Antibody Isotypes, Including IgG Subclasses, in Ecuadorian Patients with Pulmonary Paragonimiasis

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An ELISA test was developed to detect Paragonimus-specific antibodies, including IgG subclasses, using P. mexicanus crude water-soluble antigens. The test was standardized to detect antibodies in sera of Ecuadorian patients with pulmonary paragonimiasis and negative controls from the endemic area. The detected mean levels of IgG (0.753, SEM: 0.074) and IgM (0.303, SEM: 0.033) were significantly elevated (P<0.05). Within the IgG subclasses, IgG_4 showed the highest detected mean level (0.365, SEM: 0.116) and the other three subclasses showed considerably lower mean levels (IgG_1, 0.186 SEM: 0.06; IgG_2, 0.046 SEM: 0.01; IgG_3, 0.123 SEM: 0.047). The number of P. mexicanus eggs found in sputum of infected individuals showed a positive correlation with the level of antibodies detected for IgM, IgG and its subclasses (P<0.001). The relevance of these findings in Ecuadorian patients suffering from pulmonary paragonimiasis is discussed.

Key words: Ecuador - pulmonary paragonimiasis - IgM - IgG - IgG subclasses

Paragonimiasis, the infection by the lung fluke of the genus Paragonimus is essentially a zoonotic disease. Man generally becomes infected by ingesting uncooked infected crab, the second intermediate host. Foci of human infection are found in Asia, Africa and Latin America. In South America, autochthonous human paragonimiasis has been reported in Ecuador (Arzube & Voelker 1978), Peru (Ibanez & Fernandez 1980), Colombia (Buitriago et al. 1981), and Venezuela (Alarcon de Noya et al. 1985). The disease is endemic in several localities in Ecuador (Diaz 1991). It presents a spectrum of clinical manifestations ranging from assymptomatic individuals to patients with severe pulmonary disease (Fernandez 1990).

The variability and severity of the clinical manifestations are probably due to the duration and intensity of infection and to the degree and nature of the host immune response against the parasite. Clearance of the parasite is mediated by antibody dependent cell cytotoxicity mechanism (ADCC) as shown with other parasites (Greene et al. 1981). Although the isotypes involved in the ADCC and the immunopathogenesis of the disease have not been defined, the direct protective role of IgE in other parasitic infections has been demonstrated (Hagan et al. 1991, Dunne et al.1992). Some parasite target antigens responsible for eliciting these

mechanisms have been characterized (Grzych et al. 1993). Except for IgG_4, which has been associated with the inhibition of IgE specific activity in other parasitic diseases (Rihet et al. 1992, Aceti et al. 1993), the precise role of other IgG subclasses remains to be elucidated. The results of an ELISA test to detect specific P. mexicanus antibodies, including IgG subclasses, are reported and the immunological response in clinically and parasitologically defined groups of patients are compared. The data were analyzed to determine if a relationship between these levels and the degree of parasite load exists. The role that IgG isotype and its subclasses may have in relation to parasite load and immunopathology is discussed.

MATERIALS AND METHODS

Study population - The study population was comprised of 30 individuals infected with P. mexicanus from the endemic foci in the northeastern amazonic province of Sucumbios (Amunarriz 1991). A history was taken of each individual to determine the geographical areas and endemic diseases with which they may have been in contact. Included were serum controls from 14 individuals from a Paragonimus free area (PFA) in the Andean region near Quito, province of Pichincha. The study population was classified as proven pulmonary paragonimiasis (PPP) through positive sputums for P. mexicanus eggs. The Paragonimus negative control (PNC) group, individuals from areas free of paragonimiasis, consisted of persons infected with schistosomiasis

(6), fascioliasis (5), Chagas' disease (5), cestode infection (6), leishmaniasis (8), lymphatic filariasis (8) and toxocariasis (8). The sera were supplied by Prof. Y Carlier, Universite Libre de Bruxelles.

