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ANTIBODY ISOTYPE RESPONSES TO PARAMYOSIN, A VACCINE CANDIDATE FOR SCHISTOSOMIASIS, AND THEIR CORRELATIONS WITH RESISTANCE AND FIBROSIS IN PATIENTS INFECTED WITH *SCHISTOSOMA JAPONICUM* IN LEYTE, THE PHILIPPINES

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Abstract. We examined whether antibody isotype responses to paramyosin (PM), a vaccine candidate for schistosomiasis, are associated with age-dependent resistance and pathology in liver fibrosis using human sera collected from 139 individuals infected with *Schistosoma japonicum* in Leyte, The Philippines. We report that IgA and IgG3 responses to PM showed a positive correlation with age and that the epitopes responsible were localized predominantly within the N-terminal half of PM. In addition, the IgG3 response to PM was associated with serum level of procollagen-III-peptide (P-III-P), an indicator of progression of liver fibrosis. These results imply that IgG3 against PM may not only provoke age-dependent resistance to *S. japonicum* infection but also enhance liver fibrosis. In contrast, levels of IgE to PM and to multiple PM fragments showed a negative correlation with P-III-P level. Thus, in contrast to IgG3, increases in PM-specific IgE may contribute to suppression of liver pathogenesis in schistosomiasis.

INTRODUCTION

A number of epidemiologic studies have suggested the occurrence of age-dependent, acquired resistance to reinfection with *Schistosoma mansoni*,[1] *S. haematobium*,[2] and *S. japonicum*.[3,4] Age-dependent resistance is correlated with specific antibody isotype responses to the schistosome antigens, especially IgE responses to adult worm antigen (AWA).[5–8] In addition, IgA specific to parasite antigens was shown to be associated with resistance.[9,10] Thus, IgE and IgA may play a role in mediating protective immunity. Conversely, IgM, IgG2, and IgG4 have been suggested to block killing by antibody-dependent cellular cytotoxicity (ADCC) of the parasites, acting as a blocking antibody.[6,11] Nevertheless, the responses of various isotypes are controversial in their ability to provoke an immune effector mechanism.

Paramyosin (PM) is an invertebrate myofibrillar protein and is one of six candidate vaccines against schistosomiasis.[12] Vaccination with recombinant PM induced a significant reduction in worm recovery after challenge infection with *S. japonicum* in mice, pigs, and water buffaloes as experimental animal models.[13,14] Immunohistochemical and immunoelectron microscopic analyses indicated that PM is localized on the surface of cercaria, schistosomula, and adult *S. japonicum*, as well as in the muscle layers, suggesting that the surface PM could evoke ADCC.[15,16] Passive transfer of PM-specific monoclonal IgE in mice at an early stage of challenge infection resulted in reduction of worm burden.[17]

In humans, antibody isotype responses against *S. japonicum* PM have been reported. A study in The Philippines showed that IgA titers to AWA are correlated with age and the major target of IgA was PM, suggesting a role of anti-PM IgA in acquired immunity.[9] In contrast, antibody responses to PM were not correlated with susceptibility in another study in China.[18] These discrepancies may have been due to geographic differences of both human and parasite populations and differences in the PM epitopes recognized by the specific antibody isotypes, some of which would be protective with others acting as blocking antibodies.

The major pathologic lesion of *S. japonicum* infection is periportal fibrosis, which is a consequence of prolonged granuloma formation surrounding the deposited parasite eggs in the liver. From the practical view of vaccine development, schistosome vaccines are required not only to reduce worm burden but also to ameliorate liver fibrosis. With regard to the roles of isotype responses to parasite antigens in fibrosis, analyses of IgE-deficient mice infected with either *S. japonicum* or *S. mansoni* indicated that IgE modifies granuloma formation.[19,20] In addition, increased levels of IgG4 to parasite egg antigens in schistosomiasis mansoni patients with and liver fibrosis have been demonstrated.[21] Interestingly, PM has been suggested to be involved in granuloma formation in mice infected with *S. mansoni*.[22,23] Thus, it is important to examine the role of isotype responses to PM in liver fibrosis for schistosome vaccine development.

The present study was performed to determine whether isotype responses against PM are involved in age-dependent resistance and liver fibrosis in human *S. japonicum* infection. We demonstrate that IgG3 and IgA against PM were correlated positively with age, and the epitopes recognized varied among isotypes. In addition, we observed a positive correlation between IgG3 responses to PM and serum level of procollagen-III-peptide (P-III-P), an indicator of progression of liver fibrosis. Surprisingly, IgE specific to PM showed a negative correlation with P-III-P level, suggesting the involvement of IgE-PM interactions in liver fibrosis. The possibility of using PM as a schistosome vaccine is also discussed.

