

EPIDEMIOLOGY OF HUMAN ANCYLOSTOMIASIS AMONG RURAL VILLAGERS IN NANLIN COUNTY (ZHONGZHOU VILLAGE), ANHUI PROVINCE, CHINA:

II. SEROEPIDEMIOLOGICAL STUDIES OF THE AGE RELATIONSHIPS OF SERUM ANTIBODY LEVELS AND INFECTION STATUS

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Abstract. Anti-hookworm antibody serologic responses were measured in residents of an Anhui provincial Chinese village where *Ancylostoma duodenale* is the predominant hookworm. Antibody responses were measured against either soluble infective third-stage larval (L3) or adult antigens. Immunoglobulins of the IgG class, especially IgG4 correlated with both the prevalence and intensity of *A. duodenale* hookworm infections. In contrast, there was an inverse correlation with IgM, but no correlation with IgA or IgE. Circulating IgG4 antibody responses might serve as a surrogate marker for active *A. duodenale* hookworm infection.

INTRODUCTION

Hookworm infection is one of China's leading public health problems with an estimated 194 million cases (Hotez *et al*, 1997). Most of these infections are distributed throughout the Yangtze River Valley or in Hainan Province in the South China Sea (Sun *et al*, 1998; Liu *et al*, 1999; Wang *et al*, 1999). Anhui Province ranks highly among the Yangtze River provinces with respect to hookworm endemicity (Xu *et al*, 1995). Based on 54,392 fecal examinations conducted between 1988-93, it was noted that 33.4% of the population harbors hookworm (Hotez *et al*, 1997). In April and May of 1998, we reinvestigated the epidemiology of hookworm infection among a rural population living in Zhongzhou Village. The village is located in Nanting County, in southwestern Anhui Province (lat.30°55'; long 118°17') close to the Yangtze river. It was noted that *Ancylostoma duodenale* is by far the predominant cause of hookworm infection in this village. Based on the recovery of either third-stage infective larvae (L3) or adult hookworms

from infected resident villagers, *A. duodenale* predominated over *Necator americanus* in ratios of 35:1 and 21:1, respectively. The overall prevalence of hookworm infection in Zhongzhou was 33%, with a peak age-associated prevalence among adults aged 41-50.

It was previously shown for human infections with *Necator americanus* that levels of specific antibodies correlate to age-related changes in infection intensity (Pritchard *et al*, 1990; 1992). It was further shown that IgG4 responses correlated particularly well with prevalence and intensity of *Necator* infections, and may even be a useful marker for this purpose (Palmer *et al*, 1996). There are no corresponding data for human *Ancylostoma* infections. Therefore studies were undertaken to determine if there are similar relationships between the prevalence and intensity of *A. duodenale* infections and isotype-specific antibody responses.

MATERIALS AND METHODS

Patient sample selection and fecal examination

Fecal examinations were performed on 380 local residents of Zhongzhou. All of the residents selected were between 6 and 65 years old. Non-written informed consent was obtained according

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to Protocol 10453 approved by the Yale University School of Medicine Human Investigation Committee and a similar protocol approved by the National Institutes of Health. Identification of intestinal nematode eggs (*Ascaris*, *Trichuris*, or hookworm) was obtained by brine floatation. Quantitative hookworm egg counts were determined in 115 residents whose fecal exams were positive for hookworm using the Kato-Katz method (Katz *et al.*, 1972) and expressed as eggs per gram of feces (EPG) (Wang *et al.*, 1999).

Sample collection

Peripheral blood samples were collected by venipuncture from 380 residents of Zhongzhou. The sera were separated and stored at -20°C. Blood was also obtained by venipuncture from 29 hookworm uninfected residents of Shanghai who were entering the first year class at Shanghai Second Medical University. The sera were pooled and served as a negative reference sample.

Antigen preparation

Two different hookworms were used to prepare sources of soluble antigen. Third stage infective larvae (L3) of the canine hookworm *Ancylostoma caninum* were obtained from the feces of infected dogs by the filter paper method (Xiao *et al.*, 1998). Adult hookworms of *Necator americanus* were obtained from the intestines of infected golden hamsters (Ren *et al.*, 1994). Soluble antigen was prepared as described previously (Xue *et al.*, 1992). Briefly the worms were rinsed extensively with water, and then ground with normal saline in a pestle prior to further disruption by sonication. The homogenate was centrifuged at 10,000g for 20 minutes and the supernatant collected. The soluble antigen was stored at -70°C.