Parasitological and clinical examination - Demographic data were gathered and parasitological and clinical examinations were performed on all individuals from the endemic area. Parasite egg quantification in sputum was performed in all individuals using the standard concentration technique (Sachs et al. 1986).

Antigen - Adult *P. mexicanus* parasites were obtained from experimentally infected cats, which were infected with metacercariae, freshly isolated from the second intermediate crab host, *Hypolobocera aequatorialis,* collected from the endemic foci in the province of Esmeraldas (Vieira et al. 1992). The flukes were washed thoroughly with 0.01 M Phosphate buffered saline (PBS), pH 7.4, and snap frozen in liquid nitrogen, and lyophilized.

To prepare crude water-soluble antigens, six lyophilized adult flukes were homogenized in ether at 0°C. The homogenate was centrifuged for 30 min at 10,000 g at 4°C. The pellet was extracted with 0.02M Veronal buffered saline, pH 7.4, containing protein inhibitors (0.5 mM TLCK, 12.5 mM PMSF, 500 U/ml Trasylol, 2.4 mg/ml EDTA) for 24 hr at 4°C. The supernatant was dialysed against 0.02M NH_4HCO_3 for 12 hr at 4°C and then concentrated. The protein content was determined by Spector's method (Spector 1978). The crude water soluble antigens were then stored in aliquots at -70°C until used.

ELISA - The *P. mexicanus* reactive antibodies, IgG, IgG subclasses and IgM levels in all the individuals included in this study were measured by ELISA, utilizing monoclonal anti-human antibodies (mAbs). The ELISA test was performed according to the procedure of Voller et al. (1979). Briefly, polystyrene microtitre flatbottom plates (Nunc-Immuno Plates Maxisorp. Inter Med. Denmark) were coated overnight at 4°C with *P. mexicanus* crude extract (1μg protein ml-[1]) diluted in carbonate-bicarbonate buffer, pH 9.6. The appropriate concentrations of antigen, human serum and anti-human antibodies were determined by checkerboard titration. The unbound antigen was discarded and the unoccupied sites in the wells were blocked with 1% bovine serum albumin (BSA) (Sigma Chemical CO. St. Louis, MO). The plates were incubated at 37°C for 1 hr. After five washings with PBS, pH 7.4, containing 0.05% Tween 20, serum samples diluted in PBS with 0.3% BSA and 0.05% Tween 20, were added and incubated at 37°C for 90 min. To test for total IgG, IgG_1 and IgG_4 antibody isotypes, serums

were diluted 1:500, whereas they were diluted 100 fold for IgM, and IgG_2, IgG_3 isotypes. After washing, mAb anti-human IgG_1 (1:3000), mAb anti-human IgG_2, mAb anti-human IgG_3 (1:5000), mAb anti-human IgG_4, IgM (1:500) were added to the appropriate plates and incubated at 37°C for 1 hr. To determine the IgG subclasses, monoclonal mouse anti-human isotypes were used (Oxoid Ltd. Hampshire, England), while to test IgM monoclonal rat anti-human were employed (kindly provided by Prof. Bazin, Catholic University of Leuven, Belgium). The following types of horseradish peroxidase (POD) labelled conjugates were used (at a dilution of 1:1000) in the determination of the different antibodies isotypes: POD anti-goat conjugate (Diagnostic Pasteur, France) for total IgG; POD anti-mouse conjugate (Diagnostic Pasteur, France) for IgG subclasses; POD anti-rat conjugate (Amersham International, UK) for IgM. They were all incubated for 1 hr at 37°C. ABTS (2,2'- azino-di-3-ethylbenzthiazoline sulfonic acid, Boheringer Manheimn GmbH) substrate was used. The reaction was stopped by the addition of 0.1 M citric acid to each well. The period of substrate reaction was determined according to the rate of color development under dark conditions at room temperature. The rates varied with the different antibodies tested, but no reactions were allowed to exceed 30 min. The absorbance (Abs) was measured against the blank wells at 405 nm by means of a Titertek Multiscan[R] (MCC). All the assays were carried out in duplicate. The cut-off levels were defined by the mean value of the PFA group for each antibody and IgG subclass plus two standard deviations (SD).