MATERIALS AND METHODS

Study design and evaluation of liver fibrosis. The study was carried out in villages in Leyte, The Philippines, where schis-

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tosomiasis japonica is endemic. In this area, mass screening by semi-quantitative stool examination using Kato-Katz method, followed by treatment with praziquantel against *S. japonicum* infection, was conducted from 1981 to 1999, as part of the National Schistosomiasis Control Program of the Philippines. In July and August 1999, outpatients from Schistosomiasis Research Hospital, who were diagnosed as having *S. japonicum* infection by detection of the parasite eggs in their feces, were enrolled in the present study. The purpose and protocols of the study were explained and written consent obtained from all the patients. All enrolled patients underwent serologic and ultrasonographic examinations. Patients positive for hepatitis B surface antigen by radioimmunoassay (cut-off index > 2.0) and/or antibody to hepatitis C virus (second generation) and persons with alcoholism with bright liver by ultrasonography (alcohol consumption > 80 mL/day for ≥ 5 years) were excluded from the study.

A total of 139 patients were selected for further analyses. The degree of liver fibrosis was estimated by ultrasonography and classified into four stages (type 0: normal pattern; type 1: linear pattern; type 2: tubular pattern; type 3: Network pattern) as described.[24,25] Serum levels of P-III-P, type IV collagen, and total bile acids (TBAs) were measured in only 133 of the 139 blood specimens because the other six specimens were lost during analyses. Eight control sera were collected from healthy adult volunteers who lived in Japan and were free from *S. japonicum* infection.

Schistosome antigens and recombinant paramyosins. Soluble AWA was extracted from adult worms of the Yamanashi strain of *S. japonicum* by repeated freezing and thawing.[17] After centrifugation at 10,000 × *g* for 30 minutes at 4°C, the supernatant was recovered and cryopreserved at –80°C until use. Full-length *S. japonicum* PM and six truncated forms were designated as PM (1–866 amino acids), PM1 (1–164 amino acids), PM2 (157–302 amino acids), PM3 (297–451 amino acids), PM4 (447–602 amino acids), PM5 (597–742 amino acids), and PM6 (734–866 amino acids). The PM cDNAs were amplified by a polymerase chain reaction using the *S. japonicum* PM cDNA[16] as a template and the following primers: PM, 5′-CGGGATCCCATATGATGAATCACGATACAG-3′ and 5′-GCGGATCCTACATCATACTTGTTGC-3′; PM1, 5′-CGGGATCCCATATGATGAATCACGATACAG-3′ and 5′-CGGGATCCCCGGGTACCGAGCTCGACTTTTGATTCAGCTGATTG-3′: PM2, 5′-CGGGATCCATATGGTCGACGAATTCGCTAAGCAATCAGCTGAATC-3′ and 5′-CGGGATCCCTCGAGAAGCTTGAATTCCTCTGTTTTACTC-3′; PM3, 5′-CGGGATCCGAGTAAAACAGAGGAATTC-3′ and 5′-CGGGATCCCAGCTTCTAATTGAGACCA-3′; PM4, 5′-CGGGATCCGTCTCAATTAGAAGCTGAA-3′ and 5′-CGGGATCCCAACTTCATTTGCCAGCTG-3′. The amplified cDNAs were digested with *Nde* I/*Bam* HI (PM, PM1, and PM2) or *Bam* HI (PM3 and PM4) and subcloned into the expression vector pET14b. cDNA for PM5 was derived by *Pvu* II/*Eco* RI digestion of the PM cDNA, end-filled, and subcloned into the *Eco* RV site of the pT7Blue-T vector (Novagen Inc., Madison, WI). The *Nde* I/*Bam* HI fragment carrying the PM5 cDNA was subcloned into pET14b. The cDNA of PM6 was derived by *Pst* I/*Bam* HI digestion of the PM cDNA, end-filled, and subcloned into the end-filled *Xho* I site of pET14b. Transformation of bacteria, induction of expression, and purification of recombinant PMs with an N-terminal histidine 6-tag were carried out as described.[13] The PM was found to contain many degraded forms and was purified further by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by electro-elution. The recombinant PMs were stored in 10 mM sodium phosphate (pH 7.2), 1 M NaCl, and 4 M urea at -80°C until use.

