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Exposure to hookworms in patients with Crohn's disease: a case-control study

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SUMMARY

Background
Helminths have been used to inhibit intestinal inflammation in patients with Crohn's disease.

Aim
This study was undertaken to determine if there is a protective association of prior hookworm infection with Crohn's disease, in a region where there is epidemiological transition from parasitic and infectious diseases to increased auto-inflammatory diseases.

Methods
Hookworm exposure was assessed by peripheral blood mononuclear cell (PBMC) activation by hookworm antigens in 78 patients with Crohn's disease and 75 healthy control participants. The change in proportion of T cells exhibiting CD69 after exposure to crude hookworm antigens was measured. Interferon-γ ELISPOT response to a panel of six recombinant hookworm antigens was analysed.

Results
Patients with Crohn's disease were more often from an urban background ($P = 0.005$) compared to controls, while their socioeconomic status was not significantly different. T cell activation (increase in $CD3^+CD69^+$ population) by hookworm antigen was significantly higher in controls compared to Crohn's disease patients ($P = 0.017$), while activation by the nonspecific mitogen phytohemagglutinin was similar in both groups. Circulating T memory cells ($CD3^+CD45RO^+$) after exposure to hookworm antigens were not significantly different between the two groups. Mirroring these changes, interferon-γ ELISPOT responses to hookworm antigens were seen in 36 of 75 controls compared to 20 of 78 Crohn's disease patients (Fisher's exact $P = 0.005$). Multivariate analysis indicated that CD3CD69 shifts ($P = 0.019$), ELISPOT reactivity ($P = 0.039$) and place of residence ($P = 0.024$) were all independently associated with Crohn's disease.

Conclusion
The inverse association between Crohn's disease and hookworm antigen reactivity is consistent with the hygiene hypothesis, but requires further exploration.

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INTRODUCTION

Crohn's disease is an inflammatory disease of the bowel caused by an abnormal immune response to luminal commensal bacteria in a genetically predisposed host. The incidence of Crohn's disease has significantly increased in the industrialised world from the 1950s to the mid 1980s.[1, 2] Crohn's disease was believed to be rare in India until about two decades ago, but is now apparently increasing in prevalence.[3] Immigrants from the Indian subcontinent to the United Kingdom provided a particularly fascinating insight into the effect of environment on the development of this disease. The incidence of Crohn's disease in the 1980s increased in immigrants to the Leicester from the Indian subcontinent, and the increase in incidence was particularly marked in the children of first generation immigrants.[4] A comparison of 25-year-old individuals born in a particular year in the United Kingdom revealed that those of south Asian ethnic origin had a significantly higher risk of developing inflammatory bowel disease than their peers of Caucasian or European descent.[5] These studies suggested that environmental changes influence the development of Crohn's disease in susceptible individuals.

The steady increase in incidence of Crohn's disease and other auto-inflammatory diseases in many developed countries in the last three decades coincided with a steady decrease in the incidence of infectious diseases in the same countries.[6–8] A case-control study showed that patients with CD were more likely to have had hot water taps and a separate bathroom in the first house they ever lived in.[9] A 'failure to acquire intestinal parasites' is one possible factor that could explain the increasing incidence of Crohn's disease in developed countries.[10] In the original explanation for this observation, it was hypothesised that the strong T helper type 2 (Th2) reaction induced by the helminth parasites neutralised the unbalanced T helper type 1 (Th1)-directed inflammation in the gut in Crohn's disease.[11] However, increasing evidence suggests that the Th1/Th2 balance has been overemphasised in the hygiene hypothesis, and that helminths may in fact have an immunoregulatory role through expansion of the population of circulating regulatory T (T_{reg}) cells and activated macrophages, associated with increased production of interleukin-10 and transforming growth factor-β.[12, 13]

Hookworm infects nearly 750 million persons worldwide.[14] In the early and mid-1980s, between 60% and 90% of apparently healthy villagers in rural south India were observed to have hookworm infection.[15, 16] The prevalence has now decreased to between 10% and 20% in this region.[17, 18] Multiple factors, including a regular deworming programme since the mid-1990s and BCG immunisation, may be responsible for this decrease.[18–20] The present case-control study was undertaken to test the hypothesis that Crohn's disease in Indian patients would be inversely associated with exposure to hookworm infection.

