inoculated, immature hookworms (black bars; week 4 and week 6) were prone to drift distally. By week 15, all hookworms appeared mature. Of 50 L3i inoculated, 44 were located. The 6 worms unaccounted for were likely either overlooked during the capsule transit or, as suggested by the week 6 profile, already expelled from the small intestine. Some immature hookworms appear to have established in the duodenum by replacing mature worms (week 4).

To test that successful migration was not idiosyncratic for RD2, RD1 with a stable population of 17 hookworms measured 72 weeks after an initial inoculation of 100 L3i had capsule endoscopy performed 4 and 6 weeks after a second inoculation of 50 L3i. On day 41 postinoculation, 44 immature worms and 17 easily distinguished mature worms were identified (Figure 3). In both researchers, the colony appeared to contract to a constitutively determined status quo; in RD1, the week-0 count was 16, and, at week-21, it was 15; and, in RD2, the respective counts were 6 and 9.

### Host Response

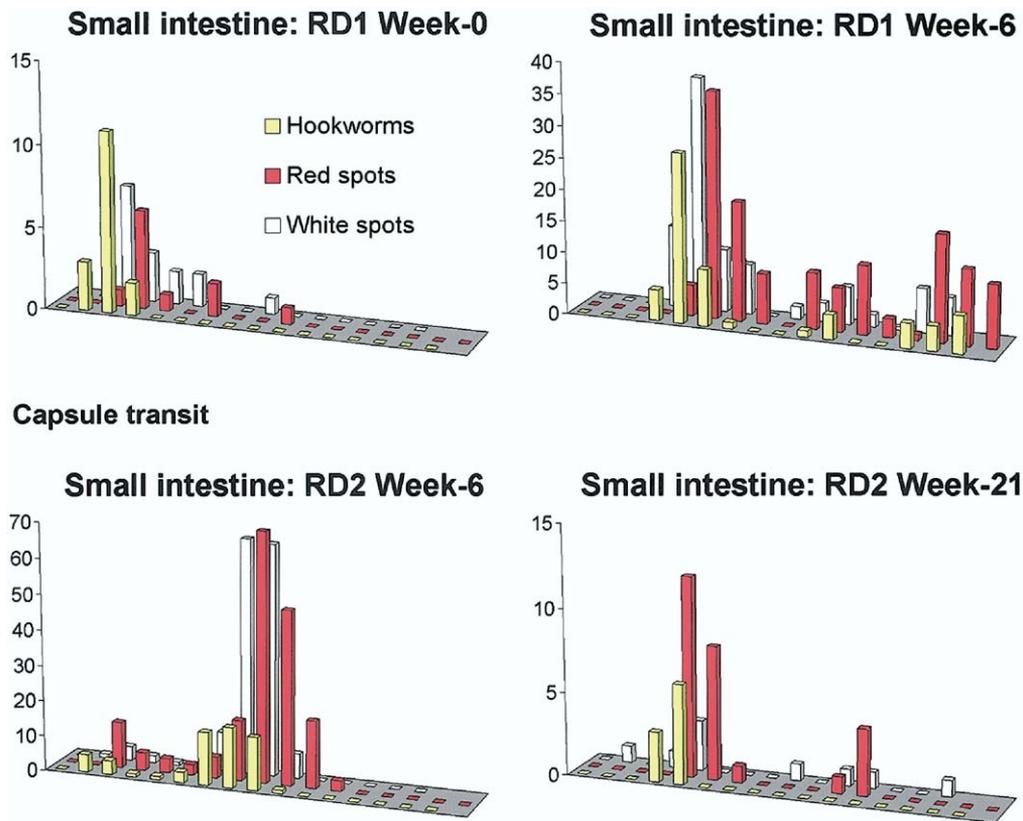
Four to 6 weeks after the initial inoculation, allergic enteritis developed in all subjects. The researchers (RD1–3) each experienced abdominal pain, and RD2 and RD3 had diarrhea, whereas, in the Crohn's disease subjects, the condition was subclinical. Peripheral blood eosinophil (PBE) counts ( $<.50 \times 10^9/L$ ) were normal in all subjects before inoculation, but rose after week 4, more so in the researchers than the Crohn's disease subjects (RD1–3: mean maximum PBE,  $4.65 \times 10^9/L$ ; range, 3.20–7.36 vs CD1-2 mean,  $.70 \times 10^9/L$ ; range, .60–.80), and Charcot–Leyden crystals indicative of eosinophil degranulation were present in feces from all subjects. In RD3, intense eosinophilic inflammation was apparent in biopsy specimens collected at 15 cm beyond the duodenal-jejunal junction at week 8 (PBE,  $2.90 \times 10^9/L$ ) after a first inoculation with 50 L3i, but biopsy specimens similarly collected after the second inoculation from RD1 at week 4 (PBE,  $1.6 \times 10^9/L$ ) and week 5 (PBE  $1.99 \times 10^9/L$ ) and RD2 at week 8 (PBE,  $3.69 \times 10^9/L$ ) were normal. In RD1, biopsy specimens were seen to be from tissue immediately

