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Effects of 5 rounds of mass drug administration with diethylcarbamazine and albendazole on filaria-specific IgG4 titers in urine: 6-year follow-up study in Sri Lanka

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ABSTRACT

ELISA for filaria-specific IgG4 in urine (urine ELISA) was applied to children in 7 schools in Sri Lanka, before and after 5 rounds of annual mass drug administration (MDA). The pre-treatment IgG4 prevalence in 2002 was 3.20%, which decreased to 0.91% in 2003 after the first MDA ($P<0.001$), and finally to 0.36% in 2007 after the 5th MDA. Among 5–10 year-old children, the prevalence decreased from 3.37% in 2002 to 0.51% in 2003 ($P=0.009$). A pattern of IgG4 titer distribution according to age and its yearly change could also provide useful information in drug efficacy analysis. In 2008, new samples from eleven 2006/07 urine ELISA-positive students and their family members (total n=56) were examined by ICT antigen test, microfilaria test, and urine ELISA. No infection was confirmed among them. Urine ELISA will be useful in monitoring elimination/resurgence in a post-MDA low endemic situation.

1. Introduction

The Global Programme to Eliminate Lymphatic Filariasis (GPELF) was launched in 2000 with the target of elimination by 2020. The strategy consists of an annual single-dose mass drug administration (MDA), conducted for 4 to 6 years, covering all people in an endemic area except pregnant women, children under 2 years of age, sick people, etc. The drugs used are a combination of diethylcarbamazine (DEC) and albendazole (ALB) in most endemic areas, except in parts of Africa where bancroftian filariasis is co-endemic with onchocerciasis, in which case, ivermectin and ALB regimen is used [1]. After 10 years since the start of GPELF, many endemic countries have or will soon have completed planned MDAs [2], and are hoping to end the annual treatments.

In Sri Lanka, bancroftian filariasis has been endemic in the Southern, Western and North-western coastal provinces, consisting of 8 administrative districts (or 8 implementation units) in total [3]. The endemicity situation in the Eastern and Northern provinces (8 districts), where social turmoil had continued until recently, is classified as 'uncertain', and the remaining 4 landlocked provinces (9 districts) have been judged non-endemic (Annual Report for the National Programme to Eliminate Lymphatic Filariasis, Sri Lanka, 2005). The first MDA covering entire endemic population with DEC and ALB was conducted in July 2002, and was followed by 4 annual MDAs. The treatments were successful, with high treatment coverage rates (no. people treated/total population) of more than 80% each time [4–7], though a 2003 study reported that only 71.4% of people consumed the drugs [3]. The follow-up blood surveys in 2004 revealed the reduction of microfilaria (mf) prevalence to less than 0.07% in all implementation units surveyed (total n=598,369; Annual Report from Sri Lanka, 2005, ibid.). However, possibility still remains that some endemic foci maintain a mf rate of more than 1%.

When the prevalence of microfilaremia has been reduced to less than 1% level, a national program has to make a decision whether MDAs can be concluded. Or, if a resurgence of infection is suspected, restart of MDAs and/or implementation of other control measures have to be considered. For such decision making, monitoring the change in once-reduced endemicity level using a highly sensitive diagnostic method will be useful. In this case, young children can play an indispensable role as a sentinel population to indicate new infection/transmission. However, it is important to recognize that the monitoring will have to be repeated with subjects who are now predominantly healthy, and in an environment where filariasis has become a less important public health problem than before. It is

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possible that much less people will be willing to submit to the invasive examination of night time finger pricking, thus compromising compliance with surveillance.

We previously reported an enzyme-linked immunosorbent assay using urine samples (urine ELISA) for the detection of filaria-specific IgG4 [8]. The ELISA showed high sensitivity (95.6%) with 91 Sri Lankan subjects who were mf and/or filarial antigen positive, and high specificity (99.0%) with 298 non-endemic controls from Thailand, Lao PDR and Japan. Due to the ease of collecting samples, the method has been well accepted by local people and employed in a study requiring repeated examinations, or that targeting children under 5 years old [9,10]. It also showed higher sensitivity than filarial antigen tests, suggesting its effectiveness in detecting low-level infection among children [11]. In the present study, the urine ELISA was applied to schoolchildren in Deniyaya, Sri Lanka to monitor the effect of 5 rounds of MDA with regard to IgG4 prevalence and titers.

2. Materials and methods

2.1. Study area and subjects

Deniyaya region is located in the northernmost part of Matara district in the Southern province. The coastal 'belt' of the district has been a well established endemic area of filariasis, but Deniyaya, which is some 40–50 km inland from the coast and a mostly mountainous tea plantation area, had been believed to be free of filariasis. However, a 1999 study covering the whole Matara district (population: 761,236) by questioning village leaders for the presence of clinical symptoms revealed an unexpected accumulation of hydrocele cases in Deniyaya region [12]. This result triggered an epidemiological survey in February, 2002 with urine ELISA and Og4C3 ELISA for *Wuchereria bancrofti* circulating antigen. The survey detected 104 (4.3%) IgG4 positives out of 2436 people examined, and more importantly, 16 (2.6%) positives among 607 children aged ≤10 years, suggesting that a low-level transmission was occurring. A portion of these Deniyaya data was reanalyzed and used in this study as the pre-treatment baseline in 2002.

Seven schools, Handford Primary School (P.S.), Kannangara P.S., Morawaka P.S., Kolawenigama Maha Vidyalaya (M.V.), Rajapaksha M.V., St. Matthews Bilingual M.V., and Deniyaya Central College, were selected for the present study, which was conducted yearly in 2002–2007, except 2005, when the aftermath of 2004 tsunami disaster persisted. The students were in grades 1 to 12, and generally aged 6 to 18 years old, with a few students aged 5 or 19 years. With assistance of teachers, study subjects were selected by matching the number, sex and grade, regarding the 2002 results as a standard (n = 1095; sex ratio: 0.94). However, in 2006 and 2007 surveys, proportionally more number of students were included, as the prevalence rate was expected to be very low.

In September/October, 2008, in order to study whether infected individuals could still be found 25 months after the last MDA, a separate study was conducted, with 11 urinary IgG4 positive school children found in the surveys after the 4th and/or 5th MDAs. They and their 45 family members were examined with ICT card test to detect *W. bancrofti* antigen, 60 μL night blood smear for mf, and urine ELISA for IgG4.

The study had been approved by the ethical committees of the Faculty of Medicine, University of Ruhuna, Sri Lanka, and Aichi Medical University, Japan. The purpose and methods of the study were explained to the children, their guardians, and teachers in advance, and study participation was voluntary. Urine ELISA positives detected in the study received treatment through the national MDA, which was active at that time.

2.2. Urine samples and ELISA

At school, each student was registered, and then requested to collect urine in a paper cup and transfer 5 mL of it into a plastic sample tube. Just after the collection, the samples were mixed with sodium azide at the concentration of 0.1% and kept, most of the time, in a refrigerator until their IgG4 titers were measured in Aichi, Japan.

The previously detailed method for urine ELISA [8] was modified slightly. In brief, urine (100 μL) was added into a well of the microtiter plate pre-coated with female *Brugia pahangi* adult antigens (5 μg/mL), and then incubated overnight at 25 °C. After washing, peroxidase-conjugated mouse monoclonal antibody to human IgG4 (Clone: HP6023, Southern Biotechnology Associates, Inc., AL, USA) was reacted for 1 hour at 37 °C. For color reaction, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) peroxidase substrate was used. Serum samples with predetermined concentrations were prepared for each microtiter plate to construct the standard curve, based on which IgG4 levels were quantified as antibody units (U) (range: 0–7290 U). The cutoff value was 54.7 U.

2.3. Statistical analysis

Prevalence rates were compared with χ^2 test or Fisher's exact test, for which P value is noted in a parenthesis. The mean ages in different survey years and geometric mean IgG4 titers in different age groups were compared with one-way ANOVA and Scheffe's test. $P<0.05$ was considered significant.

3. Results

3.1. Changes of the prevalence after MDAs

In the present study, seven schools were examined before, during, and after 5 rounds of MDA. The results are summarized in Table 1. The

Table 1
Results of urine ELISA surveys carried out before and after 5 annual mass drug administration.