Measurement of antibody titer specific to the schistosome antigens in human sera. An enzyme-linked immunosorbent assay (ELISA) was carried out using AWA, the full-length PM, and a series of recombinant PMs. Briefly, 96-well microtiter plates were coated with 5 μg/ml of AWA or 1 μg/ml of PMs. After washing out the unbound antigens three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST), the plates were blocked with blocking solution containing 0.5% bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, MO) in PBST for 30 minutes at room temperature. The plates were then washed three times with PBST. Human sera were diluted 1:100 with blocking solution for detection of IgG, IgG1, IgG2, and IgG3 and 1:50 for detection of IgG4, IgE, and IgA, and then incubated overnight at 4°C. The plates were washed five times with PBST and incubated with horseradish peroxidase–conjugated anti-human IgG1, IgG2, IgG3, IgG4, and IgA (anti-IgG; EY Laboratories, Inc., San Mateo, CA; IgG1, IgG2, IgG3, and IgG4; Southern Biotechnology Associates Inc., Birmingham, AL; and IgA; ICN Biomedicals, Costa Mesa, CA) or biotinylated anti-human IgE (Vector Laboratories Inc., Burlingame, CA) at a dilution of 1:1,000 for one hour at room temperature. The plates were then washed five times with PBST. For detection of IgE, the plates were further treated with a VECTASTAIN® Elite ABC standard kit under the conditions recommended by the manufacturer (Vector Laboratories Inc.). The assays were developed with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and the optical density was measured at 405 nm using a microplate reader (Model MTP-22; Corona Electrics Co. Ltd., Ibaraki, Japan) with a reference measured at 492 nm.

Statistical analysis. StatView™ version 4.0 (Abacus Concepts Inc., Berkeley, CA) and HALWIN version 6.2 (Gendai-Sugakusha Co. Ltd., Kyoto, Japan) were used for data analyses. Optical densities of serum concentrations of P-III-P and type IV collagen and the antibody titers were log-transformed before analyses. We used Student's *t*-test to evaluate differences of log-transformed means of antibody titers between the study and control groups. Pearson's correlation coefficient was used to quantify associations between age, ultrasonographic evaluation, and log-transformed data for P-III-P, type IV collagen, and antibody titers. In the present study, no correction has been made for multiple comparisons between levels of antibodies to AWA and PM in correlation analyses with epidemiologic indicators because PM is present in the AWA preparation and, therefore, anti-AWA responses include the responses to PM. Multiple regression analysis was used for comparisons of isotype response levels against the PM fragments and their correlations with age and markers of fibrosis.

RESULTS

Epidemiologic outcomes. The cohort of 139 subjects ranged in age from 9 to 69 years old, and the male:female sex ratio was 92:47. Table 1 shows the relationships between age

TABLE 1
Correlations between age and markers of fibrosis in schistosomiasis japonica patients in Leyte, The Philippines*

Markers	Correlation coefficient (*R*)		
	US score	P-III-P†	Type IV†
Age	0.488 (< 0.001)	0.039 (0.655)	0.126 (0.147)
US score		0.306 (0.003)	0.278 (0.001)
P-III-P			0.670 (< 0.001)

* US = ultrasound. Values in parentheses are *P* values.
† Transformed into $\log_{10}$.

and markers of fibrosis in our patient population. We adopted four indicators to estimate the degree of liver fibrosis: ultrasonographic score and serum levels of P-III-P, type IV collagen, and TBA.

Age showed a strong correlation with ultrasonographic score ($R = 0.488$, $P < 0.001$), but was not correlated with P-III-P or type IV collagen levels. In addition, ultrasonographic score was correlated with P-III-P and type IV collagen levels ($R = 0.306$, $P = 0.003$ and $R = 0.278$, $P = 0.011$, respectively). In contrast, the TBA level was not correlated with age, ultrasonographic score, or other serologic markers.

Relationships between age and antibody isotype responses against PM. To determine whether isotype responses against PM are associated with age-dependent resistance in Filipino patients, we measured the serum levels of IgA, IgE, IgG1, IgG2, IgG3, and IgG4 against PM and AWA (Figure 1). Because the reactivity of secondary antibodies used for the ELISA varied, it was difficult to determine the amounts of antibody among the isotypes. With the exception of IgG2, the levels of all antibody isotypes against PM and AWA increased significantly in patients infected with *S. japonicum*. The unresponsiveness of IgG2 production against AWA in Filipino patients was consistent with previous findings in Chinese patients with schistosomiasis japonica.[18]

We selected IgA, IgE, IgG1, IgG3, and IgG4 isotypes to examine the relationships between age and their responses against AWA and PM (Table 2). Age showed a positive correlation with serum levels of IgG3 against both AWA ($R = 0.216$, $P = 0.014$) and PM ($R = 0.325$, $P = 0.001$) and with the level of IgA against PM ($R = 0.226$, $P = 0.007$). This was in part consistent with the findings of a previous report, in which the anti-AWA IgA level was correlated with age and PM was a major target of the IgA response in The Philippines.[9] The IgE, IgG1, and IgG4 responses did not show such correlations with age.