MATERIALS AND METHODS

This was a case-control study utilising newly diagnosed and prevalent Crohn's disease patients as case subjects who were compared with a group of healthy control subjects. Both cases and controls were recruited in the outpatient clinics run by the Department of Gastrointestinal Sciences of the Christian Medical College, Vellore. Cases were recruited from patients attending the Inflammatory Bowel Disease clinic of the Department of Gastrointestinal Sciences during the period March 2006 to December 2007. Patients with Crohn's disease under follow-up for at least 3 months at this institution were recruited for the case group if they had been diagnosed to have Crohn's disease within 2 years before enrolment into the study. Diagnosis of Crohn's disease was based on satisfying standard clinical, histological and radiological criteria and confirmed by response to therapy over at least 3 months of follow-up.[21, 22] Exclusion criteria were: (i) Demonstration of acid fast bacilli on biopsy by histology or culture, or demonstration of pulmonary infiltrates on chest radiographs, (ii) Past history of tuberculosis and/or response to anti-tuberculous therapy, (iii) Disease confined to the colon with continuous involvement (no skip lesions) beginning in the rectum, (iv) History of steroid use within the last 1 month. The control participants were recruited from healthy individuals, unrelated to the Crohn's disease patients, who were accompanying patients attending the Gastroenterology outpatient clinic for some illness other than Crohn's disease. None of the controls had clinical evidence of gastrointestinal disease. Patients with Crohn's disease underwent investigation according to the standard clinical protocol of the department. Patients and controls underwent microscopic examination of three freshly passed samples of stool for parasite ova and cysts using concentration methods. The demographic, clinical, laboratory and treatment data were noted in predesigned forms. Hookworm infection could be influenced by a number of factors including socioeconomic status, rural residence and age, and hence data on these potentially confounding variables was collected. Urban or rural residence was defined as per criteria set out in the census definitions of the Government

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of India.[23] Socioeconomic status was calculated according to the modified Kuppuswamy scoring system, with the economic score being modified for 2007.[24] A venous blood sample of 20 mL of whole blood was collected from all study participants after obtaining informed written consent.

Crude extracts of adult (adult worm soluble extract, AE) and third stage larvae (L3E) of hookworm antigens as well as recombinant hookworm antigens were kindly provided by Dr Jeffrey Bethony (George Washington University, Washington DC, USA). The following recombinant hookworm antigens were used in the study – Na-ASP-1 (*Ancylostoma* Secreted Protein-1 of *Necator americanus*, expressed in *Escherichia coli*), Na-ASP-2 (*Ancylostoma* Secreted Protein-2 of *N. americanus*, expressed in *Pichia pastoris*), Ac-MTP-1 (*Ancylostoma caninum* L3 secreted metalloprotease-1, expressed in *E. coli*), Ac-FAR-1 (*A. caninum* adult secreted fatty acid and retinoic acid binding protein, expressed in *E. coli*), Ac-GST-1 (*A. caninum* adult secreted glutathione S-transferase, expressed in *P. pastoris*), and Ac-TIMP-1 (*A. caninum* adult secreted tissue inhibitor of metalloprotease, expressed in *P. pastoris*).