	Year of survey				
	2002 (pre-MDA)	2003 (post-MDA 1)	2004 (post-MDA 2)	2006 (post-MDA 4)	2007 (post-MDA 5)
Study subjects					
No. examined	1095	1094	1032	1623	1647
Male/female ratio	0.94	0.93	0.89	0.90	0.90
Mean age	12.3	12.0	12.4	11.4	11.5
(Range)	(5.2–19.0)	(5.1–19.9)	(5.2–18.2)	(5.1–19.0)	(5.1–18.8)
Urine ELISA					
All subjects					
No. pos. (%)	35 (3.20)	10 (0.91)	4 (0.39)	6 (0.37)	6 (0.36)
Children, 10 years or less					
No. pos./no. exam.	12/356	2/391	0/304	2/729	1/732
(%)	(3.37)	(0.51)	(0.00)	(0.27)	(0.14)
Age of youngest pos.	6.8	9.9	11.2	9.5	10.5

A survey in 2005 was not done.

sex ratio was not different by year ($P=0.96$) but mean ages in 2002–2004 were higher than those in 2006–2007 (Scheffe's test, $P<0.01$). The pre-treatment prevalence of urine ELISA in 2002 was 3.20% (n=1095), which decreased significantly to 0.91% (n=1094) in 2003 ($P<0.001$), to 0.39% (n=1032) in 2004, and finally to 0.36% (n=1647) in 2007 after the 5th MDA. The rates obtained in 2003–2007 were not statistically different ($P=0.15$). In the age group 5–10 years, which would indicate recent filarial transmission, the prevalence decreased significantly from 3.37% (12/356) in 2002 to 0.51% (2/391) in 2003 ($P=0.009$), and finally to 0.14% (1/732) in 2007. The rates in years 2003–2007 were not different ($P=0.50$).

The pre-MDA data of this study were obtained in a separate epidemiological study in 2002 [12], in which children from 15 schools together with adult people were examined. Subgroup analysis of the data from school-age children (6–18 years, n=1574) was carried out to assess differences in pre-treatment urinary IgG4 prevalence by sex and age. The prevalence rates for females and males were 2.1% and 3.5%, respectively, which was not different statistically ($P=0.09$). Five age groups, 6–9 (n=428), 10–11 (n=374), 12–13 (n=332), 14–15 (n=314), and 16–18 (n=126) years, had the rates ranging from 1.9% to 4.1%, which were also not different significantly ($P=0.38$), suggesting that sampling bias in age would not influence the prevalence results significantly.

3.2. Changes of the IgG4 titers after MDAs

Ages of the youngest IgG4 positives are also shown in Table 1. The age in 2002 was 6.8 years old, which increased to 9.9 years in 2003, and ranged thereafter between 9.5 and 11.2 years.

The distribution of IgG4 titers according to age and its change before and after MDAs are shown in Fig. 1. In 2002 (Fig. 1[a]), using only positive IgG4 titers (n=35), the geometric mean titers for the age groups ≤9 years (n=10), 10–13 years (n=11) and ≥14 years (n=14) were 122.7 U, 153.5 U and 295.5 U, respectively, showing a trend of increase by age, though this is not significant (ANOVA, $P=0.11$). This trend was clarified by a different way of analysis: in the ≤9 years group, none had IgG4 titers of more than 200 U, while in the 10–13 years and ≥14 years groups, 4 (36%) and 7 (50%) had titers higher than 200 U, respectively ($P=0.03$). After 2002, no analysis was possible due to a small number of positives. Additionally, the distribution of titers under the cutoff line showed an interesting change. In the space between the cutoff line (54.7 U) and the level of IgG4 titer of 10 U (vertical scale=1), 363 titer dots out of 1095 examined (33.2%) are found in 2002, which decreased to 71 of 1094 (6.5%) in 2003, 48 of 1032 (4.7%) in 2004, 66 of 1623 (4.1%) in 2006 and 39 of 1647 (2.4%) in 2007. The 2002 rate was higher than the 2003–2007 rates ($P<0.001$), and the 2007 rate was lower than the

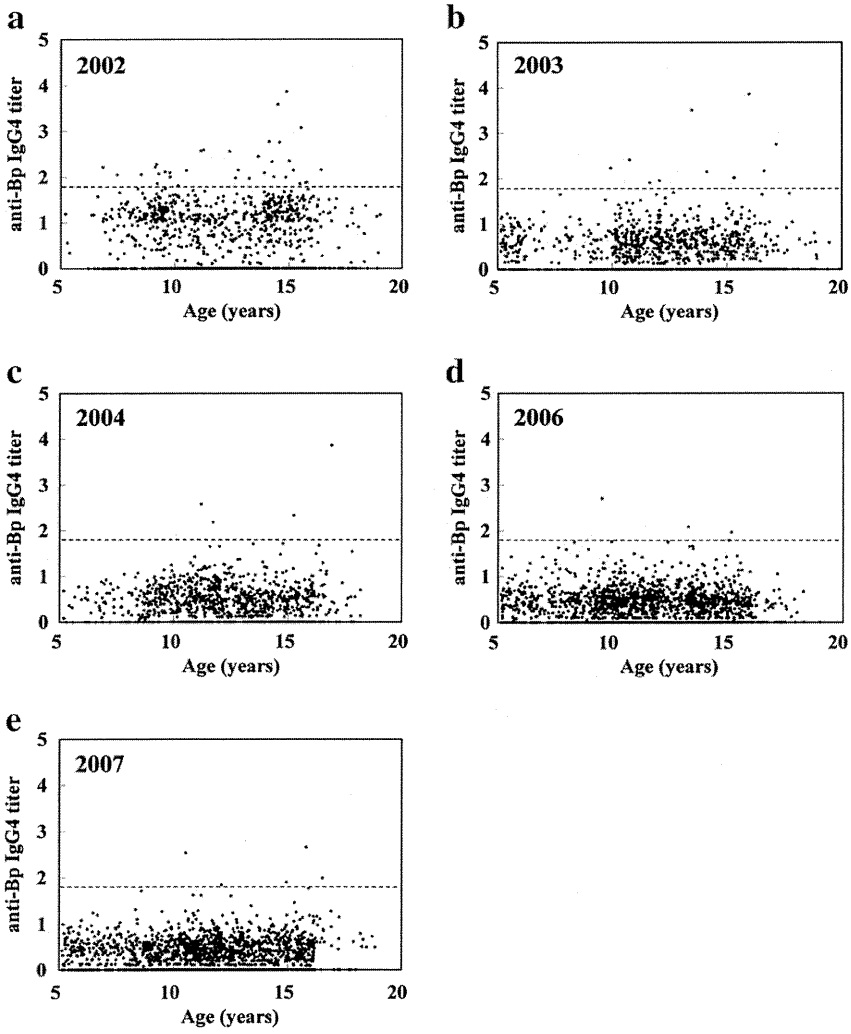

Fig. 1. Change in the distribution of urinary IgG4 titers analyzed by age before and after 5 rounds of annual mass drug administration. Note 1: the vertical axis is in log scale. Note 2: the dotted line indicates the cutoff level.

2002–2006 rates ($P<0.006$). The effect of this change is illustrated most clearly in Fig. 1[e], which shows a clear dot-sparse space under the cutoff line.

Eleven urine ELISA-positive students in the 2006 and/or 2007 surveys, and their 45 family members were examined 25 months after the last MDA with at least one of 3 different diagnostic methods: all examined with ICT (n = 54) were negative, all examined for mf (n = 46) were negative, and 3 of 49 were urine ELISA positive. The 3 ELISA positives were negative both with ICT and mf tests.

4. Discussion

The worldwide elimination program of lymphatic filariasis (GPELF) based on annual single-dose MDAs has made remarkable achievements since its inauguration in 2000. In 8 years (2000–2007), 570 million individuals were treated in 48 of the 83 endemic countries [13]. China in 2007 and Korea in 2008 declared the elimination of filariasis, and additional 5 countries were reported to no longer have any active transmission foci [14]. In a study covering 68 predetermined sentinel sites, 5 rounds of MDA reduced mf prevalence by approximately 85% overall and cleared mf from 63% of the sites [13], implying that the traditional night blood test for mf has become much less sensitive as a tool for monitoring the post-MDA filariasis situation.

Now in many endemic countries, the complicated question of how to confirm the elimination has come to the fore. Extinction/resurgence of filariasis will be influenced by many factors such as the size of residual microfilaremia after MDAs, species and abundance of vector mosquitoes, protective measures against vectors, overlooked foci in remote areas or in urban slums, migration of people, etc. As their influence is variable by endemic area, it will be difficult to apply common criteria for elimination to all endemic areas.