adjacent to mature (copulating) hookworms. Both RD1 and RD2 had a more severe skin reaction to the second inoculation than initially. In RD2, 47–49 papules identifying larval entry points that lasted 3 weeks were counted. In RD1, papules coalesced forming a blister, with changes lasting for 8 weeks. Painful enteritis similar to the initial exposure was experienced by RD2, but colonization with new hookworms in RD1 caused a barely noticeable abdominal discomfort.

Total IgE ( $<100$  IU/mL) was  $<40$  IU/mL in each subject prior to inoculation. No increase had occurred in the Crohn's disease subjects by week 20. In RD2, the levels increased to 214 IU/mL at week 20 and 404 IU/mL after the second inoculation but, in RD1, remained normal with a maximum recorded level of 53 IU/mL after the second inoculation.

### *Necator americanus* Distribution

The distribution of hookworms was influenced by the parasite's maturity. Mature hookworms populated the distal duodenum and proximal jejunum, a finding confirmed by conventional endoscopy (Figure 3). At week 6, a bimodal distribution was evident, with newly arrived worms composing the distal colony (Figure 4). Some new hookworms appeared to stick proximally, but this was quantitatively different between hosts. Taking the capsule transit point of .3 to arbitrarily separate the colonies, a seemingly more tolerant RD1 accommodated 25 new worms additional to the established colony of 17 mature worms compared with 3 in RD2, who had 7 mature worms still resident ( $P = .001$ ). Serial studies



**Figure 4.** The floor of the *graph* locates the capsules transit within the small intestine. Hookworms (yellow bars) that survive to maturity colonized the proximal small bowel, as shown in the capsule endoscopies RD1 week 0 and RD2 week 21. RD1 is a healthy researcher with a stable infection who had not been in contact with L3i for over a year, and RD2 is a different researcher whose last contact was 21 weeks prior. A bimodal distribution of hookworms, feeding spots (red bars), and aphthous ulcers (white bars) is evident by week 6 in both researchers. The red spot per hookworm ratio was higher in mixed colonies of mature and immature parasites vs colonies composed of mature worms only, although not all differences reached statistical significance. This pattern suggests that the immature worms had a shortened attachment, possibly because of less control of the allergic reaction caused by the bite.

demonstrated a drift distally of immature worms resulting in ultimate expulsion (Figure 3).