Enzyme linked immunosorbent assay (ELISA)

The optimal concentrations of antigen, antibody and enzyme-conjugated antibody were determined using checkerboard titrations. All assays were carried out at a single serum dilution, with the optimal dilution selected for testing being that for which there was good discrimination between positive and negative samples (Palmer *et al.*, 1996). The indirect ELISA assay was used to measure parasite-specific antibody responses. Linbro/Titertek EIA plates (Flow Laboratories) were coated with either *A. caninum* L3 or *N. americanus* adult parasite extracts (0.6 µg/well) in 0.05M carbonate buffer

pH 9.6 overnight at 4°C. Due to potential competition between IgG and IgE for binding sites, high binding Nunc Immuno plates and higher antigen coating concentrations were used for IgE and IgA detection (0.75 µg/well). In order to detect an IgG2 response, it was necessary to treat the plates with 100 µg/ml poly-L-lysine prior to antigen coating. All plates were blocked for 1 hour with 5% non-fat milk in phosphate buffered saline (pH 7.4). The plates were rinsed with PBS/0.05% Tween 20, and incubated for 2 hours with test sera diluted in PBS/0.05% Tween 20 at 1:800 for IgG, 1:400 for IgM, 1:200 for IgA and IgG subclasses, and 1:50 for IgE. Replicate pooled positive (from 32 Zhongzhou Village residents infected with *A. duodenale*) and negative sera (either pooled sera from 29 physically normal students from Shanghai Medical University, or pooled sera from 38 Zhongzhou Village residents who were *A. duodenale*-negative as determined by fecal examination) were included on each plate. These controls were incorporated to confirm the specificity of the reagents. After extensive washing with PBS/0.05% Tween 20, peroxidase-conjugated anti-human IgG (1:2,000 and IgM (Dako Ltd, High Wycombe, England) at dilutions of 1:2,000 and 1:1,000, respectively, and then incubated 3 hours at room temperature. Unlabelled polyclonal anti-human IgE (Biognostic Ltd) at a dilution of 1:1,000, and monoclonal IgG subclass antibodies (Biognostic Ltd) at dilutions of 1:1,000 for IgG1 and IgG4, and 1:500 for IgG2 and IgG3 were incubated for 2 hours followed by an overnight incubation with peroxidase-labelled anti-rabbit or anti-mouse antibodies (Dako) or IgE and IgG subclass assays respectively. All plates were washed as before and developed with O-phenylenediamine (OPD)/H₂O₂ substrate for 20 minutes, after which color development was stopped by the addition of 25 µl 2M H₂SO₄ to each well. Optical densities were measured photometrically at 492 nm.

RESULTS

Comparison of serologic responses in hookworm infected residents compared with uninfected individuals

Of the 380 residents of Zhongzhou Village who underwent fecal examination for this study there were 115 who were positive for *A. duodenale* infection and 265 that were negative. The parasitologic findings from Zhongzhou were reported previously from Wang *et al.* (1999). Fig 1 shows