In terms of GPELF field activity, monitoring the level of transmission after MDAs and its change over time are most essential. Young children as a sentinel population to detect new infections will play an indispensable role in such studies. As a monitoring tool, urine ELISA has advantages: sample collection in school under supervision by teachers was simple, reliable and timesaving. In addition, the ELISA was shown to be more sensitive than antigen tests: in Walgama (Sri Lanka), where the reported mf rate was 5.7% [15], urine ELISA detected 2.1 times more infections than ICT test in a sample of 68 children aged 1–10 years [11], and in Deniyaya, where endemicity is much lower, urine ELISA detected 4.0 times more infections than Og4C3 ELISA with 445 subjects aged <9 years (recomputed from Ref. [12]).

The present study confirmed the effectiveness of urine ELISA to detect small changes in infection prevalence, specifically, a statistically significant decrease from 3.20% (pre-MDA) to 0.91% (after 1st MDA), which mf or antigen test would not recognize. The sampling of students was not satisfactory as observed in Fig. 1[b], where ages 6–9 years were apparently underrepresented. However, as the pre-MDA prevalence rates in different age groups were not different ($P = 0.38$), the effect of sampling bias would be minimal. Further analysis with children aged 5 to 10 years revealed that the prevalence decreased significantly from 3.37% in 2002 to 0.51% in 2003, providing further confirmation of the ability of urine ELISA to recognize subtle DEC effects on current or very recent infections.

The distribution of IgG4 titers according to age and its change over time could provide information on the effects of MDAs and levels of transmission. Generally, in endemic areas, the titers among very young positives are low, and increase gradually in older ages. This pattern was observed in the pre-MDA study, suggesting a low level of transmission (Fig. 1[a]). Similar findings were obtained in Thailand [16]. Interestingly, the frequency of titer dots found between the cutoff line and the IgG4 10 U line decreased after MDAs, producing a clear dot-sparse space under the cutoff line (Fig. 1[e]). It seems that even under the cutoff level, *B. pahangi*-reactive IgG4 was elicited and sustained by invasion of filarial infective larvae, very light or latent infection, and also likely by various cross-reactive nematode infections. In the case of soil-transmitted helminths, the use of ALB in MDAs could contribute to the result. Whether this phenomenon can be utilized to evaluate MDA effect needs further studies.

The age of the youngest IgG4 positive will increase when transmission is reduced or stopped by treatment. In this study, the youngest was 6.8 years old in 2002, and 9.5–11.2 years in 2003–2007. As a relatively large number of children aged ≤10 years were examined in each survey (n = 304–732), the age increase could suggest the possible cessation of transmission. This result is in accordance with the finding that no infection was found with 11 previously known ELISA-positive students and their 45 family members examined by ICT test, mf test, and urine ELISA. The combined use of these tests could be useful and practical as a way to confirm elimination. In China, a similar approach was applied to confirm elimination on a larger scale [17]. As for the age of the youngest positive, it could be as low as 1 year old in high endemic areas [10,18]. In such a case, children aged 1 to 5 years may have to be examined to find out new infection/transmission. Urine ELISA was shown to be easily applicable to these ages [9,10].

Urine ELISA has several disadvantages: (1) an ELISA system is often not a practical method in field conditions, (2) the present urine ELISA using crude *B. pahangi* antigen cannot be employed on a large scale due to difficulty to obtain the antigen, and (3) IgG4 ELISA will cross react with other species of human filariae. To address (1), we have developed a visual diagnosis that detects urinary IgG4 (manuscript in preparation). It is a qualitative test and can be used for or in combination with urine ELISA. Concerning (2), we started using a recombinant *W. bancrofti* SXP-1 antigen, and the new urine ELISA has been employed in our recent studies (manuscript in preparation). More studies are required in relation to cross reactivity of IgG4 urine tests, especially for use in Africa.

Urine-based tests have a definite advantage of non-invasive sample collection. By standardizing sampling methods, they can be utilized more effectively to confirm elimination or to detect remaining/resurging infections without disturbing endemic people much.

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Development of loop-mediated isothermal amplification method for detecting *Wuchereria bancrofti* DNA in human blood and vector mosquitoes

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ABSTRACT

We have developed loop-mediated isothermal amplification (LAMP) method to detect *Wuchereria bancrofti* DNA. The sensitivity and specificity of LAMP method were equivalent to those of PCR method which detects *Sspl* repeat sequence in *W. bancrofti* genomic DNA: both methods detected one thousandth of *W. bancrofti* DNA from one microfilaria (Mf), and did not cross-react with DNAs of *Brugia malayi*, *B. pahangi*, *Dirofilaria immitis*, human and *Culex quinquefasciatus*. We also examined the sensitivity of LAMP using the mimic samples of patient's blood or blood-fed mosquitoes containing one *W. bancrofti* Mf per sample. The LAMP method was able to detect *W. bancrofti* DNA in 1000 μl of blood or in a pool of 60 mosquitoes, indicating its usefulness in detecting/monitoring *W. bancrofti* infection in humans and vector mosquitoes in endemic areas.

1. Introduction

Lymphatic filariasis is caused by three species of parasite, *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*, and transmitted by mosquitoes. An estimated 120 million people in 81 countries are infected, and 1.34 billion live in endemic areas [1]. The disease is ranked by WHO as the second important cause of permanent and long-term disability, and since year 2000 it has been targeted for elimination by 2020 under the Global Program to Eliminate Lymphatic Filariasis. The annual mass drug administration (MDA) with two drugs (diethylcarbamazine or ivermectin in combination with albendazole) given for 4–6 years is the basic strategy [2,3].

For monitoring and evaluating the global program, the microfilaria (Mf) test has been used for years. However, the sensitivity had been reduced significantly after successful MDAs, and more usefulness of immunodiagnoses and PCR has been reported in the post-MDA stage. Immunochromatographic card tests (ICT) have high efficiency but are expensive and often not easily available. Anti-filarial IgG4 antibody detection systems have high sensitivity, but cross-reactivity with other human filariae is reported [4,5]. Recent reports have shown that PCR is a powerful tool for detecting filarial infection with patients' blood and blood-fed mosquitoes [5]. In particular, detection of *W. bancrofti* DNA in pooled wild-caught mosquitoes can be useful as a non-invasive method for detecting and quantifying filarial transmission in endemic areas [6,7].

In recent years, loop-mediated isothermal amplification (LAMP) has been utilized as a novel method to amplify DNA with high specificity and simplicity [8,9]. This method employs *Bst* DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. The reaction proceeds to an autocycling strand displacement mechanism and produces a various size of amplicons consisting of alternately inverted repeats of the target sequence on the same strand. Thus, the amplified product is not a single band but a ladder-like pattern on agarose gel analysis. The significant advantages of the method are high amplification efficiency under isothermal conditions (60–65 °C) and visual judgment based on the turbidity or fluorescence of the reaction mixture [10]. LAMP methods have been applied to amplify DNA or RNA from human pathogens including viruses, bacteria, protozoa, fungi and helminthic parasites [11,12]. In this paper, we report a newly developed LAMP method for *W. bancrofti* infection.

2. Materials and methods

2.1. Parasites and mosquitoes

W. bancrofti microfilariae were obtained from frozen Mf-positive blood collected in Sri Lanka. They were purified by Percoll gradient centrifugation [13]. *B. malayi* and *B. pahangi* adults were kindly provided by the Institute of Tropical Medicine, Nagasaki University.

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Dirofilaria immitis adults were obtained from a euthanized dog at the animal shelter in Nagoya, Japan. *Culex quinquefasciatus* Say female adults were supplied by the National Institute of Infectious Diseases, Japan [14]. This study was reviewed and approved by the ethical committees of University of Ruhuna, Sri Lanka and Aichi Medical University School of Medicine, Japan.

2.2. Samples and DNA extraction

2.2.1. Sensitivity study with diluted *W. bancrofti* DNA

One hundred microfilariae were collected with a micropipet under the microscope. DNA of 100 microfilariae was purified using QIAamp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's manual. DNA was eluted with 200 μl of AE buffer. Two microliter of this extracted DNA sample was considered as the amount of DNA from one Mf. DNA was serially diluted from 1 to 100,000-fold (equivalent to 1, 1/10, $1/10^2$, $1/10^3$, $1/10^4$, and $1/10^5$ Mf) for the sensitivity test for LAMP and PCR.