Relationships between fibrosis and antibody isotype responses against PM. To determine whether isotype response levels against PM are associated with fibrosis, we examined the relationships between fibrosis and levels of IgA, IgE, IgG1, IgG3, and IgG4 against PM in patients with schistosomiasis japonica. We observed that correlations of isotype responses with fibrosis were different among the indicators of fibrosis, ultrasonographic score and serum levels of P-III-P and type IV collagen (Table 3). With ultrasonographic score, positive correlations were observed for IgA, IgG3, and IgG4 levels against PM. In contrast, the P-III-P level showed a positive correlation only with IgG3 to PM, and type IV collagen level was not associated with isotype responses to PM.

Unexpectedly, IgE levels against PM showed a negative correlation with serum P-III-P level ($R = -0.260$, $P = 0.003$; Table 3), in which individuals with high IgE titers developed lower levels of serum P-III-P. Such trends were also observed between ultrasonographic score and level of IgE to PM ($R = -0.069$, $P = 0.441$), and between type IV collagen and IgE levels ($R = -0.107$, $P = 0.228$).

The IgG1 responses to AWA showed a positive correlation with the ultrasonographic score ($R = 0.320$, $P < 0.001$), but no positive correlations were observed against PM. These results suggest that fibrosis associated with IgG1 responses may be attributable to other parasite antigens.

Epitope analyses of PM recognized by isotypes. In light of these findings, we attempted to identify the epitopes recognized by IgA, IgE, IgG3, and IgG4 isotypes that are associated with age-dependent resistance or fibrosis in *S. japonicum* infection. We constructed a series of deletion mutants, PM1 (1–164 amino acids), PM2 (157–302 amino acids), PM3 (297–451 amino acids), PM4 (447–602 amino acids), PM5 (597–742 amino acids), and PM6 (734–866 amino acids). These truncated mutants had an average length of 150 amino acids and provided a sequential overlap of at least five residues (Figure 2).

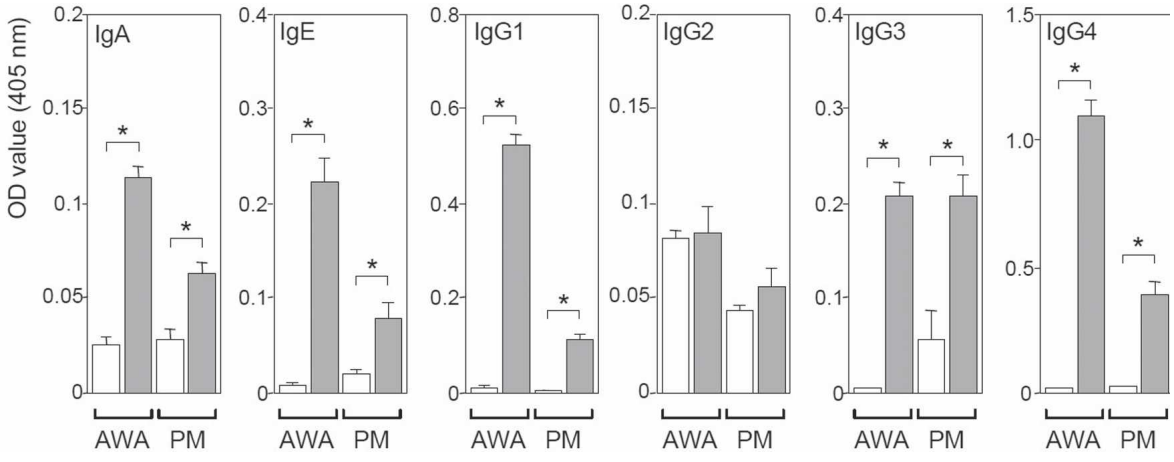

FIGURE 1. Antibody isotype levels (geometric mean ± SE) against *Schistosoma japonicum* adult worm antigens (AWA) and paramyosin (PM) in 8 healthy (open bars) and 139 infected (gray bars) individuals OD = optical density. **P* < 0.01.