Lymphocyte proliferation in response to crude hookworm antigens was used as the primary marker of prior encounter with hookworm, and was measured as a shift in the population of cells expressing the early activation antigen CD69. Lymphocyte phenotyping was done for evaluation of membrane expression of activation markers and markers of memory cells. Monoclonal antibodies conjugated with one of the following fluorescence compounds Phycoerythrin (PE), Allophycocyanin (APC), Peridinin-chlorophyll proteins (PerCP) were obtained from BD Biosciences (San Diego, CA, USA) Whole blood was cultured with and without crude hookworm antigens (mixed, 35 μg/mL) at 37 °C in a humidified CO_2 incubator for 48 h. The optimal concentration of the hookworm crude antigens was determined by initial standardisation experiments. Phytohemagglutinin (*Phaseolus vulgaris* lectin 10 μg/mL, L8902, Sigma-Aldrich, St. Louis, MO, USA), a nonspecific mitogen, was used as a control for these studies. After 48 h, 200 μL of activated and nonactivated blood cells were incubated with 5 μL of monoclonal antibody in the dark at room temperature for 30 min, lysed with 1x FACS Lysing Solution (BD Biosciences, San Diego, CA, USA), washed twice in wash buffer containing 0.5% bovine serum albumin and 0.1% sodium azide in PBS (pH 7.2), and re-suspended in 200 μL of wash buffer. The following combinations of monoclonal antibodies were used CD3 (PE)/CD45RO (APC)/CD69 (PerCP). Appropriate isotype controls were used with these fluorescent antibodies. Cell counts were acquired in a four colour flow cytometer (FACSCalibur, BD, San Jose, CA, USA) and analysed using the FlowJo software package (Tree Star, Ashland, OR, USA). 50 000 events were analysed and the cells were gated on lymphocytes and subsequently on CD3. The values shown in this paper, of early activation or memory cells, are expressed as a percentage of the $CD3^+$ population. Changes in cell populations in response to hookworm antigens were derived by subtracting the cell percentage in tubes incubated without hookworm antigens from the cell percentage in tubes stimulated with hookworm antigens.

IFN-γ cytokine responses to recombinant hookworm antigens were assessed using ELISPOT (BD Biosciences, San Diego, CA, USA). Peripheral blood mononuclear cells were separated from blood by density gradient centrifugation using Histopaque 1077 (Sigma) and resuspended in RPMI medium with 10% fetal bovine serum. They were added in amounts of 2.5×10^5 cells per well to 96 well ELISPOT cell culture plates (Millipore, Catalog number S2EM004M99, Billerica, MA, USA). Incubation was done in duplicate for control wells, phytohemagglutinin, and the six different recombinant hookworm antigens in a final volume per well of 200 μL of RPMI-1640 medium (Gibco, Grand Island, NY, USA). Final concentrations of stimulants, determined by initial standardisation experiments and optimal concentration in cell culture were 30 μg/mL for recombinant hookworm antigens and 10 μg/mL for phytohemagglutinin (lectin from *Phaseolus vulgaris*, L8902, Sigma-Aldrich). Cells were cultured at 37 °C in a humidified CO_2 incubator for 48 h. After 48 h, the cells were washed and developed for IFN-γ following the manufacturer's instructions. The spots were documented in a Chemi-Smart 3000 (Vilber-Lourmat, Marne-la-Vallee, France) reader and counted manually. ELISPOT responses were recorded as positive if the control well had at least 10 spots, and wells with antigen had at least twice the number of spots as control wells. All individuals were checked to determine that they responded to phytohemagglutinin (the positive control) to ensure that the responses were not negative due to nonspecific anergy.

Sample size calculations and statistical analysis:

Sample size calculations were based on the assumption that 50% of the control group would have been exposed to hookworm infection. Using a categorical definition of exposure, we calculated that a sample size of 85 partici-

pants per group would have 90% power to detect a 50% reduction in hookworm exposure in the Crohn's disease group. We recruited 91 patients with Crohn's disease and 82 controls, but due to a technical error, ELISPOT results could not be interpreted in 20 participants (13 Crohn's disease, 7 controls).

Categorical values are shown as numbers or proportions while continuous variables are shown as mean (SD). Significance of differences for individual variables between the two groups was calculated using the Mann–Whitney test in case of continuous variables and the Fisher exact test in the case of categorical variables. Odds ratios (OR) were obtained and their 95% confidence interval (CI) were used to test the association of Crohn's disease with the respective independent variable. A P-value < 0.05 was considered significant. Multivariate analysis using forward conditional logistic regression was undertaken to evaluate the association between Crohn's disease and immune responses to hookworms adjusting for socioeconomic status and residence. The model included the CD3CD69 response, ELISPOT, socioeconomic status and residence, with the latter three being treated as categorical variables.

ETHICAL CONSIDERATIONS

The study protocol and consent forms were approved by the Institutional Review Board of the Christian Medical College, Vellore.