2.2.2. Specificity study with different species of filarial parasites

DNAs from *B. malayi*, *B. pahangi* and *D. immitis* adults were purified as above. For DNA purification from *C. quinquefasciatus*, 20–60 adult whole bodies were added with 180 μl of 1× PBS(−) and homogenized using a disposable plastic homogenizer (Nippi Co., Ltd. Japan). The sludge was added to 20 μl of proteinase K and 200 μl of Buffer AL (QIAGEN, Germany). After incubation at 70 °C for 10 min, the mixture was added with additional 20 μl of proteinase K and incubated at 56 °C for 3 h. Next, the sample was centrifuged at 14,000 rpm (17,000×*g*) for 5 min to sediment mosquito debris. DNA in the supernatant was purified using the same QIAGEN kit as in 2.2.1. DNA concentration and DNA purity of the samples were measured by the spectrophotometer. To confirm the specificity, 100 pg of DNA/sample was used for LAMP and PCR.

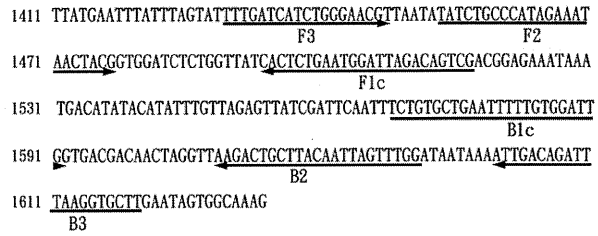
2.2.3. Detection of *W. bancrofti* DNA in human blood or in a pool of *Culex mosquitoes* using mimic samples

For mimicking *W. bancrofti* carrier's blood, one Mf was mixed in 200 μl or 1000 μl of human blood. DNA was purified using the same QIAGEN kit. For mimicking blood-fed *W. bancrofti* infected mosquitoes, one Mf was added with 180 μl of 1× PBS(−) to a pool of 20, 40 or 60 adult mosquitoes. After being homogenized by a disposable plastic homogenizer, 100 μl of human blood was added, and then DNA was purified using the QIAGEN kit. DNA was eluted twice from the column with 125 μl of Buffer AE (final sample volume was 250 μl). Two microliter of DNA sample was subjected to LAMP or PCR.

2.3. Loop-mediated isothermal amplification (LAMP)

The LAMP reaction was performed according to our previous report [15]. Primers for *W. bancrofti* DNA detection were designed against *W. bancrofti* nuclear scaffold/matrix attachment region (GenBank accession no. AY297458) by using Primer Explorer software (http://primerexplorer.jp/). The targeted location and sequence of each primer are shown in Fig. 1. The reaction mixtures in a total volume of 25 μl contained 40 pmol (each) of FIP and BIP, 5 pmol (each) of F3 and B3c, 1.4 mM of each deoxynucleoside triphosphate, 0.8 M betaine, 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% TritonX-100, 8 U of *Bst* DNA polymerase large Fragment (New England Biolabs, MA), and 2 μl of sample DNA. The mixture was incubated at 62 °C for 60–90 min by using a heat block. As the LAMP reaction progresses, the reaction by-product (pyrophosphate ions) binds to magnesium ions and forms white precipitates of magnesium pyrophosphate. After incubation, the turbidity of reaction fluid was inspected visually. Furthermore, LAMP products were subjected to electrophoresis on a 2% agarose gel in TAE buffer (40 mM Tris acetate and 1 mM EDTA) and visualized under UV light after ethidium bromide (5 μg/ml) staining.

A



B

primer	sequence (5'→3')
F3	TTTGATCATCTGGGAACGT
B3c	AAGCACCTTAAATCTGTCAAT
FIP	CGACTGTCTAATCCATTCAGAGTG-TATCTGCCCATAGAAATAACTACG
BIP	TCTGTGCTGAATTTTTGTGGATTG-CCAAACTAATTGTAAGCAGTCTT

Fig. 1. The target sequence on *W. bancrofti* DNA and the primer set used for amplification in LAMP. (A) Locations and names of target sequences on *W. bancrofti* nuclear scaffold/matrix attachment region (GenBank accession no. AY297458). Numbers on the left margin indicate the nucleotide position. (B) Sequences of four primers, F3, B3c, FIP (F1c-F2) and BIP (B1c-B2), used in the LAMP reaction.

2.4. Polymerase chain reaction (PCR)

The PCR reaction was performed according to the original report [16,17]. The two primers NV-1 and NV-2 were used. The sequences of these primers are NV-1: 5′-CGTGATGGCATCAAAGTAGCG-3′; NV-2: 5′-CCCTCACTTACCATAAGACAAC-3′. These primers amplify a fragment of 188 bps, a highly repeated DNA sequence in *W. bancrofti* genome (*Ssp*I repeat). The reaction mixtures in a total volume of 50 μl contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (W/V) gelatin, 200 μM of each deoxynucleoside triphosphate, 0.5 μM of each primer, and 1.25 U of AmpliTaq Gold (Applied Biosystems, CA). PCR amplification was performed using a thermal cycler (Takara, Japan) programmed for 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, preceded by an initial denaturation of 5 min at 94 °C. After completion of all cycles, the final extension reaction was for 10 min at 72 °C. The products were analyzed by electrophoresis on a 2% agarose gel.

3. Results

3.1. Sensitivity and specificity of LAMP method

Aiming at developing a new diagnostic assay that is more sensitive, specific and convenient than PCR, we designed the oligonucleotide primers for LAMP in *W. bancrofti* DNA (Fig. 1). To estimate the sensitivity, *W. bancrofti* DNA diluted tenfold serially (range from 1 Mf to $1/10^5$ Mf) were used as templates for LAMP reaction. The amplification product was detectable with the samples containing≥ one thousandth of the amount of DNA from one Mf (Fig. 2(B), lanes 1–4). The sensitivity was equivalent to that of PCR (Fig. 2(C)). The same PCR method used in the present study was reported to detect 0.1 pg of *W. bancrofti* genomic DNA, equivalent to 0.1% of the DNA found in one Mf [16]. When the reaction fluid was inspected for white turbidity, it was visually detected in all electrophoresis positive samples (Fig. 2(A), lanes 1–4), indicating that the time-consuming confirmation with electrophoresis is unnecessary.

For specificity, the LAMP was tested with DNAs of *B. malayi*, *B. pahangi*, *D. immitis*, human and *C. quinquefasciatus*. Even when

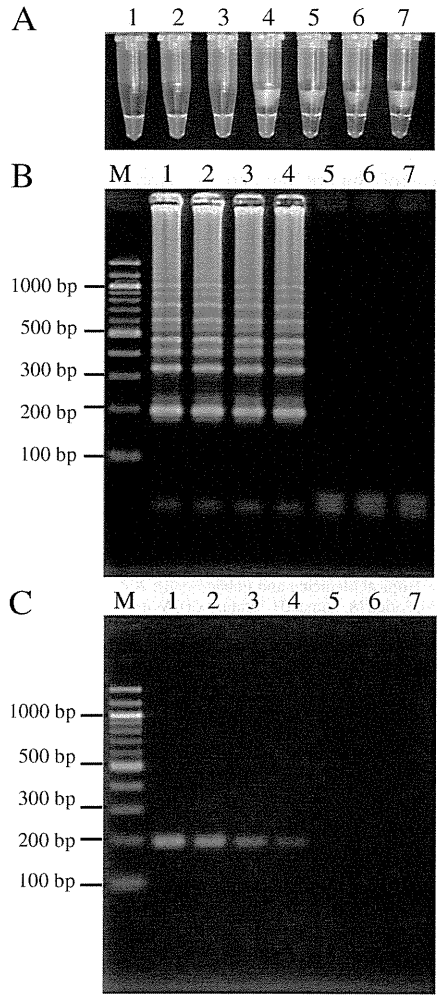


Fig. 2. Sensitivity of LAMP and PCR for *W. bancrofti* DNA detection. (A) The tubes after LAMP reaction. The reaction mixtures of positive samples turned white, the mixtures of negative samples are clear. LAMP (B) and PCR (C) were carried out using the serial 10-fold dilution of *W. bancrofti* DNA and analyzed on agarose gels. Lane 1: the amount of DNA of one Mf, lane 2: DNA of 1/10 Mf, lane 3: DNA of $1/10^2$ Mf, lane 4: DNA of $1/10^3$ Mf, lane 5: DNA of $1/10^4$ Mf, lane 6: DNA of $1/10^5$ Mf, lane 7: water (negative control), lane M: 100 bp DNA Ladder (New England Bio labs. MA).

