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Specific IgG Antibody Subclasses to *Angiostrongylus cantonensis* in Patients with Angiostrongyliasis

Pewpan M. Intapan[1], Wanchai Maleewong[1], Yada Polsan[1], Kittisak Sawanyawisuth[2] and Verajit Chotmongkol[2]

SUMMARY **Total IgG, IgG1, IgG2, IgG3, IgG4, IgA and IgM specific antibodies against *Angiostrongylus cantonensis* somatic antigen were determined by enzyme-linked immunosorbent assay (ELISA) in sera from proven human angiostrongyliasis (PA) cases, clinically suspected angiostrongyliasis cases with eosinophilic meningitis (EM) and healthy control (HC). The specific IgA antibody in each of the patient groups was significantly higher than those of the HC group ($p < 0.05$). The mean ELISA value of the specific IgM in the PA group was not significantly different from that of the HC group ($p > 0.05$). However, the mean specific IgM ELISA value in the EM group was significantly higher than that of the HC group ($p < 0.05$). The levels of the specific IgG and IgG subclasses in both patient groups were significantly higher than in the healthy control (HC) group ($p < 0.001$). Major differences were evident in the distribution of the IgG subclass antibodies between the patient groups. The IgG1 antibody demonstrated the highest sensitivity and specificity while the IgM and IgA responses were generally poor in both patient groups. The levels of the specific IgG antibody subclasses possibly explain immune responses to the parasite.**

Angiostrongylus cantonensis, the rat lungworm, is considered the main etiologic agent in human eosinophilic meningitis and meningoencephalitis from Southeast Asia to the Pacific islands, and in Africa, India, the Caribbean, Australia and North America. Hundreds of cases infected with this parasite have been recorded from these areas.[1-8] Man is accidental "dead–end" host who becomes infected by ingesting the larvae in infected snails, slugs or contaminated uncooked vegetables. These larvae cause eosinophilia in the spinal fluid and peripheral blood. Humans rarely harbor adult parasites; mainly, rats carry the sexually mature worms in their pulmonary arteries and heart. Juvenile worms, however, have been found in the eyes, brain and spinal cord of infected individuals. The disease presents with severe headaches, vomiting, paresthesia, weakness and, occasionally, visual disturbances and extraocular muscle weakness. Most patients fully recover from the disease, although heavy infections can lead to chronic, disabling disease and even death.[9] Much is now known about the antibody responses in *A. cantonensis* infected hosts,[10-15] but little is known of the different IgG antibody subclass responses to *A. cantonensis* antigen in the infected individuals. We report a detailed examination of specific antibody profiles by classes (IgM, IgA and IgG) and the different IgG subclasses in proven human angiostrongyliasis (PA) and clinically suspected angiostrongyliasis with eosinophilic meningitis (EM). The ratios of antibody responses of the infected groups and a healthy control (HC) group were compared. Diagnostic sensitivity and specificity of the assays were also evaluated.

MATERIALS AND METHODS

Animals

Female rats (*Rattus norvegicus*) aged 8 weeks, were obtained from the Animal Section of the Faculty of Medicine, Khon Kaen University, northeastern Thailand. Rats were housed in groups of 4 or 5 in plastic cages, maintained under conventional conditions and provided with rodent chow and water

From the [1]Department of Parasitology and [2]Department of Medicine, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand.
Correspondence: Pewpan M. Intapan

ad libitum. The maintenance and care of all animal experiments complied with the ethic guidelines set by the Animal Experimentation Committee, Khon Kaen University.