RESULTS

A total of 104 patients who were diagnosed with Crohn's disease were recruited for the study. Thirteen of these were excluded either because of change in diagnosis (six patients) or lack of follow-up to confirm the diagnosis of Crohn's disease (seven patients). Thirteen patients with Crohn's disease and 7 of 82 control participants were excluded because of inability to do ELISPOT analysis for technical reasons. Hence the final analysis included 78 patients with Crohn's disease and 75 control healthy volunteers. Their demographic characteristics are listed in Table 1. Patients with Crohn's disease were significantly more often from an urban background compared to control participants. Hookworm ova were detected on stool examination in two patients and in five controls (P = NS).

T cell activation ($CD3^+CD69^+$ cell shift) in response to crude hookworm antigens was significantly less in patients with Crohn's disease than in controls (Table 2). The shifts in memory T ($CD3^+CD45RO^+$) cells following exposure to hookworm antigens was not significantly different between controls and patients with Crohn's disease (Table 2).

Table 1 | Demographic characteristics of the study participants

	Crohn's disease	Controls
Age in years, mean (SD)	27.0 (8.8)	26.0 (4.4)
Gender (M:F)	51:27	53:22
Socioeconomic class		
Median	II	II
Range	I–III	I–III
Area of residence		
Rural	21	37
Urban	57	38

Socioeconomic class (I – upper, II – upper middle, III – middle) was assigned from a composite score based on educational achievement, occupation, and family income. Residence was significantly different between control participants and patients with Crohn's disease (P = 0.005).

Table 2 | Immune responses to hookworm antigens in patients with Crohn's disease and controls

	Crohn's disease	Controls	P-value
IFN-γ ELISPOT	20 / 78	36 / 75	0.005
Δ $CD3^+CD45RO^+$ (%)	0.52 (5.04)	0.66 (3.46)	0.846
Δ $CD3^+CD69^+$ (%)	0.903 (4.20)	3.16 (6.44)	0.001

ELISPOT reactivity was defined as the presence of a positive test in response to stimulation with one or more of six recombinant hookworm antigens; significance of difference between groups was tested using the Fisher exact test. T cell activation and change in memory T cell populations was measured as the change in $CD3^+CD69^+$ and $CD3^+CD45RO^+$ ratio, respectively, in peripheral blood mononuclear cells exposed or unexposed to crude hookworm antigens, are expressed as mean (SD), and significance of difference was analysed using the Mann–Whitney U test.

Both patients and controls showed equal numbers of ELISPOT spot forming units when stimulated with the mitogen phytohemagglutinin (Figure 1). Interferon-γ ELISPOT responses to hookworm antigens were noted in 36 (48%) of controls and in 20 (25%) of patients with Crohn's disease, which was a statistically highly significant difference. Figure 2 shows the ELISPOT responses to each of the six recombinant hookworm antigens. The greatest number of responses was noted to the antigens Ac-FAR and Ac-TIMP. Responses to these antigens tended to occur in fewer patients with Crohn's disease

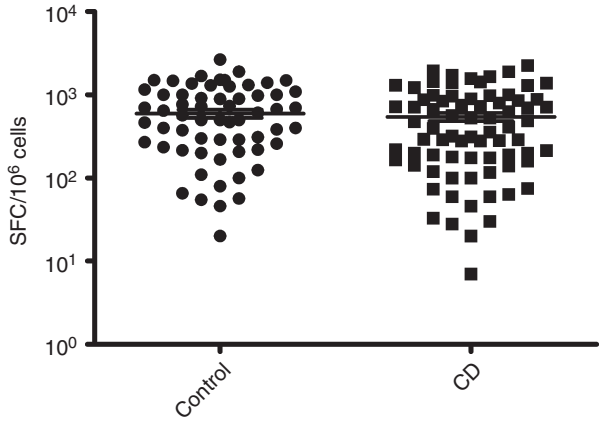

Figure 1 | Number of spot forming cells (normalised to number of cells incubated) in the ELISPOT when peripheral blood mononuclear cells from control volunteers and patients with Crohn's disease (CD) were treated with phytohemagglutinin. Horizontal bars indicate the median values, there was no significant difference between the two groups. Y axis shows number of spot forming cells formed per million cells (10^5 cells were usually plated per well) and is logarithmic.