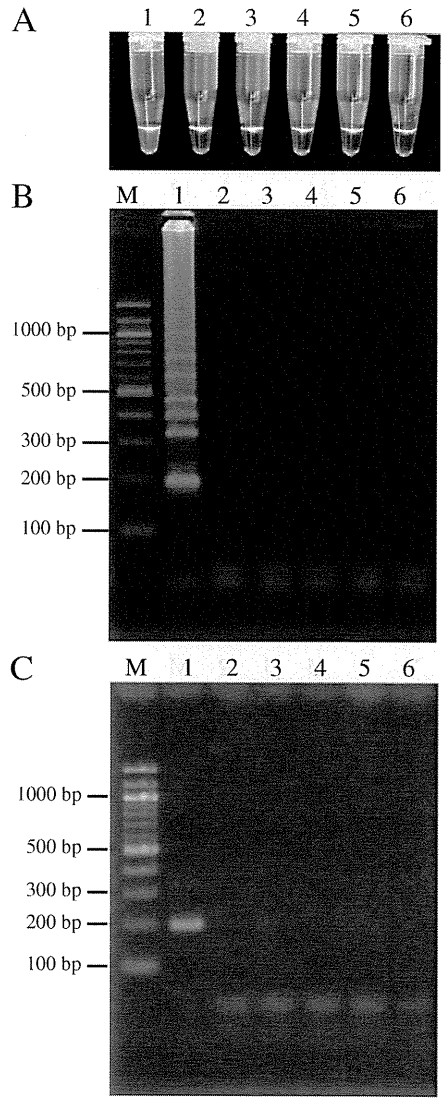


Fig. 3. Specificity of LAMP and PCR for *W. bancrofti* DNA. (A) The tubes after LAMP reaction. (B) LAMP and (C) PCR products detection by electrophoresis. Lane 1: *W. bancrofti*, lane 2: *B. malayi*, lane 3: *B. pahangi*, lane 4: *D. immitis*, lane 5: human, lane 6: *Culex quinquefasciatus*, lane M: 100 bp DNA ladder.

100 pg DNA was used for each, no amplification product could be detected (Fig. 3(B), lanes 2–6), indicating that these DNAs were not cross-reactive. Corresponding to the negative results, the turbidity of reaction fluid was not observed (Fig. 3(A), 2–6). The results of PCR were also negative (Fig. 3(C), lanes 2–6). In a separate study, DNA samples (100 pg each) from 11 healthy persons and *Plasmodium falciparum* parasite were tested by the same protocol, which were all negative (data not shown).

3.2. Detection of W. bancrofti DNA in mimicry patients' blood and blood-fed mosquitoes

To evaluate the clinical and mosquito screening utility of the LAMP method, it was applied to the mimic positive samples: 200 or 1000 μl human blood containing one Mf, and a pool of 20, 40 or 60 *Culex* mosquitoes containing 100 μl human blood and one Mf. The LAMP detected *W. bancrofti* DNA in all the samples (Fig. 4(B)), which was confirmed with agarose electrophoresis. All reaction fluids were visually turbid (Fig. 4(A)). As to PCR, all samples were positive (Fig. 4(C)). To confirm the reliability of LAMP, we repeated the same experiment with mimicry samples 7 times. LAMP detected *W. bancrofti* DNA in all samples in all occasions (data not shown). Negative control experiments with DNA from a pool of 20 mosquitoes mixed with 100 μl human blood were all negative (data not shown).

4. Discussion

In recent years, it has been increasingly reported that LAMP technique was successfully applied to the rapid and specific detection of protozoa and helminth infections [12,15,18–20]. In a more recent report, the technique detected *D. immitis* in wild-caught mosquitoes [21]. We reported here the development of a LAMP method for a human lymphatic filarial nematode *W. bancrofti*. As to the sensitivity and specificity, the LAMP reaction was equivalent to those of PCR which targets *Ssp*I repeat, detecting 1/1000 of the DNA amount of one Mf. This target sequence of LAMP is located in the nuclear scaffold/matrix attachment region. The same regions abound in genomic DNA and have many important functions in eukaryotes [22]. Thus, the sequence would not be very variable

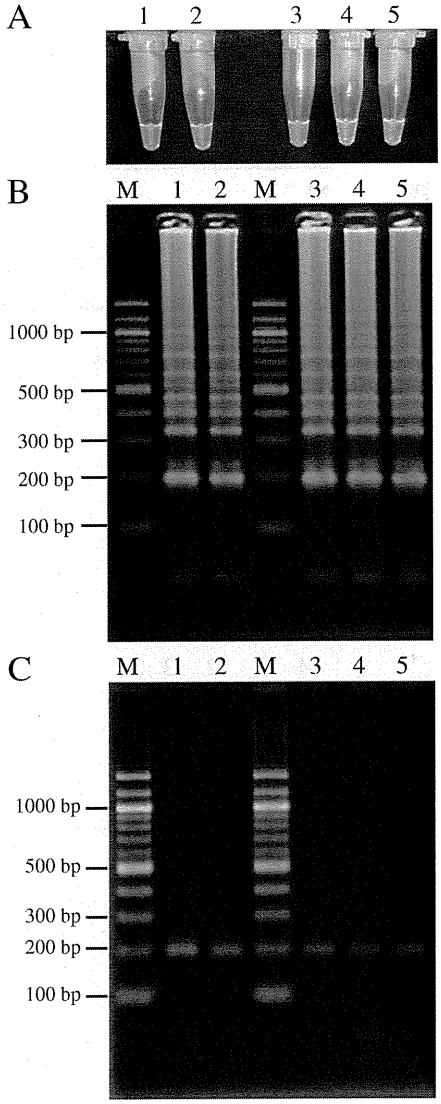


Fig. 4. Diagnosis by LAMP and PCR with the mimic samples. (A) The tubes after LAMP reaction. (B) LAMP and (C) PCR products detection by electrophoresis. Lane 1: DNA extracted from one Mf in 200 μl blood, lane 2: DNA extracted from one Mf in 1000 μl blood, lane 3: DNA extracted from 20 mosquitoes, one Mf and blood, lane 4: DNA extracted from 40 mosquitoes, one Mf and blood, lane 5: DNA extracted from 60 mosquitoes, one Mf and blood, lane M: 100 bp DNA Ladder.

among microfilariae from different endemic areas. However, without confirmation, it is necessary to evaluate our LAMP using different isolates.

To diagnose or detect *W. bancrofti*-infected individuals and mosquito vectors, many DNA-based techniques have been developed: PCR for *Ssp*I repeat [16,17], IWb 35 repeat [23] and pWb12 repeat [24], multiplex-PCR [25], PCR-restriction fragment length polymorphism [26,27], PCR-enzyme linked immunosorbent assay [28], nested PCR [29] and real time quantitative PCR [30]. Although these methods have high sensitivity and specificity, PCR has disadvantages: the agarose electrophoresis is a necessary step after PCR reaction with a thermal cycler for 2–3 h. The step is definitely inconvenient to process many samples as in mosquito studies for *W. bancrofti* infection. While with LAMP, diagnosis can be made visually after the reaction for 60–90 min using a heat block or a water bath. Moreover, the cost per LAMP test is less than US$ 0.82 compared with more than US$ 2.2 of PCR, including the cost of electrophoresis. It is expected that our LAMP can replace labor-intensive mosquito dissection for finding filarial larvae, and facilitate quantifying *W. bancrofti* transmission status in an endemic area, especially in the post-MDA stage.