Parasite antigen preparations

The life cycle of a Thai strain of *A. cantonensis* has been maintained in our laboratory in both rats and *Achatina fulica* snails. Digestion of infected snails with pepsin yielded third-stage *A. cantonensis* larvae, and these larvae were given to rats by stomach intubation. Rats infected for 3 weeks were killed with ether anesthesia and young adult worms were removed from their brains. The young adult female worms were thoroughly washed with normal saline solution (0.85% NaCl in distilled water). Young adult, female worm somatic extract (FSE) was prepared with modifications of a method described elsewhere.[15] The worms were homogenized with a tissue grinder in a small volume of normal saline solution (NSS) containing 0.1 mM phenylmethyl sulfonyl fluoride, 0.1 mM tosylamide-2-phenylethyl-chloromethylketone and 1μM L-trans-3-carboxyoxiran-2-carbonyl–L-leucylagmatine. The preparation was then sonicated with an ultrasonic disintegrator and centrifuged at 10,000 x *g* for 30 minutes at 4°C. The protein concentration of the supernatant fluid was determined by the standard method.[16]

Sera

The PA sera were obtained from 7 parasitologically confirmed patients. Among these patients, 4 had the worms recovered by lumbar punctures and 3 from the eyeballs. Eighty-five cases of EM patients were obtained from Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. The diagnosis of EM was based on previously established criteria.[6] All of them had a history of eating raw aquatic snails, *Pila* spp., within 1 to 6 weeks before the onset of the disease. Twenty-seven HC serum samples (obtained from healthy adults whose stool examinations at the time of blood collection gave no evidence of any intestinal parasitic infection) were employed as control cases and provided the baseline for antibody detection among infected subjects. Pooled positive and negative reference sera were prepared by combining equal volumes of PA or HC sera and were used for observation of the day-to-day variation in the antibody detection.

The Scientific-Ethical Committee of Khon Kaen University approved the study protocol. Informed consent was obtained from each patient using a standard approval procedure.

Enzyme-linked immunosorbent assay (ELISA)

For determination of parasite-specific IgG, IgA and IgM, microtiter plates (Costar, Cambridge, MA, USA) were coated with FSE diluted to 5 μg/ml in 0.05 M carbonate buffer, pH 9.6, incubated overnight at 4°C, and blocked with 0.25 ml/well of 1% bovine serum albumin (Frac V, Sigma, St. Louis, MO, USA; BSA) in 0.1 M phosphate buffered saline, pH 7.4-0.05% Tween-20 (PBS-T). After incubation at room temperature for 1 hour, the plates were washed five times with PBS-T. The serum samples were diluted to 1:200 in 1% BSA in PBS-T, then incubated in triplicate (0.1 ml/well) for 1 hour at 37°C. After washing, horseradish peroxidase (HRP) conjugated monoclonal anti-human immunoglobulin antibodies (DAKO A/S, Glostrup, Denmark), diluted 1:20,000 (for IgG), 1:10,000 (for IgA) and 1:500 (for IgM) in 1% BSA in PBS-T (according to the result of the checkerboard titration) were added. After 1 hour of incubation at 37°C and washing five times, substrate solution (2.2 mM *o*-phenylene-diamine dihydrochloride in 0.1 M phosphate/citrate buffer, pH 5.0, with 0.02 ml of H_2O_2/50 ml of solution) was added (0.1 ml/well), and the reaction stopped with 8 N H_2SO_4 (0.05 ml/well) after 30 minutes of incubation. The optical density (OD) was read at 490 nm with an ELISA reader (E_{LX} 800 Biotek Instrument, INC., Winooski, VT, USA). The precision of the ELISA was investigated by performing the test on different days using the same pooled positive and negative sera, the same batch of antigen and under the same conditions. Consistent data were obtained from all the tests indicating no day-to-day variation.

Determination of parasite-specific IgG subclasses

ELISA was performed as described with the following modifications: after incubation of the serum samples, HRP conjugated monoclonal anti human IgGi (i = 1, 2, 3, 4) subclasses (Zymed, South San Francisco, CA, USA), diluted 1:1,000 in 1% BSA in PBS-T (0.1 ml/well) was added. After 1 hour incubation at 37°C, the assay was continued as described above.

