INTRODUCTION

A number of epidemiological 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 antigens (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. On the other hand, 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] Indeed, 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 geographical 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 etiology of schistosomiasis 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 improve 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 induces granuloma formation.[19,20] In addition, increased levels of IgG4 to parasite egg antigens in schistosomiasis mansoni patients with 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 aging, while 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 negative correlation negatively 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 on Leyte, the Philippines, in which schistosomiasis japonica is endemic. In this area, mass screening by stool examination 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 stool examination, were enrolled in the present study. The purpose and protocols of the study were explained to and written consent obtained from all the patients. All enrolled patients underwent serological and ultrasonographic (US) examinations. Patients positive for hepatitis B surface antigen on radioimmunoassay (RIA; cut off index > 2.0) and/or anti-HCV antibody (second generation) and alcoholics with bright liver on ultrasonography (US; alcohol consumption > 80 ml/d for 5 yrs or more) were excluded from the study.

A total of 139 patients were selected for further analyses. The degree of liver fibrosis was estimated by US 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 procollagen-III-peptide (P-III-P), type-IV collagen (Type-IV), and total bile acids (TBA) were measured in only 133 of the 139 blood specimens, the other six specimens having been 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. The soluble adult worm antigens (AWA) were 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 min 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 PCR 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 His6-tag were carried out as described.[13] PM was found to contain many degraded forms and was purified further using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electro-elution. Finally, the recombinant PMs were stored in a

solution of 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. Enzyme-linked immunosorbent assay (ELISA) was carried out using SWA, the full-length PM, and a series of recombinant PMs. Briefly, 96-well microtiter plates were coated with 5 μg/ml of SWAP or 1 μg/ml of PMs. After washing out the unbound antigens 3 times with 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 min at room temperature. The plates were further washed 3 times with PBST. The human sera were diluted 1:100 with blocking solution for detection of IgG, IgG1, IgG2, and IgG3, and to 1:50 for IgG4, IgE, and IgA, and then incubated overnight at 4°C. The plates were washed 5 times with PBST and incubated with HRP-conjugated anti-human IgG1, IgG2, IgG3, IgG4, IgA (anti-IgG: EY Laboratories, Inc., San Mateo, CA; IgG1, IgG2, IgG3, and IgG4: Southern Biotechnology Associates Inc., Birmingham, AL; IgA: ICN Biomedicals, Costa Mesa, CA), or biotinylated anti-human IgE (Vector Laboratories, Inc., Burlingame, CA) at 1:1000 for 1 hr at room temperature. The plates were then washed 5 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). 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 reference at 492 nm.

Statistical analysis. StatView™ version 4.0 (Abacus Concepts Inc., Berkeley, CA) was used for all data analyses. Optical densities of serum concentrations of P-III-P and Type-IV and the antibody titers were log transformed before analyses. We used Student's *t*-test to evaluate differences between log-transformed means and Pearson's correlation coefficient to quantify associations between age, ultrasonographic evaluation, and log transformed data for P-III-P,

Type-IV, and antibody titers.

RESULTS

Epidemiological 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 aging and markers of fibrosis in our patient population. We adopted four indicators to estimate the degree of liver fibrosis: ultrasonographic (US) score, and serum levels of procollagen-III-peptide (P-III-P), type-IV collagen (Type-IV), and total bile acids (TBA).

Age showed a strong correlation with US score ($R = 0.488$, $p < 0.001$), but was not correlated with P-III-P or Type-IV level. In addition, US score was correlated with P-III-P and Type-IV levels ($R = 0.306$, $p = 0.003$; $R = 0.278$, $p = 0.011$, respectively). On the other hand, TBA level was not correlated with aging, US score, or other serological markers (data not shown).

Relationships between aging 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 (Fig. 1). As the reactivity of secondary antibodies used for ELISA varied, it was difficult to determine the amounts of antibody among the isotypes. With the exception of IgG2, the levels of all of the antibody isotype 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 aging and their responses against AWA and PM (Table 2). Aging was correlated positively with serum levels of IgG3 against both AWA ($R = 0.313$, $p = 0.0003$) and PM ($R = 0.241$, $p = 0.0057$) and with the level of IgA against PM ($R = 0.180$, $p = 0.034$). This was, in part, consistent with the findings of a previous report, in which anti-AWA IgA level was correlated with aging and PM was a major target of the IgA response in the Philippines.[9] IgE, IgG1, and IgG4 responses did not show such correlations with aging.