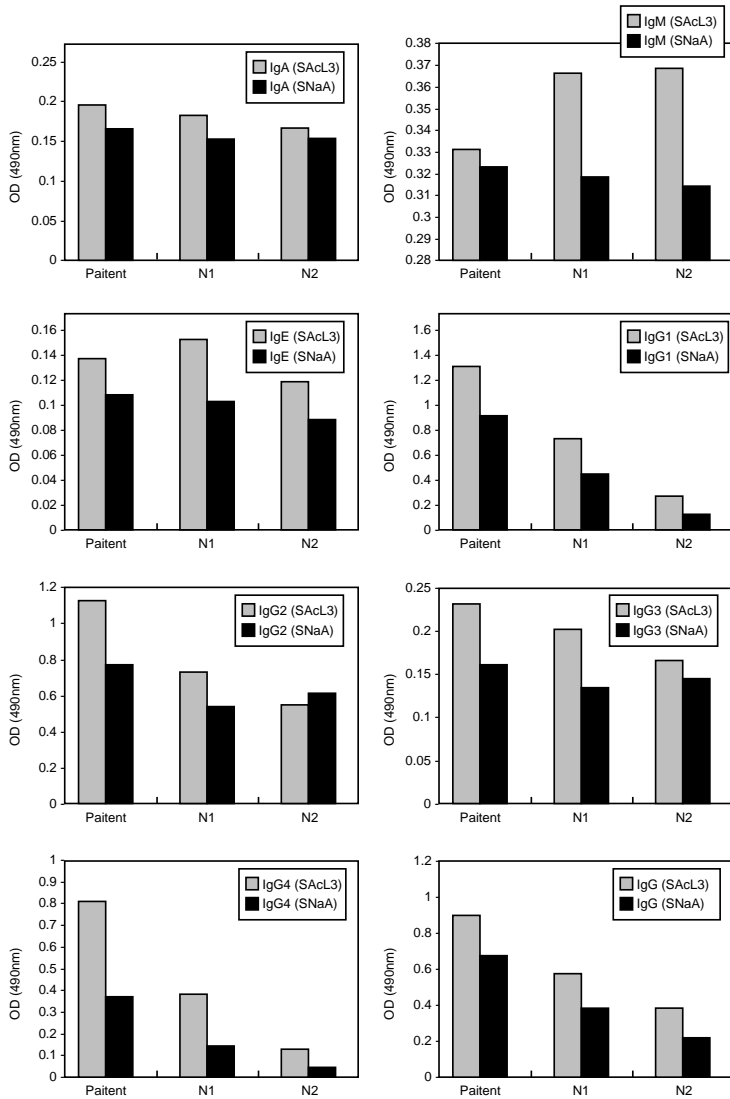


Fig 1—The antibody response(Ig class and subclass) in the different groups people.

Patient: worm or egg positive cases).

N1: Sera of egg and worm negative control for Zhongzhou.

N2: Sera of normal control from Shanghai.

the mean ELISA OD values obtained using sera from either 115 hookworm-infected residents of Zhongzhou Village, 265 residents of Zhongzhou who were determined to be negative for hookworm infection by fecal examination, or 29 normal control sera from medical students attending Shanghai Second Medical University. The Shanghai medical students have no known history of exposure to hookworm. The hookworm-infected Zhongzhou residents exhibited greater IgG and IgG subclass

responses to both L3 and adult hookworm antigens than either the hookworm-uninfected residents of Zhongzhou or Shanghai residents. The most striking difference was in the IgG4 responses relative to the uninfected individuals, whereas there were or no significant differences in the IgG3 responses. Among the uninfected individuals, the Zhongzhou residents exhibited greater IgG antibody responses compared to the Shanghai residents. In contrast, there were no significant differences in IgE, IgA and IgM responses between the hookworm-infected and uninfected residents. There were no significant differences in antibody responses between male and female residents (data not shown).

Age-dependent prevalence and antibody responses

The prevalence of hookworm in Zhongzhou increases with age so that the highest prevalence occurs among adults between the ages of 41-50 (Wang *et al*, 1999). As shown in Fig 2 the IgG and IgG subclass responses to either L3 or adult hookworm antigens correlate with the age-dependent prevalence of *A. duodenale* infections in Zhongzhou. The IgG4 responses in particular exhibited a strong correlation with increasing age ($r = 0.91$ using L3 as antigen, and $r = 0.96$ using adult hookworms as antigen). The IgG4 responses most closely mirror the prevalence. In contrast the IgM and IgE antibody responses exhibit a distinctly different age-dependent pattern. The IgM responses were at their highest among children aged 6-20 years and then subsequently declined. A similar (but less pronounced pattern) was also seen with IgE responses. IgA antibody responses remained constant for all age groups.

Age dependent intensity and antibody responses

The intensity of *A. duodenale* hookworm infections was also the highest among adults over the age of 30 with a peak in adults aged 31-40. However, the overall intensity of ancylostomiasis in this region was low and comprised of predominantly light infections. As shown in Fig 3 the patterns between antibody responses and intensity were similar to the relationship between antibody