TABLE 2
Correlations between antibody isotype levels (log_{10}) to *Schistosoma japonicum* antigens and age*

Antibody	Correlation coefficient (*R*) by antigen							
	AWA	PM	PM1	PM2	PM3	PM4	PM5	PM6
IgA	0.138	0.226	0.150	0.234	0.335	0.117	0.113	0.004
	(0.105)	(0.007)	(0.077)	(0.006)	(< 0.001)	(0.169)	(0.185)	(0.966)
IgE	–0.064	0.031	0.113	0.041	–0.004	–0.060	–0.027	0.016
	(0.473)	(0.731)	(0.204)	(0.648)	(0.964)	(0.502)	(0.762)	(0.860)
IgG1	0.093	0.113						
	(0.287)	(0.198)						
IgG3	0.216	0.325	0.268	0.254	0.183	0.030	0.223	0.090
	(0.014)	(< 0.001)	(0.002)	(0.003)	(0.037)	(0.733)	(0.011)	(0.307)
IgG4	0.092	0.152	0.126	0.108	0.231	0.114	0.239	0.084
	(0.306)	(0.088)	(0.158)	(0.226)	(0.009)	(0.201)	(0.007)	(0.346)

* Values in parentheses are *P* values. *R* values for IgG1 responses to the truncated PMs are not shown. AWA = adult worm antigen; PM = paramyosin.

Box and whisker plots of isotype responses demonstrated the presence of low responders and high responders for antibody production against the full-length PM and its deletion mutants (Figure 3). Among the deletion mutants, PM6 hardly evoked any antibody production for any antibody isotype. The IgA and IgG3 isotypes reacted predominantly with the PM1, PM2, and PM3 mutants. In contrast, IgG4 appeared to react predominantly with PM2, PM3, PM4, and PM5. IgE did not show such specificity.

The IgA and IgG3 response levels against PM1, PM2, and PM3, and IgG3 levels against PM5 showed a positive correlation with age (Table 2). Multiple regression analysis was carried out to specify the responsible PM epitope(s) and showed correlations of age with IgA levels against PM3 ($R = 0.356$, $P < 0.001$) and with IgG3 levels against PM2 and PM3 ($R = 0.318$, $P < 0.001$ and $R = 0.307$, $P < 0.001$, respectively). These results suggest that levels of anti-PM3 IgA and levels of anti-PM2 and anti-PM3 IgG3 are likely to be associated with age-dependent resistance.

With regard to fibrosis, IgG3 levels against any of the de-

TABLE 3
Correlations between antibody isotype levels (log_{10}) to antigens of *Schistosoma japonicum* and various markers of fibrosis*

Antibody	Correlation coefficient (*R*) by antigen							
	AWA	PM	PM1	PM2	PM3	PM4	PM5	PM6
US score								
IgA	0.143	0.180	0.109	0.280	0.180	0.128	0.124	0.111
	(0.094)	(0.034)	(0.204)	(< 0.001)	(0.034)	(0.134)	(0.146)	(0.193)
IgE	0.035	–0.069	0.031	–0.020	–0.111	–0.124	–0.130	–0.204
	(0.693)	(0.441)	(0.726)	(0.821)	(0.210)	(0.163)	(0.141)	(0.020)
IgG1	0.320	0.081						
	(< 0.001)	(0.352)						
IgG3	0.313	0.241	0.017	0.151	0.156	0.084	0.142	–0.084
	(< 0.001)	(0.006)	(0.845)	(0.086)	(0.076)	(0.344)	(0.107)	(0.344)
IgG4	0.198	0.246	0.196	0.137	0.106	0.186	0.256	0.128
	(0.026)	(0.005)	(0.027)	(0.124)	(0.235)	(0.186)	(0.004)	(0.151)
P-III-P								
IgA	0.027	–0.048	–0.025	0.263	0.194	–0.013	0.295	0.238
	(0.756)	(0.587)	(0.77)	(0.002)	(0.025)	(0.880)	(< 0.001)	(0.006)
IgE	–0.049	–0.260	0.051	–0.056	–0.178	–0.304	–0.260	–0.194
	(0.581)	(0.003)	(0.564)	(0.531)	(0.043)	(< 0.001)	(0.003)	(0.027)
IgG1	0.097	–0.156						
	(0.267)	(0.074)						
IgG3	0.215	0.292	0.025	0.188	0.216	0.011	0.286	0.182
	(0.014)	(< 0.001)	(0.776)	(0.033)	(0.014)	(0.900)	(< 0.001)	(0.038)
IgG4	0.028	0.076	0.120	0.056	0.025	0.028	0.085	0.085
	(0.751)	(0.397)	(0.178)	(0.532)	(0.777)	(0.755)	(0.342)	(0.342)
Type IV								
IgA	–0.079	–0.067	0.032	0.038	–0.014	0.027	0.088	0.084
	(0.306)	(0.447)	(0.719)	(0.666)	(0.869)	(0.755)	(0.316)	(0.337)
IgE	–0.030	–0.107	–0.041	–0.093	–0.062	–0.140	–0.072	0.002
	(0.734)	(0.228)	(0.645)	(0.296)	(0.488)	(0.114)	(0.416)	(0.978)
IgG1	0.072	–0.057						
	(0.413)	(0.514)						
IgG3	0.174	0.150	0.073	0.053	0.020	–0.037	0.088	0.177
	(0.047)	(0.088)	(0.413)	(0.551)	(0.825)	(0.675)	(0.321)	(0.044)
IgG4	–0.032	–0.035	0.100	–0.031	–0.034	–0.007	0.032	–0.013
	(0.719)	(0.693)	(0.266)	(0.731)	(0.706)	(0.940)	(0.718)	(0.884)