Statistical analysis - The non-parametric Mann-Withney-Wilcoxon test was used to compare groups of data. Differences between groups, given a p value of <0.05, were considered as significant. Correlation analysis (r) was also applied.

RESULTS

Diagnostic ability of ELISA - The ELISA positivity expressed as a percentage varied within the different antibodies in the two study populations. The percent positivity (ELISA sensitivity) was near 50%, with no significant differences observed between any of the antibodies studied (Table I). All individuals in the PFA group were negative for all antibodies studied. In the PNC group, cross reactions were only detected in sera from individuals infected with *Fasciola hepatica* and schistosomiasis. However, in Ecuador, schistosomiasis does not exist and previous studies have shown the study area to be free of fascioliasis. The histories taken indicated that none of the individuals studied had been in contact with areas endemic for fascioliasis.

TABLE I

Percent positivity[a] of the two study groups

Group	IgG	IgG_1	IgG_2	IgG_3	IgG_4	IgM	Total (n)
PPP	46.6	40.0	50.0	53.0	40.0	43.0	30
PFA	0.0	0.0	0.0	0.0	0.0	0.0	14

PPP: proven pulmonary paragonimiasis; PFA: *Paragonimus* free area; a: positive individuals were determined according to the ELISA cut-off values of 0.300 (IgG), 0.040 (IgG1), 0.026 (IgG_2), 0.029 (IgG_3), 0.027 (IgG_4), 0.232 (IgM)

TABLE II

Mean values (absorbance) of the two study groups

Group	IgG	IgG_1	IgG_2	IgG_3	IgG_4	IgM
PPP	0.500	0.186	0.046	0.123	0.365	0.303
PFA	0.207	0.018	0.006	0.012	0.006	0.167
p^a	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

PPP: proven pulmonary paragonimiasis; PFA: *Paragonimus* free area; p: significance; [a]: Mann-Withney-Wilcoxon test

Antibody response in the two study groups - There were differences in the various antibody responses in the two groups studied (Fig.). Within all the antibody isotypes tested in the PPP group, all the antibody mean levels of IgG and IgM were significantly different (P<0.05) from those of the PFA group (Table II).

In relation to the IgG subclasses tested, IgG_4 had the highest detected antibody mean level (0.365 SEM: 0.116), although the antibody mean level for all classes, IgG_1, IgG_2, IgG_3 and IgG_4 in the PPP group were significantly different (P<0.05) than those of the PFA group. For IgG_1, 70% (21/30) of the indiviuals from the PPP group showed a higher response than the mean of the individuals from the PFA group, while for IgG_2 and IgG_3, 73.3% (21/30) of the patients in the PPP group showed a higher response. For IgG_4, fewer individuals in the PPP group, 53.3% (16/30) showed a response higher than the control group.

Relationship between the antibody levels and the density of Paragonimus *eggs in sputum* - There was a direct relationship between the number of eggs detected in the sputum of individuals and the level of antibodies detected for both isotypes and IgG subclasses (Table III). The mean average of the egg count in sputum of the individuals studied was 102 eggs/cc of sputum, ranging from 14-400 eggs/cc of sputum. The correlation coefficients were statistically significant (p<0.001) for IgM, IgG and all subclasses.