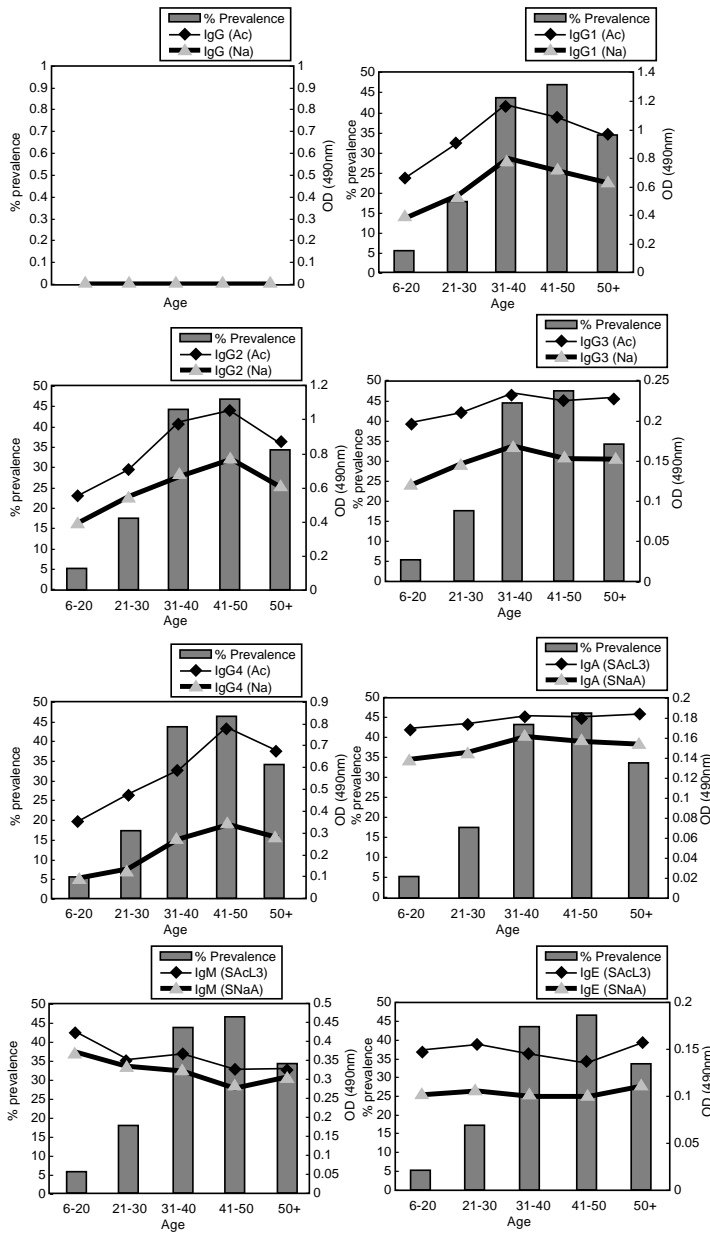


Fig 2—The responses of antibody (Ig class and subclass) versus prevalence.

responses and prevalence.

DISCUSSION

Our ELISA studies indicate that Chinese residents who live in a village with endemic ancylostomiasis will acquire circulating IgG antibody responses to the parasite. *A. duodenale* is almost the exclusive hookworm of this village (Wang *et al*, 1999). Although *A. duodenale* is the

predominant geohelminth in Zhongzhou with an overall prevalence of 33%, an estimated 25% and 9% of the population is also infected with *Ascaris lumbricoides* and *Trichuris trichiura* (Wang *et al*, 1999). The observation that uninfected residents of Zhongzhou also have higher circulating IgG antibodies to *A. duodenale* antigens than the Shanghai residents to the likelihood that they were exposed previously to hookworm. Anthelmintics are widely available and affordable for some of the Zhongzhou residents. Anti-hookworm antibodies of the IgG class correlated with levels of *A. duodenale* prevalence and infection. The highest correlation was associated with circulating IgG4 responses. This subtype was reported previously to correlate with the prevalence and intensity of *N. americanus* hookworm infections in Zimbabwe (Palmer *et al*, 1996), as it has for other helminthiases (Ottesen *et al*, 1985). It has been proposed by others that IgG4 responses can serve as a surrogate marker for the prevalence and intensity of *Necator* infections (Palmer *et al*, 1996). Presumably, IgG4 antibodies do not have a role in protective immunity against hookworm or related nematodes. This is the first report of IgG4 as a marker for *A. duodenale* infections.