* Values in parentheses are *P* values. *R* values for IgG1 responses to the truncated PMs are not shown. US = ultrasound; AWA = adult worm antigen; PM = paramyosin.

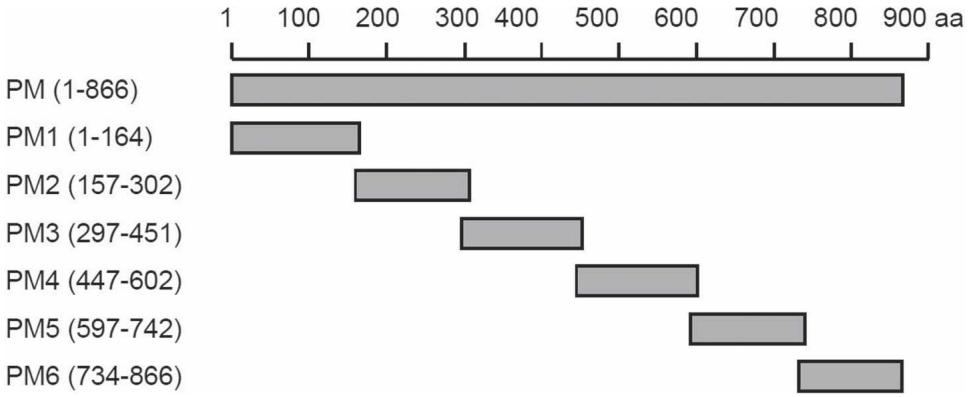

FIGURE 2. Schematic representation of recombinant *Schistosoma japonicum* paramyosin (PM) and its deletion derivatives used in this study. The scale and numbers indicate the amino acid (aa) positions. The full-length PM consists of 866 amino acids. The deletion mutants and their amino acid positions were as follows; PM1 (1–164 amino acids), PM2 (157–302 amino acids), PM3 (297–451 amino acids), PM4 (447–602 amino acids), PM5 (597–742 amino acids), and PM6 (734–866 amino acids).

letion mutants showed no significant correlations with ultrasonographic score and P-III-P levels, despite the positive correlation of anti-PM IgG3 levels with these markers (Table 3). Similarly, multiple regression analysis did not show significant correlations of IgG3 levels against any PM fragments with ultrasonographic score and P-III-P levels. It is likely that the multiple PM epitopes were associated with fibrosis and recognized differently by the patients.

The PM epitopes associated with fibrosis were recognized differently by IgA and IgG4. The IgA levels to PM2 and PM3 and IgG4 levels to PM1, PM4, and PM5 showed positive correlations with ultrasonographic score (Table 3). Multiple regression analysis showed correlations of ultrasonographic score with IgA levels against PM2 ($R = 0.292$, $P < 0.001$) and with IgG4 against PM1 and PM5 ($R = 0.196$, $P = 0.028$ and $R = 0.258$, $P = 0.004$, respectively).

Negative correlations between IgE titers and P-III-P levels were observed for PM3, PM4, PM5, and PM6, but the IgE responses against these deletion mutants were weak. Multiple regression analysis showed a correlation of P-III-P levels with anti-PM4 IgE levels ($R = -0.309$, $P < 0.001$). These results were consistent with the relationship between IgE levels and full-length PM, and suggested that PM4 recognized by IgE has a role in suppression of the progression of fibrosis.

DISCUSSION

Schistosome vaccines are expected to show both effects against infection and disease. In the present study, we addressed the relationships of antibody isotype responses to PM, not only with age-dependent resistance but also with fibrosis, because liver fibrosis is the most important lesion in schistosomiasis japonica.