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Longistatin, a novel plasminogen activator from vector ticks, is resistant to plasminogen activator inhibitor-1

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ABSTRACT

Thrombo-occlusive diseases are major causes of morbidity and mortality, and tissue-type plasminogen activator (t-PA) is recommended for the treatment of the maladies. However, both t-PA and u-PA are rapidly inactivated by plasminogen activator inhibitor-1 (PAI-1). Here, we show that longistatin, a novel plasminogen activator isolated from the ixodid tick, *Haemaphysalis longicornis* is resistant to PAI-1. Longistatin was relatively less susceptible to the inhibitory effect of SDS-treated platelet lysate than physiologic PAs. Platelet lysate inhibited t-PA and tcu-PA with the IC_{50} of 7.7 and 9.1 μg/ml, respectively, whereas for longistatin inhibition IC_{50} was 20.1 μg/ml ($p < 0.01$). Similarly, activated PAI-1 (20 nM) inhibited only 21.47% activity of longistatin but almost completely inhibited t-PA (99.17%) and tcu-PA (96.84%). Interestingly, longistatin retained 76.73% initial activity even after 3 h of incubation with 20 nM of PAI-1. IC_{50} of PAI-1 during longistatin inhibition was 88.3 nM while it was 3.9 and 3.2 nM in t-PA and tcu-PA inhibition, respectively. Longistatin completely hydrolyzed fibrin clot by activating plasminogen efficiently in the presence of 20 nM of PAI-1. Importantly, unlike t-PA, longistatin did not form complex with PAI-1. Collectively, our results suggest that longistatin is resistant to PAI-1 and maybe an interesting tool for the development of a PAI-1 resistant effective thrombolytic agent.

1. Introduction

Thrombo-occlusive events including myocardial infarction, ischemic stroke, obstructive pulmonary disease and peripheral thromboembolism are major causes of morbidity and mortality in humans. Most countries are facing a high and increasing rate of cardiovascular diseases. In each year, heart diseases kill more Americans than does cancer. In women, death due to cardiovascular diseases is higher than that caused by breast cancer [1–4]. Treatment of thrombo-occlusive diseases is one of the most challenging areas in medicine. Plasminogen activators (PAs) have great clinical significance as thrombolytic agents in the managements of cerebral and cardiac attacks [5]. PAs such as tissue-type plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA) and others activate plasminogen, a circulating plasma zymogen, into its active form plasmin, a powerful serine protease, which in turn dissolves thrombi and restores circulation in thrombosed vasculature; thus, rescue the affected tissues/organs from the devitalizing events of thrombosis [1,6–8]. Among the PAs recommended for clinical use, t-PA is generally preferred for its relatively higher efficacy and wider safety window compared to u-PA and streptokinase. However, both t-PA and u-PA are rapidly inactivated by the fast-acting serine protease inhibitor (serpin) plasminogen activator inhibitor-1 (PAI-1), a molecular switch of physiologic PAs, and is the major PAI in the serum [9,10]. PAI-1 plays crucial roles in the regulation of PA mediated thrombolysis [1,6]. PAI-1 is synthesized and secreted by a variety of cells such as endothelium, adipocytes, monocytes/macrophages, fibroblasts, cardiomyocytes, hepatocytes, smooth muscle cells, granulosa cells and megakaryocytes, and stored in the α-granules of platelets, and is released following activation of platelets [10,11]. PAI-1 forms a tight complex with PAs and rapidly neutralizes them. In fact, exogenous PAs have to work after saturating the PAI-1 and other serpins present in serum

Abbreviations: t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; tcu-PA, two chain u-PA; serpin, serine protease inhibitor; PAI-1, plasminogen activator inhibitor-1; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

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that hinder thrombolytic therapy to some extent [6,12]. Therefore, development of PAI-1 resistant PA is essential for the betterment of effective thrombolytic therapy.

Recently, we have isolated and identified longistatin, a novel PA from salivary glands of the ixodid tick, *Haemaphysalis longicornis*, a common vector of many deadly diseases of humans and animals [13,14]. Longistatin is a 17.8-kDa secretory protein with an N-terminal sequence of unknown structure and two calmodulin-like functional EF-hand domains at the C-terminus. Longistatin is synthesized in, and secreted from the salivary glands with saliva and injected into host tissues during acquisition of blood-meal [15]. Longistatin, like physiologic PAs can efficiently activate plasminogen and cause lysis of fibrin clot and fresh thrombi; thus, plays crucial roles in the development and maintenance of the blood pool, the essential feeding lesion of hard ticks, throughout the entire feeding period keeping blood in a fluid state. Longistatin binds with fibrin meshwork with higher affinity than t-PA, and fibrin acts as co-factor during plasminogen activation processes [16]. Here, we show that longistatin is relatively resistant to SDS-treated platelet lysate. Longistatin is refractory to the inhibitory effects of activated, purified and commercially available PAI-1. Longistatin sufficiently activated plasminogen into plasmin in the presence of PAI-1 (20 nM), and completely hydrolyzed fibrin clot. Furthermore, longistatin does not form any complex with PAI-1. To our knowledge, longistatin is the first PA identified and characterized from arthropods.

2. Materials and methods

2.1. Production of recombinant longistatin

Recombinant longistatin was produced and purified as described previously [15]. Protein was concentrated using Centrisart® (Sartorius) having a mol. wt cut-off of 10 kDa. His-tag was removed from longistatin by incubating with enterokinase (EKMax™, Invitrogen) and purified using the enterokinase-eliminating column (Ek-Away™ Resin, Invitrogen) following the manufacturer's protocol. The concentrated protein was dialyzed extensively at 4 °C with successive changes of 20 mM Tris–HCl (pH 7) and a decreasing concentration of NaCl (500–250 mM) using a Slide-A-Lyser Dialysis Cassette (Pierce) with a mol. wt cut-off of 10 kDa. N-terminal and internal amino acid sequences of purified longistatin were confirmed by automated Edman's degradation. Purity of the recombinant was judged employing one and two dimensional electrophoresis followed by silver staining technique. The concentration of longistatin was determined using micro-BCA reagents (Pierce) as described previously [15].

2.2. Blood collection and preparation of plasma and serum

Blood samples were collected from fully consented healthy volunteers into EDTA-treated tubes. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were produced following the procedures as described [6,17] with slight modifications. Briefly, PRP was produced by centrifuging blood at 100*g* and 23 °C for 10 min, and supernatant was collected. To prepare PPP, PRP was chilled at 4 °C for 15 min and centrifuged at 2000*g* and 4 °C for 30 min, and supernatant was carefully collected without disturbing the pellet. To prepare serum, 1 ml of either whole blood, PRP or PPP was treated with 5 μl of thrombin (0.1 NIH unit/μl, Sigma) and incubated at 37 °C for 1 h. The clot was centrifuged at 10,000*g* and 4 °C for 30 min and supernatant was collected. Plasma and serum were stored at −20 °C until further use.

2.3. Preparation of platelet lysate

PRP (1 ml) from human blood was chilled at 4 °C for 15 min and centrifuged at 2000*g* and 4 °C for 30 min; then, PPP was aspirated. The pellet was extensively washed with PBS. The platelets were incubated with thrombin (0.5 U/ml, final concentration) at 37 °C for 6 h in Tyrode's buffer (pH 6.5). The platelets were sonicated with an ultrasonic processor (VP-5S, TAITEC) for 2 min on ice with three pauses each of 25 s, and centrifuged at 22,300*g* and 4 °C for 1 h. Supernatant was collected and treated with 0.1% SDS at 37 °C for 1 h; then, SDS was neutralized treating with 1% tritonX-100 at 4 °C for 1 h as described previously [17]. SDS-treated platelet lysate was stored at −20 °C until further use.

2.4. Treatment of longistatin with platelet lysate

An equal amount (1 μg) of longistatin, t-PA (Calbiochem) or tcu-PA (CosmoBio Co. LTD) was reacted with various amount of SDS-treated platelet lysate (0–30 μg/ml) in a total volume of 25 μl of buffer A (50 mM Tris–HCl, pH 7; 100 mM NaCl and 5 mM $CaCl_2$) at 37 °C for 15 min. Then, the mixture was immediately diluted with buffer A. Plasminogen (5 μl, 0.2 μg/μl, Sigma) was added in the reaction mixtures and incubated at 37 °C for further 1 h in the absence (incase of tcu-PA) or presence (incase of t-PA/longistatin) of soluble fibrin (8 μg/ml, Technoclone). Residual activity of plasminogen activator was determined employing indirect fluorogenic assays using a plasmin-specific synthetic substrate (Boc-2Glu-Lys-Lys-MCA, Peptide Institute). Substrate hydrolysis was monitored by measuring excitation and emission wavelengths of 360 nm and 460 nm, respectively, using a Spectra Fluor fluorometer (TECAN). Percentage of inhibition of the enzyme was calculated using the following formula:% inhibition = (1 − inhibited rate/uninhibited rate) × 100 and IC_{50} was determined as described previously [18].