#### Feeding Time, Inflammation, and Attrition

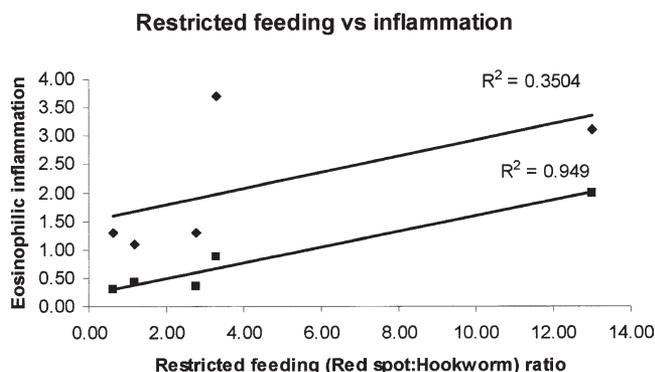
As immature hookworms were expelled in a distal direction, this drift was accompanied by evidence of restricted feeding and an enhanced inflammatory response (Figure 4). The feeding time for immature hookworms was shorter than for mature parasites. The restricted feeding indices ( $nRS:nHW$  ratio) for the total hookworm colony immediately before the second inoculation vs the colony 6 weeks later were less: 11:16 vs 142:61 ( $P = .005$ ) in RD1 and 9:6 vs 192:58 ( $P = .24$ ) in RD2. In RD2, both the eosinophil count measured at the time of capsule endoscopy and the number of aphthous ulcers relative to feeding spots, indices of eosinophilic inflammation, correlated with the number of feeding spots per hookworm (Figure 5). In RD1, who experienced a vigorous enteritis after first contact with 100 L3i (maximum PBE,  $4.00 \times 10^9/L$ ) and a barely evident response after the second with 50 L3i (maximum PBE,  $1.99$

$10^9/L$ ), a correlation between blood eosinophilia and restricted feeding ( $R^2 = .02$ ) and aphthous ulceration and feeding ( $R^2 = .00$ ) was not demonstrated.

Hookworm attrition was linked to allergic inflammation. Eight capsule endoscopies were performed 13–15 weeks after an inoculation, 7–9 weeks after L3i had theoretically completed migration and when the eosinophilic enteropathy was most intense. Hookworm attrition during this period, estimated as the difference in the maximum theoretical population (the L3i dose at the previous inoculation plus the number of hookworms already in the system as counted at the most recent, previous capsule endoscopy) minus the current count, correlated with the concurrent maximum eosinophil count ( $R^2 = .34$ ).

#### Discussion

This small, largely unplanned observational study has produced challenging results. The fortuitously com-



**Figure 5.** The results from 5 serial studies performed on RD2 are summarized. The restricted feeding index ( $nRS:nHW$ ) correlated with the propensity for the feeding spots to ulcerate ( $nWS:nRS$ ; ■), and the eosinophil count ( $n \times 10^9/L$ ) was measured at the time of capsule endoscopy. The difference in  $R^2$  likely relates to the different methodologies; the *red spot per hookworm* result would have been affected by the quality of vision available at each capsule endoscopy, an effect that would similarly influence the aphthous ulcer count but not blood eosinophilia.

plete record of all hookworms surviving migration in an already infected but healthy person related to a single observation but was validated when tested in a second volunteer. These are the only instances in which *N americanus* migration has been tested using such a reliable technology. The result is inconsistent with the concept of challenged migration, suggesting rather a protected transport. Allergic inflammation accompanied the commencement of feeding, but this was not a capricious response. Although qualitatively similar in both the tolerant and resistant host, quantitative differences were obvious. A purposeful strategy of undermining attachment and restricted feeding so as to control the hookworm colony is described.

A hookworm's success is tied to that of the permissive host. A sustainable dispersion is crucial. Adult parasites are robust and physically separated from the immune milieu. Also, established infections in endemic areas do not provoke much intestinal reaction, a finding consistent with the histologic appearance of mucosa from our chronically infected volunteers.<sup>21</sup> For these reasons, larval migration has been regarded as the more likely avenue for infection control.<sup>4</sup> Our observations suggest otherwise. Parasites and hosts share many homologous molecules.<sup>22</sup> Infective larvae discard an exterior sheath after penetrating the dermis. Perhaps surface lectins then exposed are recognized by receptors expressed by the host, setting in train a process mimicking the transport of the host's migratory cells.<sup>23,24</sup> Absence of a pulmonary response suggests that allergenic molecules are scarcely expressed during this phase of the lifecycle.<sup>10</sup>