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, US score and serum levels of P-III-P and Type-IV (Table 3). With US score, positive correlations were observed for IgA, IgG3, and IgG4 levels against PM. In contrast, P-III-P level was correlated positively only with IgG3 to PM and Type-IV level was associated with no isotype responses to PM.

Unexpectedly, IgE levels against PM correlated negatively with serum P-III-P level ($R = -0.260$, $p = 0.0028$, Table 3), in which individuals showing high IgE titers developed lower levels of serum P-III-P. Such trends were also observed between the US score and level of IgE to PM, ($R = -0.069$, $p = 0.44$) and between type-IV collagen and IgE level ($R = -0.107$, $p = 0.23$).

IgG1 responses to AWA were correlated positively with US score ($R = 0.320$, $p = 0.0002$), while no such 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 acid residues and provided a sequential overlap of at least five residues (Fig. 2). The minor bands of PM2 and PM5 were probably degraded proteins, as the mouse antisera raised against *S. japonicum* PM reacted with these bands (data not shown).

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 (Fig. 3). Among the deletion mutants, PM6 hardly evoked antibody production for any antibody isotype. IgA and IgG3 isotypes reacted predominantly with the first three PM mutants, PM1, PM2, and PM3. In contrast, IgG4 appeared to react predominantly with PM2, PM3, PM4, and PM5, while IgE did not show such specificity.

IgA and IgG3 response levels against PM1, PM2, and PM3, and IgG3 levels against PM5 were correlated positively with aging (Table 3). These results suggest that the PM epitopes recognized by IgA and IgG3 in association with age-dependent resistance are likely to be distributed within the N-terminal half of PM.

With regard to fibrosis, IgG3 levels against any of the deletion mutants showed no significant correlations with US score, despite the positive correlation between US score and IgG3 level against full-length PM. The PM epitopes associated with fibrosis were recognized differently by IgA and IgG4. That is, IgA levels to PM2 and PM3 and IgG4 levels to PM1, PM4, and PM5 showed positive correlations with US score.

Negative correlations between IgE titers and P-III-P levels were observed for PM3, PM4, PM5, and PM6, while IgE responses against these deletion mutants were very weak. These results were consistent with the relationship between IgE levels and full-length PM, and suggest that the C-terminal part of PM recognized by IgE play a role in suppression of

the progression of fibrosis.

DISCUSSION

Relationships between aging and fibrosis in patients with schistosomiasis japonica in the Philippines. Schistosome vaccines are expected to show both anti-infection and anti-disease effects. In the present study, we addressed the relationships of antibody isotype responses to paramyosin not only with age-dependent resistance but also with fibrosis, as liver fibrosis is the most important lesion in schistosomiasis japonica.

To determine the epidemiological states of schistosomiasis japonica patients in the Philippines, we first examined the relationships between aging and fibrosis (Table 1). We observed a positive correlation between aging and US score but not between aging and any serological markers of fibrosis. Correlations between aging and US score appear to reflect accumulation of fibrosis along with aging rather than the current progression of fibrosis. For example, old cases of schistosomiasis japonica in Japan, showing advanced liver fibrosis by US scoring, excrete no eggs.[26]

Positive correlations of US score with P-III-P and Type-IV levels are consistent with the previous findings in case of schistosomiasis japonica.[24,27] It is noteworthy that P-III-P level reflects mainly the progress of collagen synthesis, while the Type IV level reflects collagen degradation.[28] Thus, correlations between US score and these serological markers may reflect the current pathological progress.

TBA level has been suggested to be an good indicator of hepatic fibrosis.[25] In the present study, however, 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[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 .