2.5. Inhibition of longistatin with PAI-1

An equal amount (1 μg) of longistatin, t-PA or u-PA was incubated with commercially available, activated PAI-1 (ITSI Bioscience) at various concentrations (0–90 nM) at 37 °C for different time (0–180 min) in a total volume of 25 μl of buffer A. Then the reaction mixture was immediately diluted adding buffer A and residual activity was determined using a synthetic substrate specific for t-PA/u-PA (Pyr-Gly-Arg-MCA, Peptide Institute). Substrate hydrolysis was monitored by measuring excitation and emission wavelengths of 360 nm and 460 nm, respectively, using a Spectra Fluor fluorometer (TECAN). Percentage of inhibition of enzyme and IC_{50} were determined as described above.

2.6. Microplate clot lysis assay

We produced fibrin clot in a 96-well plate by incubating 3 μl of fibrinogen (7.5 mM in final concentration, Sigma) and 2 μl of thrombin (0.10 NIH unit/μl, Sigma) in a total volume of 100 μl of buffer A at 37 °C for 1 h. An equal amount (1 μg) of t-PA, tcu-PA or longistatin was mixed with PAI-1 (20 nM, in final concentration) in a total volume of 90 μl of buffer A and incubated for 15 min at 37 °C. Then, plasminogen (10 μl, 0.2 μg/μl, Sigma) was mixed with each reaction mixture. The mixtures were gently added to the fibrin clot separately and incubated at 37 °C for 6 h. Only buffer A was added as a negative control and longistatin alone was used as a positive control. Lysis of fibrin clot was detected visually and also by measuring changes in turbidity at 450 nm using a spectrophotometer (TECAN) up to 6 h.

2.7. Complex formation study with active PAI-1

Commercially available, activated PAI-1 was incubated with t-PA or longistatin in an equimolar concentration (666.6 nM) in a total volume of 15 μl of buffer A at 37 °C for 10 min. Aliquots were separated by 12.5% SDS–PAGE under reducing conditions and proteins were visualized by silver. Additionally, proteins were transferred onto nitrocellulose membranes and probed with either mouse anti-longistatin sera (1:200) or mouse anti-PAI-1 sera (1:500, AntibodyShop) following the same methods as described [16]. The membranes were incubated with alkaline phosphate conjugated goat anti-mouse IgG (H + L) (ZYMED) for 1 h and the bound proteins were visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT, Promega).

2.8. Statistical analysis

Data were presented as mean ± standard error, where appropriate. Statistical significance was determined using Student's *t* test with unequal variance.

3. Results

3.1. Longistatin is less susceptible to the inhibitory effect of activated platelet lysate

To determine the effect of platelet lysate on longistatin, we employed two-step indirect fluorogenic assay using plasmin-specific synthetic flourogenic substrate (Boc-Glu-Lys-Lys-MCA). In this assay, longistatin or other physiologic PAs activated plasminogen to plasmin, which in turn hydrolyzed the plasmin-specific substrate. Data revealed that longistatin was relatively less susceptible to the inhibitory effect of SDS-treated platelet lysate than physiologic PAs such as t-PA and tcu-PA. Within 15 min 30 μg/ml of activated platelet lysate almost completely inhibited the plasminogen activation potential of t-PA (98.55%) and tcu-PA (98.15%) with the estimated IC_{50} of 7.7 and 9.1 μg/ml, respectively. Whereas in the same experimental setup, the equal amount of platelet lysate caused 56.51% inhibition of longistatin showing a significantly ($p < 0.01$) higher IC_{50} (20.1 μg/ml) (Fig. 1), suggesting that longistatin is less susceptible to the inhibitor(s) released by activated platelets.

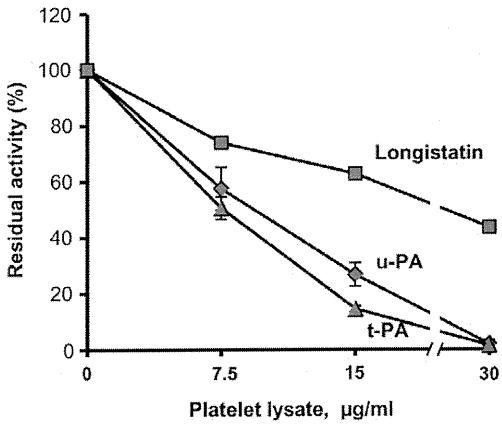

Fig. 1. Effects of activated platelet lysate on longistatin. Equal amount of longistatin/t-PA/u-PA was treated with various amount of platelet lysate (0–30 μg/ml) at 37 °C for 15 min and then diluted with buffer A (50 mM Tris–HCl, pH 7; 100 mM NaCl and 5 mM $CaCl_2$). Plasminogen (5 μl, 0.2 μg/μl) was added and incubated at 37 °C for further 1 h in the absence (incase of u-PA) or presence (incase of t-PA/longistatin) of soluble fibrin (8 μg/ml). Residual activity of longistatin, t-PA or u-PA was determined using plasmin-specific synthetic substrate (Boc-Glu-Lys-Lys-MCA) as detailed in Section 2.

3.2. Longistatin is more resistant to PAI-1 than physiologic PAs

To explore the effect PAI-1 on longistatin, we conducted direct fluorogenic assays using a t-PA-/u-PA-specific synthetic fluorogenic substrate (Pyr-Gly-Arg-MCA) since longistatin also hydrolyzed the t-PA-/u-PA-specific synthetic substrate. Activated PAI-1 at 5 nM concentration inhibited only 6.34%, 8.84% and 10.75% enzymatic activity of longistatin by 5, 10 and 15 min, respectively. At the highest physiologic concentration (20 nM) PAI-1 inhibited only 18.18%, 20.55% and 21.47% enzymatic activity of longistatin by 5, 10 and 15 min of incubation, respectively at 37 °C. Importantly, longistatin could retain its 76.73% initial activity even after very long time (3 h) of incubation with 20 nM of PAI-1 (Fig. 2 Ai). In contrast, in the same experimental conditions, PAI-1 almost completely inhibited t-PA and tcu-PA. PAI-1 at 5 nM inhibited 76.05%, 79.51% and 88.68% enzymatic activity of t-PA by 5, 10 and 15 min, respectively. And, PAI-1 at 20 nM concentration caused 83.95% inhibition of t-PA within 5 min of incubation at 37 °C and by 15 min it inhibited 99.17% enzymatic capability of t-PA (Fig. 2 Aii). PAI-1 also rapidly inactivated tcu-PA. PAI-1 at 5 nM concentration inhibited 65.46%, 74.13% and 82.4% catalytic capability of tcu-PA by 5, 10 and 15 min, respectively. PAI-1 at upper physiologic plasma level (20 nM) caused 75.71%, 93.31% and 96.84% inhibition of tcu-PA within 5, 10 and 15 min of incubation, respectively (Fig. 2 Aiii). To compare the inhibitory effect of the activated PAI-1 on longistatin, t-PA or tcu-PA, we also calculated IC_{50} in the same experimental settings. Estimated IC_{50} of PAI-1 for longistatin inhibition was 88.3 nM but for inhibition of t-PA and tcu-PA the IC_{50} was 3.9 and 3.2 nM, respectively (Fig. 2B), suggesting that longistatin is more resistant to PAI-1 than physiologic PAs.

3.3. Longistatin efficiently lyses fibrin clot by activating plasminogen in the presence of PAI-1

In addition to synthetic substrate hydrolysis, we conducted microplate fibrin clot lysis assays to judge the plasminogen activation and subsequent fibrinolytic potentiality of longistatin in presence of activated PAI-I. Longistatin in the presence of 20 nM of PAI-1 activated sufficient amount of plasminogen into plasmin which completely hydrolyzed fibrin clot produced in a 96-well plate, as it was done by longistatin in the absence of PAI-1 during the same incubation period. However, both t-PA and tcu-PA failed to activate plasminogen, and eventually to hydrolyze fibrin clot in the presence of PAI-1 even after long time (6 h) of incubation (Fig. 3). The results clearly suggest that longistatin can activate a large amount of plasminogen and induce robust fibrinolysis in the presence of PAI-1 at the highest physiologic level.