Being hematophagous, hookworms must bite the mucosa to maintain location, access a vessel, prevent clotting, feed, and eventually establish a new attachment. An expanding repertoire of hookworm-derived facilitating products is being unravelled.<sup>25</sup> Excretory-secretory molecules (ESMs) have evolved to fit reciprocal receptors in the designated host. By optimizing this relationship, hookworms in the permissive host have a mechanistic advantage over opportunist species.<sup>26</sup> For example, *A caninum*-derived cathepsins D digests canine hemoglobin more effectively than human, and the *N americanus* equivalent vice versa.<sup>27</sup> As well as antiplatelet, anticoagulant, proteolytic, and digestive functions, ESMs also regulate the host's immune response.<sup>25,28,29</sup> However, ESMs, composed of peptides and lectins, are intrinsically allergenic, irrespective of the parasite-host pairing and irrespective of IgE priming.<sup>23</sup> By cross-linking the carbohydrate component of the Fc region of IgE, mast cells and basophils can be activated independently of antigen-antibody binding.

In northern Australia, *A caninum* was proven the cause of a minor epidemic of eosinophilic enteropathy occurring in people living in developed urban environments.<sup>30–32</sup> Perhaps more remarkable, subclinical *A caninum* infection appeared to be common with several examples of a single worm being found serendipitously in healthy people undergoing colonoscopy for unrelated reasons.<sup>33</sup> Red spots interpreted as being recent feeding sites and aphthous ulcers, white spots, were found in close proximity to the attached hookworms.<sup>34</sup> The hookworms recovered were undamaged but were smaller than those recovered from dogs. The concept of challenged nutrition was proposed.<sup>35</sup> Simply, ESMs in an exotic host are suboptimally matched to designated receptors and fail to control allergy. In this setting, allergy causes a mostly controlled degradation and abbreviation of the bite.

We can now show changes in humans infected with *N americanus* that are reminiscent of our experience with canine hookworms. Red spots and aphthous ulcers were adjacent to attached hookworms, with transitional red spot-ulcers also found. This appearance was influenced by the host and by the location and maturity of the hookworms. Hookworm attrition appears linked to the vigour of eosinophil-mediated damage to the mucosa. Immature worms were vulnerable. We speculate that mature worms are better able to produce enough ESMs to check the allergic response. Parallel examples of immunity exist. In rodents and domesticated livestock, spontaneous cure of intestinal nematodes due to a *weep and sweep* mechanism triggered by enhanced Th2 immunity has been described.<sup>36,37</sup> Nematodes are not killed, but the

hostile physiologic changes result in the expulsion of vulnerable parasites.

All subjects, including the immune suppressed Crohn's disease patients, developed allergic enteritis immediately after the first exposure to *Necator*, almost certainly before an IgE response to ESMs could have occurred. Some ESMs, *superallergens*, could and probably do initiate an innate immune response.<sup>38</sup> Adaptive responses were measured in 2 reservoir donors, both healthy men of similar age and background, but who were unexpectedly constitutively different. Both responded in a qualitatively similar way but not quantitatively so. The more tolerant host (RD1) experienced virtually no symptoms of enteropathy and produced less total IgE and eosinophils following inoculation with additional hookworms.

How might this contrived infection in volunteers relate to adaptive immunity as might occur in an endemic setting? In brief, ESMs generate IgE, which in turn activates type 2 cytokines. In an endemic setting, IgE correlates with an individual's hookworm burden and negatively with the size of resident hookworms and fecundity.<sup>7,8</sup> The more severe enteropathy seen in the IgE-responsive volunteer is consistent with the adaptive enhancement of the allergic process. A new paradigm for resistance to hookworm emerges. A naturally tolerant host, a status linked to interleukin 5, sustains a large colony. To drive IgE high enough to up-regulate allergy, more ESMs are required than in the naturally resistant host. When allergic inflammation is sufficient to impact on feeding, hookworm size and fecundity are compromised. Presumably, senile hookworms eventually die or are expelled, setting in train reduced ESMs, a fall in IgE and allergy, sufficiently so to allow new hookworms to mature. A biologic balance is achieved. The corollary, a naturally resistant host requires less ESMs to trigger attrition.

Resistance against *N americanus* does occur. Two layers appear likely: an innate resistance based on the intrinsic allergenicity of ESMs and an adaptive response mediated by IgE to fine tune the colony size.

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