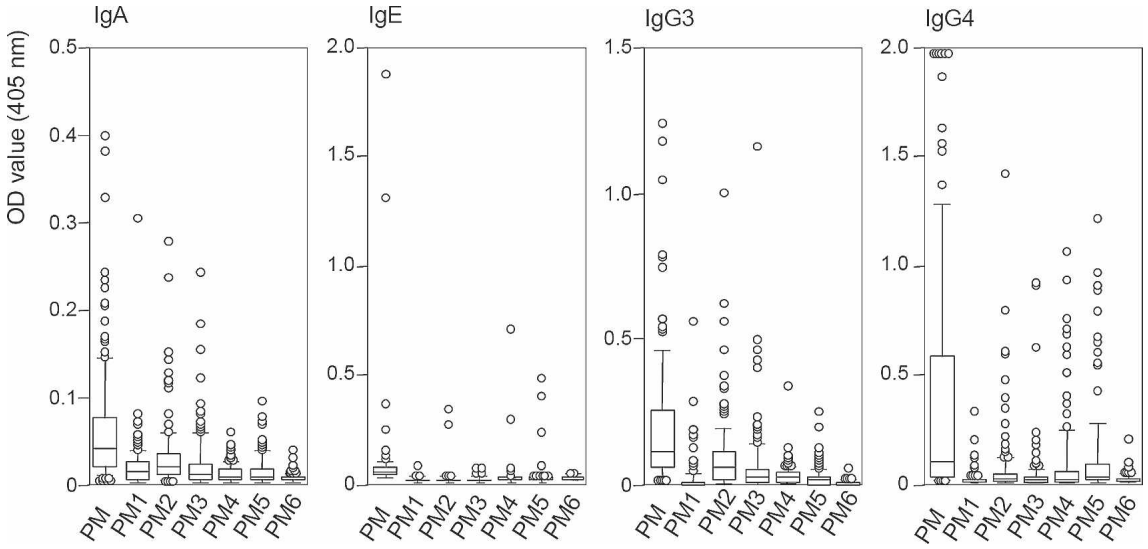

FIGURE 3. Box and whisker plots of IgA, IgE, IgG3, and IgG4 isotype responses to *Schistosoma japonicum* paramyosin (PM) and its deletion mutants. The full-length PM and a series of deletion mutants (PM1, PM2, PM3, PM4, PM5, and PM6) were used for an enzyme-linked immunosorbent assay. The box indicates the area ranging from the first to the third quartiles of each dataset and the median is indicated by the black centerline. The vertical bar represents 1.5 times the interquartile range (IQR) from the upper or lower quartile. Points at a greater distance from the IQR are plotted individually as circles. OD = optical density.

To determine the epidemiologic states of patients with schistosomiasis japonica in The Philippines, we first examined the relationships between age and fibrosis (Table 1). We observed a positive correlation between age and ultrasonographic score but not between age and any serologic markers of fibrosis. Correlations between age and ultrasonograpic score appear to reflect accumulation of fibrosis along with age rather than the current progression of fibrosis. For example, cases of schistosomiasis japonica in older persons in Japan, which showed advanced liver fibrosis by ultrasonographic scoring, did not excrete eggs.[26]

Positive correlations of ultrasonograpic score with P-III-P and type IV collagen levels are consistent with the previous findings for schistosomiasis japonica.[24,27] It is noteworthy that P-III-P level reflects mainly the progress of collagen synthesis, while the type IV collagen level reflects collagen degradation.[28] Thus, correlations between ultrasonographic score and these serologic markers may reflect the current pathologic progress.

The TBA level has been suggested to be a good indicator of hepatic fibrosis.[25] In the present study, however, the TBA level did not show any correlations with other indicators of fibrosis. This discrepancy may have been due to the difference in duration between the previous study design[25] and the present study design, in that the subjects in the present study had received mass treatment with praziquantel in the previous 10 years, which may have influenced the serum level of TBA.

The IgG3 levels to both AWA and PM showed a positive correlation with age (Table 2). A similar age-related trend with these IgG3 responses was reported previously in the human population in The Philippines.[7,29] In addition, multiple regression analysis showed a correlation of age with IgG3 levels against PM2 and PM3. These findings suggest that IgG3 responses to PM, especially anti-PM2 and anti-PM3 IgG3 levels, may be involved in protective immunity to *S. japonicum* infection.

Likewise, IgA responses to PM showed a positive correlation with age (Table 2). This was consistent with the previous report of a positive correlation between IgA levels against PM and age in Filipino patients.[9] Multiple regression analysis suggested that the anti-PM3 IgA level is likely to be associated with age-dependent resistance. In contrast, there was no correlation between any antibody responses to PM and age in China.[18] Thus, the correlation between levels of IgA to PM and age is likely to be distinctive in The Philippines, possibly because of differences in epidemiologic and immunologic features between China and The Philippines.