In contrast to some of the IgG subclasses, there were no significant differences in the IgM, IgA and IgE levels between either the Zhongzhou or Shanghai residents. Moreover, these antibody levels did not parallel either the prevalence or intensity of ancylostomiasis in Zhongzhou. What is the explanation for this finding? The IgM antibody responses were highest in children and adolescents who had the lowest prevalence of hookworm infection. Among the explanation for this finding is that IgM represents an early antibody response to initial hookworm exposure. Alternatively, IgM has been noted to be a predominant antibody in rodents immunized with live nematode L3, including *Ancylostoma* (Ghosh and Hotez, 1999) and *Strongyloides* (Abraham *et al*, 1995; Brigandi *et al*, 1996). It has been further suggested that IgM antibody responses correlate with diminished worm burdens, and have

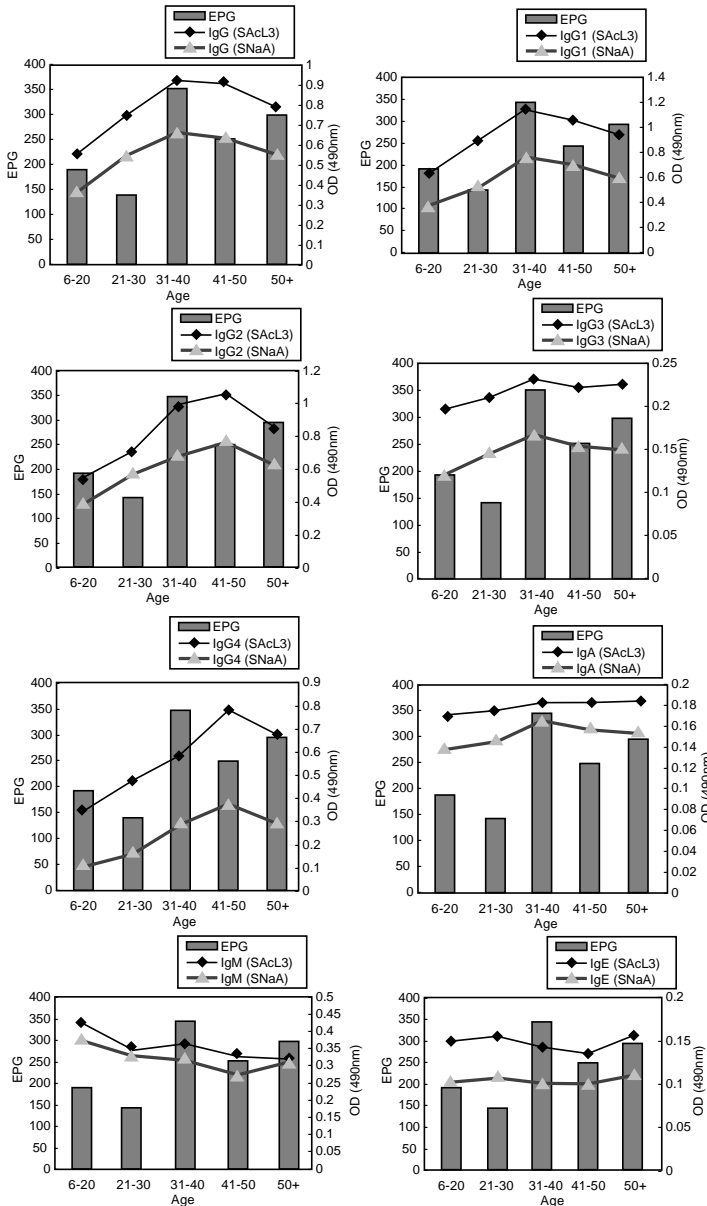


Fig 3—The responses of antibody (Ig class and subclass) ver-sus EPG.

a protective role in these systems (Abraham *et al*, 1995; Brigandi *et al*, 1996). Possibly the elevated IgM responses in the largely uninfected children and adolescents of Zhongzhou reflect this finding. Similarly, Pritchard and his colleagues (1990, 1992) have noted the protective role of circulating anti-hookworm IgE antibody responses. The studies reported here examine the circulating antibodies to whole worm larval and adult hookworm antigens. Further studies are in progress to examine immune

responses to individual larval and adult secreted antigens. These *A. duodenale* and *N. Americanus* antigens have been cloned from hookworms originating out of China; they are expressed in *Escherichia coli* (Zhan *et al*, 1999; Liu *et al*, 2000).

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