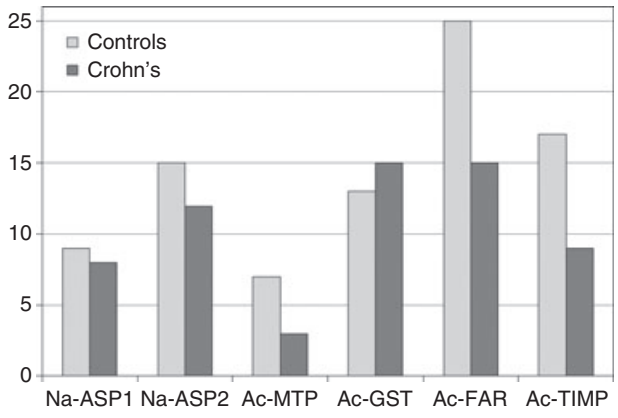

Figure 2 | ELISPOT reactivity to individual recombinant hookworm antigens in Crohn's disease patients and in controls. Bars represent absolute numbers of participants (out of a total of 75 controls and 78 Crohn's disease patients) showing responses to individual antigens. Na-ASP-1 - *Ancylostoma* Secreted Protein-1 of *N. americanus*, Na-ASP-2 - *Ancylostoma* Secreted Protein-2 of *N. americanus*, Ac-MTP-1 - *A. caninum* L3 secreted metalloprotease-1, Ac-FAR-1 – *A. caninum* adult secreted fatty acid and retinoic acid binding protein, Ac-GST-1 – *A. caninum* adult secreted glutathione S-transferase, Ac-TIMP-1 – *A. caninum* adult secreted tissue inhibitor of metalloprotease.

Table 3 | Logistic regression (forward LR) was undertaken to assess the association between Crohn's disease and the immunological parameters while controlling for area of residence and socioeconomic status

Variable	*P*-value	OR	Lower 95% CI	Upper 95% CI
CD3CD69 (% shift)	0.019	0.849	0.741	0.973
Positive ELISPOT response	0.039	0.448	0.209	0.961
Rural residence	0.024	0.421	0.199	0.890

CR, class interval; OR, odds ratio.

CD3CD69 shift, positive ELISPOT response to hookworm antigens, and rural residence were each protectively associated with Crohn's disease.

(15/78 for Ac-FAR and 9/78 for Ac-TIMP) compared to healthy controls (25/75 for Ac-FAR and 17/75 for Ac-TIMP) ($P = 0.06$ and 0.08 respectively). Responses to Na-ASP1, Na-ASP2, Ac-MTP and Ac-GST occurred in a similar proportion of individuals in both groups.

The logistic regression analysis model tested the association between Crohn's disease and the immunological responses to hookworm antigens while controlling for socioeconomic status and residence. It showed that CD3CD69 shifts in response to hookworm antigens, a positive ELISPOT response to hookworm antigens, and rural residence were all independently associated in a protective manner with Crohn's disease (Table 3).

DISCUSSION

The present study observed that patients with Crohn's disease in India demonstrated significantly less T cell activation than control participants in response to stimulation with crude hookworm antigens *in vitro*. They also reacted significantly less often to recombinant hookworm antigens in the interferon-γ ELISPOT than did healthy control participants. We interpret these observations as being indicative of reduced exposure to hookworm infection in patients with Crohn's disease in India. This negative association between exposure to hookworm infection and a diagnosis of Crohn's disease is of significant interest in relation to the hygiene hypothesis and Crohn's disease causation.

We chose to study immune responses to hookworm antigens in Crohn's disease patients as markers of whether these patients had been exposed to hookworm infection earlier in life. Periodic anti-helminthic intake, either through self-treatment or as part of a public health

programme, is not uncommon and therefore the use of stool examination for hookworm ova was considered to be insufficient to measure exposure to hookworms. Indeed three stool examinations revealed hookworm ova in only a minority of patients and controls. Lymphocyte proliferation in response to specific recall antigens is used to detect previous exposure to specific dietary or other antigens. The lymphocyte proliferative response to hookworm antigens is often suppressed during active worm infection, but increases after treatment and a robust lymphocyte proliferative response continues to be present even 1 year after eradication of the worm infestation.[25] CD69 is an early activation antigen, expressed on the surface of T (and other immune) cells, that is expressed within 2 h of T cell activation and is easily detected by flow cytometry.[26] T cells positive for this antigen ($CD3^+CD69^+$ cells) are significantly increased in patients with active hookworm infection.[27] The lower proportion of hookworm antigen-induced $CD3^+CD69^+$ cells in Crohn's disease patients than in the control group is therefore likely to reflect reduced previous exposure of Crohn's disease patients to hookworm infection.