TABLE III

Correlation of the number of *Paragonimus* eggs in sputum with the levels of IgG, IgG subclasses, and IgM in the PPP group

Antibody	Correlation coefficient (r)	Probability (p)
IgG	0.773	<0.001
IgG_1	0.715	<0.001
IgG_2	0.590	<0.001
IgG_3	0.759	<0.001
IgG_4	0.774	<0.001
IgM	0.757	<0.001

DISCUSSION

In pulmonary paragonimiasis the adult parasite reaches maturity in the lungs of the definitive host after migrating from the peritoneal cavity to the thoracic cavity. Upon maturity eggs are produced, and when detected in sputum, a clinical diagnosis of the disease can be made. During this time the host is in constant exposure to multiple antigenic substances of the parasite. As in other
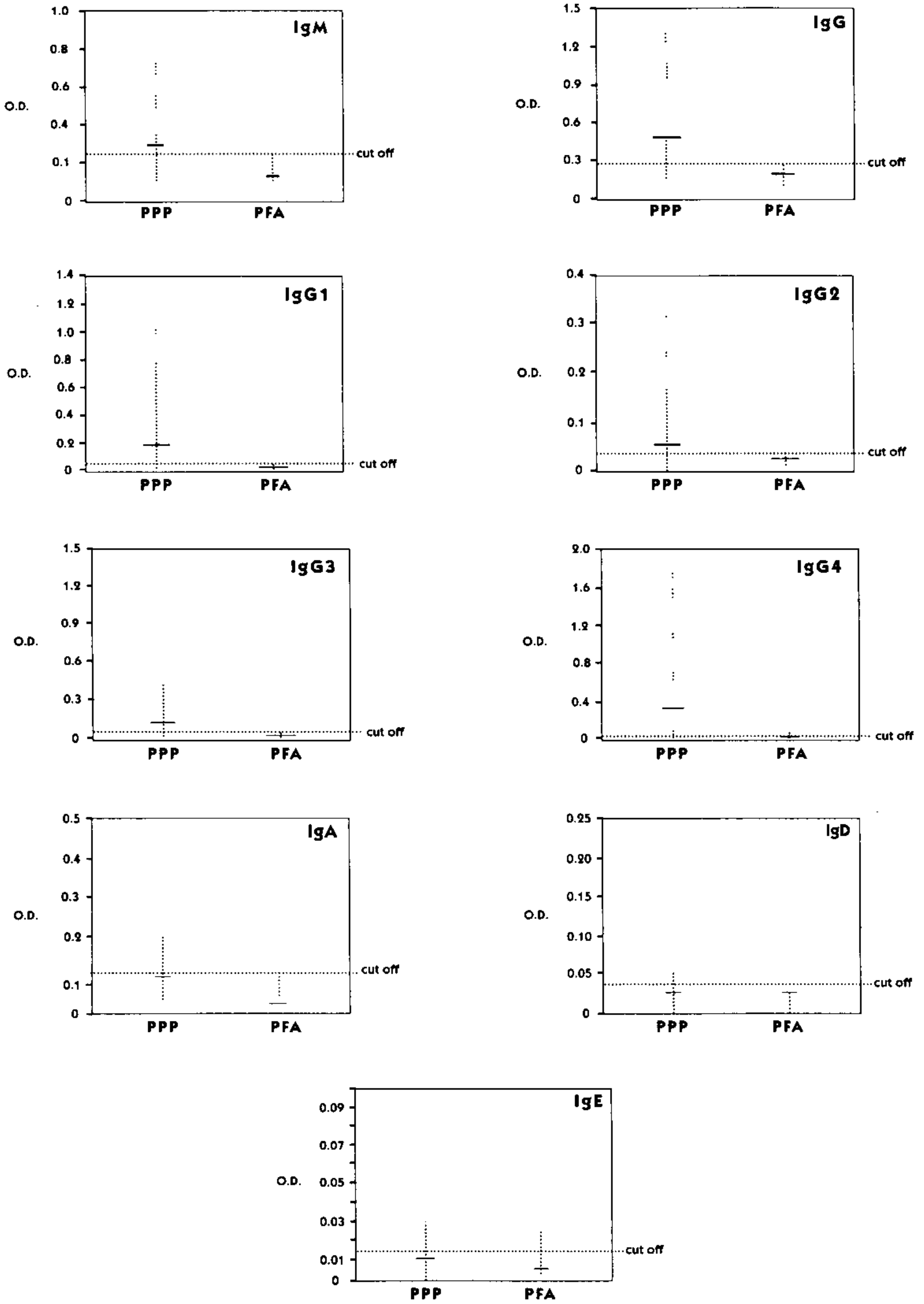
Scatter diagrams for IgG, IgG suclasses and IgM in the two study groups, those with proven pulmonary paragonimiasis (PPP) and those from a *Paragonimus* free area (PFA).

parasitic diseases, patients with active infections have specific IgG antibodies distributed across all the subclasses.