Involvement of PM-specific IgG3 and IgA in age-dependent resistance to *S. japonicum* infection. We showed that levels of IgG3 to both AWA and PM were correlated positively with aging (Table 2). A similar age-related trend with these IgG3 responses was reported previously in the human population in the Philippines.[7,29] These findings suggest that IgG3 responses to PM may be involved in protective immunity to *S. japonicum* infection.

Likewise, IgA responses to PM were correlated positively with aging (Table 2). This was consistent with the previous report of a positive correlation between IgA levels against PM and aging in Filipino patients.[9] In contrast, there was no correlation between any antibody responses to PM and aging in China.[18] Thus, the correlation between levels of IgA to PM and aging is likely to be distinctive in the Philippines, possibly due to differences in epidemiological and immunological features between China and the Philippines.

We did not find any correlations between IgE levels and aging, 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, a representative of intensity of reinfection (data not shown). These observations were consistent with the report that levels of IgE against AWA did not show correlations with aging.[18,29] In contrast, levels of IgE to AWA were higher in subjects who were unsusceptible to reinfection 2 years post-treatment in China.[8] Another group has also reported an association between IgE response to AWA and aging 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.

Different modes of PM-specific IgG3 and IgE responses in progression of fibrosis. 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 the antibody isotypic responses to PM with the degree of liver fibrosis as follows: the IgA, IgG3, and IgG4 levels with US score and IgG3 level with P-III-P (Table 3). These results suggest that PM-specific IgA, IgG3, and IgG4 are likely to be associated with granuloma formation. It is important to note that US score is likely to represent accumulation of fibrous tissues in the liver, while serum P-III-P level indicates the currently active state of fibrosis.[28] Therefore, IgG3 response to PM may predominantly enhance antibody-dependent granuloma formation.

Surprisingly, a negative correlation between IgE response and serum P-III-P level was found in the Filipino patients in the present study (Table 3), while animal experiments using a rodent model indicated a contribution of IgE in granulomatous development.[19,20] 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, while other combinations may enhance fibrosis.

Recent studies have demonstrated the roles of surface PM as immunomodulators. PM is capable of binding *in vitro* to collagen and the complement components, C1, C8, and C9, resulting in inhibition of complement activation and of menbrane attack complex (MAC) formation.[30--32] Likewise, PM can bind to the Fc domain of immunoglobulin *in vitro*.[33] 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, leading to enhanced inflammation and granuloma formation.

Importance of recombinant vaccine design. In the present study, it was difficult to identify

the epitope(s) responsible for age-dependent resistance or granuloma formation. Epitope mapping of PM recognized by the isotypes and their correlation analyses indicated some trends, in that aging 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 immunological and etiological 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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LEGENDS TO FIGURES

FIGURE 1. Antibody isotype levels (geometric mean ± SE) against *S. japonicum* adult worm antigens (AWA) and PM (PM) in healthy (open bar) and infected (gray bar) individuals (uninfected, $n = 8$; infected, $n = 139$). $*p < 0.01$.

FIGURE 2. Schematic representation of recombinant *S. japonicum* paramyosin (PM) and its deletion derivatives used in this study. The scale and numbers indicate the amino acid 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).

FIGURE 3. Box-and-whisker plots of IgA, IgE, IgG3, and IgG4 isotype responses to *S. japonicum* paramyosin and its deletion mutants. The full-length paramyosin (PM) and a series of deletion mutants (PM1, PM2, PM3, PM4, PM5, and PM6) were used for ELISA.

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 inter-quartile range (IQR) from the upper or lower quartile. Points at a greater distance from the IQR are plotted individually as circles.

TABLE 1

Correlations between aging and markers of fibrosis in schistosomiasis japonica patients in Leyte, the Philippines.

Markers	Correlation coefficient (R) (p value)			
	US score	P-III-P*	Type-IV*	TBA*
Aging	**0.488 (< 0.001)**	0.039 (0.65)	0.126 (0.14)	0.044 (0.61)
US score	-	**0.306 (0.003)**	**0.278 (0.001)**	0.023 (0.79)
P-III-P	-	-	**0.670 (< 0.001)**	-0.147 (0.09)
Type-IV	-	-	-	0.056 (0.52)

*Transformed into $\log_{10}$