Memory cells, that express CD45RO on the surface, are generated during the first response to an antigen, survive for long periods of time, and expand rapidly on re-exposure to the antigen. In the present study, $CD3^+CD45RO^+$ shifts after exposure to hookworm antigen were minimal in both study groups and were not significantly different between the two groups. Memory T cells comprise a very small proportion of the T cell population and as they expand rapidly in response to their cognate antigen, their surface expression of CD45RO may change, which may explain the lack of significant difference in their population between the two groups.

Interferon-γ ELISPOT responses to hookworm antigens were significantly more common in the control group than in patients with Crohn's disease. Interferon-γ secretion by peripheral blood mononuclear cells occurs in response to hookworm antigens in patients who had hookworm infection and have cleared it after therapy.[28–30] The presence of a positive interferon-γ ELISPOT response is likely to predict relatively recent exposure to hookworm infection. Individual hookworm proteins could potentially elicit different immune responses in the ELISPOT test. An excretory-secretory antigen of *Necator americanus* has been shown to stimulate NK cells inducing interferon-γ production,[31] while Ac-TMP-1 induced generation of IL-10-expressing T cells.[32] In the present studies, Ac-FAR and Ac-TIMP were the most potent antigens eliciting an interferon-γ ELISPOT reaction, and lesser numbers of Crohn's disease patients responded than the healthy controls. These are primarily *Ankylostoma caninum* antigens and this raises the possibility that perhaps *A. caninum* exposure is the primary protective association in our Crohn's disease patients. The latter hookworm does infect man, infection is often with a small number of worms, and the worms do not lay eggs in the stool making diagnosis of the infection quite difficult.[33, 34]

The hypothesis that parasitic infections protect against Crohn's disease is supported by several lines of circumstantial evidence. Crohn's disease is common in developed countries where intestinal nematode infections are uncommon, and uncommon in developing countries where intestinal nematode infections are common. In the United States, one in six individuals showed signs of previous *Trichinella* exposure on routine autopsy in the 1940s compared to less than 5% in the 1960s.[35] The situation in India currently with respect to helminth infections thus resembles the transition in the US between the 1940s and 1960s. Infestation with parasites produces a strong Th2 response, which can modulate the Th1 immune response to unrelated stimuli. Patients infected with *Schistosoma mansoni* mount a Th2 like response to tetanus toxoid immunization rather than the usual Th1 or Th0 type response.[36] The microfilaria *Brugia malayi*, or a soluble filarial extract, induces a Th2 response in mice that modulates the Th1 response to mycobacterial antigens.[37] There is experimental evidence that administration of helminths to patients with Crohn's disease limits intestinal inflammation. Patients with Crohn's disease have been effectively treated with oral administration of eggs from the pig whipworm, *Trichuris suis*.[38] In another pilot study, five of nine Crohn's disease patients deliberately infected with *Necator americanus* went into remission, while two others relapsed while long term immunosuppression was withdrawn.[39] The present study focused on hookworm infection because this was the most common gastrointestinal helminth infection in this region. However, it is possible that the hookworm immune responses noted in this study are only a surrogate marker for helminth infections of any kind, or more generally a marker for poor hygiene. It is interesting that there was a protective association between rural residence and Crohn's disease which would suggest that factors other than hookworm infection possibly play a significant role. Animal parasites often cause subclinical infection in man. For instance, *A. caninum* infection may occur in dog owners and infection is probably introduced into pet dogs through contact with feral dogs.[40]

In conclusion, the present study provides indirect support for the hygiene hypothesis through the finding of an association between reduced immune responses to hookworm antigens and a diagnosis of Crohn's disease. The role of helminth infection other than hookworm, as well as other factors related to rural vs. urban residence, will need to be evaluated further.

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