Statistical analysis

The Student's *t*-test or Mann-Whitney Rank Sum test was used to determine the significance of the difference between the two groups of data. The ANOVA or Kruskal-Wallis test was used to determine the significance of the difference of the immunoglobulin reactivity for 1 to 6 weeks after the disease onset. Sigma Stat (San Rafael, CA, USA) was the statistical software used. The diagnostic sensitivity and specificity were calculated using the Galen's method.[17] These values were calculated and expressed as: sensitivity = [no. of true positives/(no. of

true positives + no. of false negatives)] ×100; specificity = [no. of true negatives/(no. of false positives + no. of true negatives)] × 100; true negative = number of HC samples that were negative by ELISA; true positive = number of PA samples that were positive by ELISA; false positive = number of HC samples that were positive by ELISA; false negative = number of PA samples that were negative by ELISA.

RESULTS

Specific IgM, IgA, total IgG and IgG subclasses

The levels of the specific IgM, IgA, total IgG and IgG subclasses response to the *A. cantonensis* antigen in the PA, EM and HC groups, detected by the ELISA, are presented in Table 1 and Fig. 1. The distribution of the ELISA values for the total IgG and IgG subclasses in patients were mostly higher than those in uninfected persons, although there were a few overlapping values. The mean ELISA values from the specific total IgG and IgG subclasses in each patient group were significantly higher than those in the uninfected group ($p < 0.001$). Major differences were evident in the distribution of IgG subclasses between patient groups. The ranked order of IgG subclasses based on their proportion ratios of antibody responses between infection groups and HC group is IgG4 > IgG1 > IgG2 > IgG3 (Table 1). The IgM and IgA responses were generally poor. The mean ELISA value for the specific IgA in each patient group was significantly higher than in the HC group ($p < 0.05$). The mean ELISA value for the specific IgM in the PA patient group was not significantly different from the HC group ($p > 0.05$). The mean value for the EM patient group was significantly higher than for the HC group ($p < 0.05$).

Sensitivity and specificity

Using an absorbance value of the mean plus 2 SD of the HC group as the cut-off limit between positivity and negativity for angiostrongyliasis, the sensitivity and specificity for the detection of antibodies are shown in Table 2. The IgG1 antibody demonstrated the highest sensitivity and specificity.

DISCUSSION

The human immune responses to parasitic infections exhibits different distributions of IgG subclass antibodies between infected groups.[18-19] We here describe the antibody patterns in proven angiostrongyliasis and clinically suspected angiostrongyliasis with eosinophilic meningitis using ELISA and subclass-specific reagents.

We observed an increased antigen specific total IgG and IgG subclasses that did not apply to IgM antibody. The main protective mechanism against infection with *A. cantonensis* might involve IgG antibody acting in antibody-dependent cell-mediated cytotoxicity.[20] The serum IgA antibody responses were generally poor, but levels were significantly higher than in the healthy controls ($p < 0.05$). David[21] suggested that parasites in the intestine of the definitive hosts stimulate immune responses in the mucosa that secrete IgA antibody into the intestinal lumen. However, why the IgA antibody levels were elevated in the serum of patients with angiostrongyliasis remains obscure.

Measurement of IgG subclasses provides some particularly interesting comparisons between the infection groups and the HC group. A substantial part of the overall increase in IgG is accounted for by a seven to nine-fold elevation of IgG1, a five to six-fold increase of IgG2

Table 1 Serum antibody levels by IgM, IgA, total IgG and IgG subclasses to *A. cantonensis* antigen in sera from proven angiostrongyliasis (PA), clinical angiostrongyliasis with eosinophilic meningitis (EM) and healthy controls (HC). Measurement by ELISA

Test groups	No. of samples	IgG antibodies					IgM	IgA
		Total IgG	IgG1	IgG2	IgG3	IgG4		
		Mean ± SD*	Mean ± SD	Mean ± SD	Mean ± SD	Mean±SD	Mean ± SD	Mean ± SD
PA	7	0.601 ± 0.360	0.880 ± 0.731	0.789 ± 0.460	0.065 ± 0.050	0.341±0.230	0.090 ± 0.050	0.094 ± 0.080
EM†	85	0.599 ± 0.350	0.669 ± 0.610	0.703 ± 0.602	0.067 ± 0.100	0.312±0.490	0.218 ± 0.200	0.110 ± 0.110
HC	27	0.155 ± 0.063	0.106 ± 0.038	0.137 ± 0.081	0.020 ± 0.014	0.026±0.025	0.113 ± 0.058	0.045 ± 0.032

*Mean OD ± SD is the mean optical density reading for the number of samples given and read at 490 nm with one standard deviation (SD).