We did not find any correlations between IgE levels and age, whereas AWA- and PM-specific IgE were present in the sera of Filipino patients. Similarly, we found no significant correlations between IgE levels and frequency of treatment, which is an indication of intensity of reinfection. These observations were consistent with the report that levels of IgE against AWA did not show correlations with age.[18,29] In contrast, levels of IgE to AWA were higher in subjects who were unsusceptible to reinfection two years post-treatment in China.[8] Another group has also reported an association between IgE response to AWA and age in The Philippines.[7] Since there is no direct evidence that human IgE in combination with effector cells mediates killing of the parasites, further analyses are necessary to explain this discrepancy by verifying the precise role of IgE in age-dependent resistance.

In the present study, the relationships between antibody response levels to PM and the degree of liver fibrosis were investigated. We observed positive correlations of antibody isotypic responses to PM with the degree of liver fibrosis as follows: the IgA, IgG3, and IgG4 levels with ultrasonographic score and the IgG3 level with the P-III-P level (Table 3). Multiple regression analysis showed positive correlations of ultrasonographic score with IgA levels against PM2 and with IgG4 against PM1 and PM5, suggesting that anti-PM2 IgA and anti-PM1 and -PM5 IgG4 are likely involved in progressive fibrosis.

The anti-PM IgG3 level showed a positive correlation with ultrasonographic score and P-III-P level. It is important to note that the ultrasonographic score likely represents accumulation of fibrous tissues in the liver, while the serum P-III-P level indicates the currently active state of fibrosis.[28] Therefore, the IgG3 response to PM maybe involved in both progression and the subsequent accumulation of fibrosis, but the PM epitope(s) associated with the degree of fibrosis is unclear.

In contrast to the IgG3 response to PM, a negative correlation between IgE response and serum P-III-P level was found in the Filipino patients (Table 3). In addition, multiple regression analysis showed a negative correlation of P-III-P levels with anti-PM4 IgE levels. In the experimental rodent model, there are controversial observations concerning the role of IgE in schistosome infection: reduced granuloma formation in mice lacking IgE[19,20] and enhanced granulomatous inflammation in FcεRI-deficient mice.[30] The discrepancy in the mode of IgE in granulomatous development may suggest that the roles of IgE in fibrosis are dependent on parasite antigens; IgE to PM may interfere with the progression of fibrosis, and other combinations may enhance fibrosis in humans.

Recent studies have demonstrated the roles of surface PM as immunomodulators. Paramyosin is capable of binding *in vitro* to collagen and the complement components C1, C8, and C9, which results in inhibition of complement activation and formation of membrane attack complex.[31–33] Likewise, PM can bind to the Fc domain of immunoglobulin *in vitro*.[34] The modes of isotype responses to PM in granuloma formation are unclear. However, it is possible that the immune complex of immunoglobulins and PM released from the parasite surface binds to the endothelial or fibroblastic matrix surrounding the embolized eggs through interaction of PM with collagen, which leads to enhanced inflammation and granuloma formation.

It is commonly assumed that granulomatous pathology is $CD4^+$ T cell-mediated in mice. It is noteworthy that B cell- or FcRγ-deficient mice exposed to *S. mansoni* developed larger granulomas in the chronic stage of the infection and displayed unaltered T cell responses, which suggests a suppressive role of immunoglobulin receptor–mediated responses in granuloma formation.[35] In the present study, it is possible to speculate that PM or PM-immunoglobulin complex modulate positively immune responses at the site of lesions, which leads to accumulation of fibrous tissues, the mechanism of which remains unknown.

In the present study, it was difficult to identify the epitope(s) responsible for age-dependent resistance or granu-

loma formation. Epitope mapping of PM recognized by the isotypes and their correlation analyses indicated some trends, in that age tended to be associated with the responses to the N-terminal half of PM (Table 2). In contrast, no such tendency was observed in relationships between liver fibrosis and the epitopes. These results suggest that multiple epitopes are involved in both age-dependent resistance and liver fibrosis.

A positive correlation between IgG3 responses to PM and P-III-P levels was an undesirable finding in the context of schistosome vaccine development. In contrast, IgE response to PM is likely to play a suppressive role in the development of fibrosis, a desired feature for a schistosome vaccine. These contrasting results clearly indicate that PM has complex roles in modulating human immune responses.

Although PM can induce protective immunity against challenge parasite infection in experimental animal models, there are marked immunologic and pathologic differences between humans and animals. Our findings provide insights into the importance of combinations between PM and isotype responses for schistosome vaccine development, in which the desired immune responses should be provoked to avoid exacerbating the pathology. Further studies to characterize the precise mode of PM in antibody-dependent killing of the parasite and in granuloma formation in humans are required prior to clinical trials.

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