Of particular interest was the predominance of IgG_4 levels in relation to the IgG subclass in this disease. High levels of IgG_4 have been found in bancroftian filariasis (Ottesen et al. 1985), onchocerciasis (Dafa'Alla et al. 1992), hydatid disease (Aceti et al. 1993) as well as in untreated individuals infected with Schistoma mansoni (Jassim et al. 1987). Presently IgG_4 is preceived to have several functions. The IgG_4 synthesis seems to be dependent upon IL-4, IL-8 and Il-10, which involves the Th_2 helper cell subset in combination with a chronic or repeated antigenic exposure. This IgG_4 may be of particular benefit to the host as it may protect against anaphylactic responses in the host with concurrently high IgE levels. Or this co-expression of IgE and IgG_4 may be beneficial to the parasite as well, since IgG_4 can block the IgE-mediated allergic response to the parasite. Protective mechanisms may also be modulated by the $IgE:IgG_4$ ratio which tends to be high in heavy infections of schistosomiasis and lymphatic filariasis (Aalberse et al. 1983, Maizels et al. 1993). It could be that the subclass IgG_4 is blocking antibody adherence to the parasite and thus the ADCC mechanism involved in the parasite destruction. This antibody may have the same binding pattern as IgE and as such plays a role in obstructing the action of IgE-mediated immunopathology, and is being responsible for delaying the development of protective immunity (Hussain & Ottesen 1986, Hagan et al. 1991, Rihet et al. 1992).

There was a moderate elevation of the other IgG subclasses in patients with pulmonary paragonimiasis. This same type of response has been seen in acute infection of schistosomiasis where there was a significant IgG_2 response together with IgG_1 and IgG_3 (Jassim et al. 1987). IgM and IgG_2 isotype correlate with susceptibility to reinfections, while IgG_1 and IgG_3 correlate with resistance. It is probable that the detectable IgM, IgG_1, IgG_2 and IgG_3 responses seen in patients with Paragonimus infection have the same function as documented in patients with schistosomiasis, both being trematodes. However, further studies are needed to verify this observation.

There was a correlation of intensity of infection with all antibody and IgG subclasses studied in patients with paragonimiasis. This result is somewhat difficult to interpret since crude antigen was used in the study. Using specific isolated antigens in determining the antibody response to S. mansoni, significant positive correlations were made. IgG_4 anti-egg antibodies correlated better with intensity of infection than did other IgG subclasses. The total IgG response to polysaccharide

antigens did not correlate with intensity of infection as well as IgG responses to other antigens (Dunne et al. 1992). With paragonimiasis, further studies using specific antigens are required to better define the observed correlation.

In the control group the cross reactions with serums from patients with fascioliasis and schistosomiasis with the crude Paragonimus antigens were expected. Studies have shown that crude antigen is not species or genus specific and does not differentiate between Paragonimus, Fasciola and Schistosoma (Knobloch & Lederer 1983). A study has shown that the area was free of these infections, thus eliminating any interference with the detection of Paragominus antibodies. However, it would be of interest to study the immune response to specific Paragonimus antigens where no cross reactions would occur.

In pulmonary paragonimiasis, the continous antigenic stimulation probably is involved in the enhanced IgG_4 subclass response. However, further studies are needed to elucidate the functions of IgG and the IgG subclasses associated with pulmonary paragonimiasis. The relationship of these antibodies to specific parasite antigens needs to be defined, which may help in the understanding of their role in the pathology of this disease.

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