†Considering different immunoglobulin types (total IgG, IgG1, IgG2, IgG3, IgG4, IgM and IgA) in this patient group, the differences of antibody reactivity values between weeks from the onsets of disease (range, 1 to 6 weeks) were not significantly different ($p > 0.05$) using an ANOVA or the Kruskal-Wallis test as appropriate.

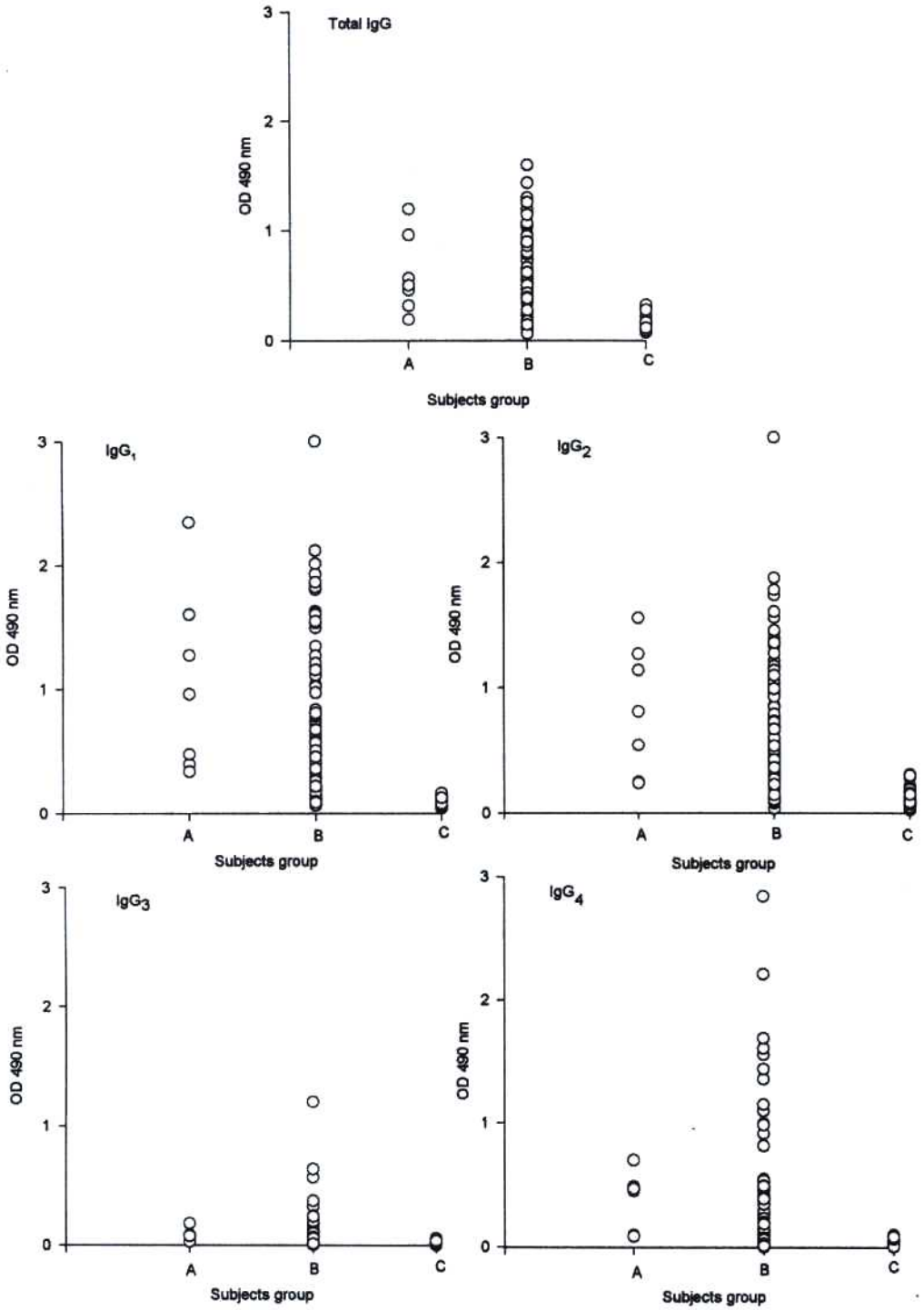

Fig. 1 Scatter diagram of the total IgG and IgG subclasses to *A. cantonensis* antigen in proven angiostrongyliasis (A), and clinically suspected angiostrongyliasis sera with eosinophilic meningitis (B), compared to the background level in the healthy control (C) sera. OD 490 nm was the optical density reading for the number given. Each dot represents one individual.

Table 2 Sensitivity and specificity of the ELISA in the detection of antibodies by immunoglobulin classes and IgG subclasses

	Immunoglobulin types (%)						
	IgM	IgA	IgG	IgG1	IgG2	IgG3	IgG4
Sensitivity	0	42.8	85.7	100	71.4	42.8	85.7
Specificity	100	96.3	88.9	100	96.3	96.3	96.3

and the most remarkable change, however, is seen in IgG4 which is raised by 12- and 13- fold in EM and PA groups, respectively. IgG3 showed a three-fold elevation in the infected groups compared to the HC group, probably due to the intrinsically low levels of this subclass in normal serum. The IgG3 molecule seems to be labile and is likely to have a short half-life.

An increase in the specific IgG1 against *A. cantonensis* antigens might be due to a host protective response just as has been observed in *Heligmosomoides polygyrus* infections.[22-23] The IgG1 subclass develops mostly in the early stage of a parasitic infection such as fascioliasis.[24] Geiger *et al.*[25] observed that the differential morbidity and mortality of *A. costaricensis* infections in BALB/c and C57BL/10 mice are correlated with total IgE and specific IgG1 production. The IgG2 and IgG3 levels were significantly increased in our patients groups compared with the healthy controls. This may be directly related to the carbohydrate antigens as in *Blastocystis hominis* infections[26] and schistosomiasis.[18,27] Development of the IgG4 subclass, due to chronic antigenic stimulation, has been demonstrated in filariasis,[28] schistosomiasis[29] and in allergic conditions.[30]

The role of IgG4 in the human immune response is poorly understood, but it appears to be generated primarily in allergic and helminthic diseases. It has been hypothesized that this antibody subclass may interfere with the complement activation by IgG1 and block mast cell degranulation by competing with the specific IgE for worm allergens.[31] Hagan and others[32] showed that the early production of IgG4 antibody in patients with schistosomiasis may block the IgE pathways responsible for the development of protective immunity for *Schistosoma haematobium*. Immunoglobulin E is protective to the host in many helminthiases; however, its production also results in immunologic responses that are harmful to the host as well as the parasite. Yen and Chen[11] reported that the IgE specific for *A. cantonensis* antigen was also found in patients with eosinophilic meningitis associated with *A. cantonensis*. This *A. cantonensis* antigen might be the allergen that caused the eosinophilic meningitis; both IgE and IgG4 may compete directly for binding to the molecule, perhaps with IgG4 being more successful in patients with less severe disease.

In conclusion, the levels of IgG subclasses in the immune response in PA and EM patients against *A. cantonensis* antigen are higher than in the HC group. In addition, the detection of IgG1 antibody to the *A. cantonensis* antigen may be the most specific serological assay available for detecting parasitic infections with *A. cantonensis* in human. The sensitivity and specificity of the IgG1 detection assay was 100% in the population we tested, but the level of sensitivity of the assay needs to be confirmed in a larger study.

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