3.4. Longistatin does not form complex with active PAI-1

To investigate whether longistatin forms stable complex with PAI-1 or not, we incubated longistatin with activated PAI-1 in an equimolar concentration. Here, we used t-PA as a control, since t-PA produced SDS stable complex with PAI-1 in an equimolar concentration [12,22]. After visualization of proteins with silver stain, we observed that longistatin neither formed complex with PAI-1 nor cleaved it. In contrast, t-PA produced a stable complex with PAI-1 at the level of ~110 kDa. Furthermore, while incubated t-PA also cleaved PAI-1, which appeared at ~40 kDa (Fig. 4A). To validate our data, we conducted immunoblot study using mouse anti-longistatin or mouse anti-PAI-1 antibody. However, we also could not detect longistatin-PAI-1 complex by immunoblotting but both longistatin and PAI-1 were detected individually by their own

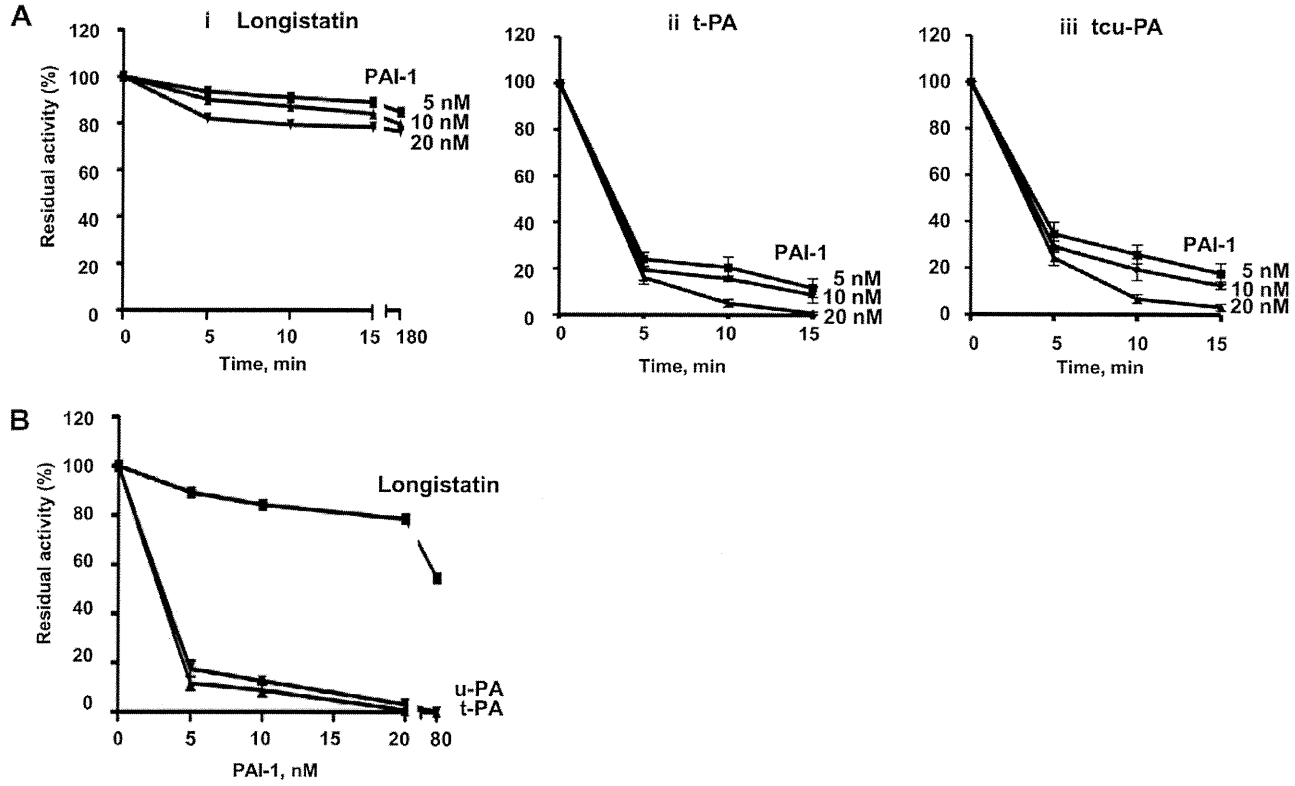

Fig. 2. Effect of PAI-1 on plasminogen activators. (A) Effects of PAI-1 on plasminogen activators at different time of incubation. Longistatin (i)/t-PA (ii)/tcu-PA (iii) was incubated without or with PAI-1 (5–20 nM) at 37 °C for different time (0–180 min) in 25 μl of buffer A (50 mM Tris–HCl, pH 7; 100 mM NaCl and 5 mM $CaCl_2$). Residual activity was determined using a synthetic substrate specific for t-PA/u-PA (Pyr-Gly-Arg-MCA) as described in Section 2. (B) Comparison of inhibitory effect of PAI-1 on plasminogen activators. Longistatin, t-PA or tcu-PA was incubated without or with PAI-1 (5–80 nM) at 37 °C for 15 min in 25 μl of buffer A. Residual activity was determined using synthetic substrate specific for t-PA/tcu-PA (Pyr-Gly-Arg-MCA) as described in Section 2.

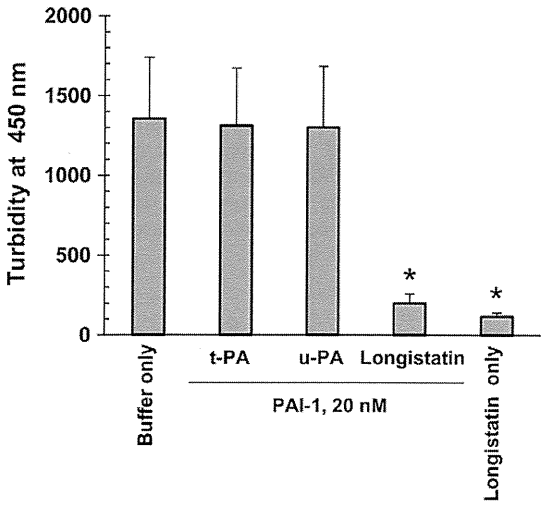

Fig. 3. Fibrinolysis by longistatin in the presence of PAI-1. Fibrin clot was produced in a 96-well plate by incubating 3 μl of fibrinogen (7.5 mM, in final concentration) and 2 μl of thrombin (0.10 NIH unit/μl) in a total volume of 100 μl of buffer as described in Section 2. The same amount (1 μg) of t-PA, tcu-PA or longistatin was incubated in the absence or presence of PAI-1 (20 nM) for 15 min and then plasminogen (10 μl, 0.2 μg/μl) was added. The mixture was added to the fibrin clot and incubated at 37 °C for further 6 h. Lysis of fibrin clot was detected as described in Section 2. Asterisks (*) indicate that the difference compared with control group is significant as determined by Student's *t*-test with unequal variance (*$p < 0.01$).

antibodies at the expected level (Fig. 4B), which further potentiates the findings that longistatin does not form any complex with PAI-1.

4. Discussion

PAI-1 is the main inhibitor of the physiologic PAs present in plasma and significantly reduces the PA-mediated thrombolysis during the conservative therapy of thrombo-occlusive disorders [6,19,20]. Therefore, PAI-1 resistant PA may be the best choice for the prompt and efficient dissolution of thrombi and subsequent restoration of circulation to the affected tissues/organs. Here, we report on longistatin, a novel plasminogen activator from the salivary glands of the hard tick, *H. longicornis*, which shows significant resistance to activated platelet lysate and commercially available PAI-1. Importantly, longistatin does not form complex with PAI-1.

We observed that following incubation with an amount of activated platelet lysate (SDS treated) which completely inhibited physiologic PAs such as t-PA and u-PA, longistatin significantly retained its functional activity. During thrombogenesis, thrombin causes limited cleavage of fibrinogen and produces fibrin meshwork, which entraps platelets and produces haemostatic plaque [1,21]. Therefore, thrombi, especially arterial thrombi, become platelet rich. PAI-1, upon production from different sources, is stored in α-granules of platelets and is released by the activated platelets during thrombi formation [11,19]. Thus, PAI-1 concentration is elevated in and around the platelet-rich thrombi and the thrombi become resistant to some extent to the PA therapy with physiologic PAs [6,19]. Our longistatin exhibits significant tolerance to the activated platelet lysate, suggesting that longistatin may be resistant to the PAI-1 released by activated platelets, which is mainly responsible for the attenuation of PA-mediated fibrinolysis system.

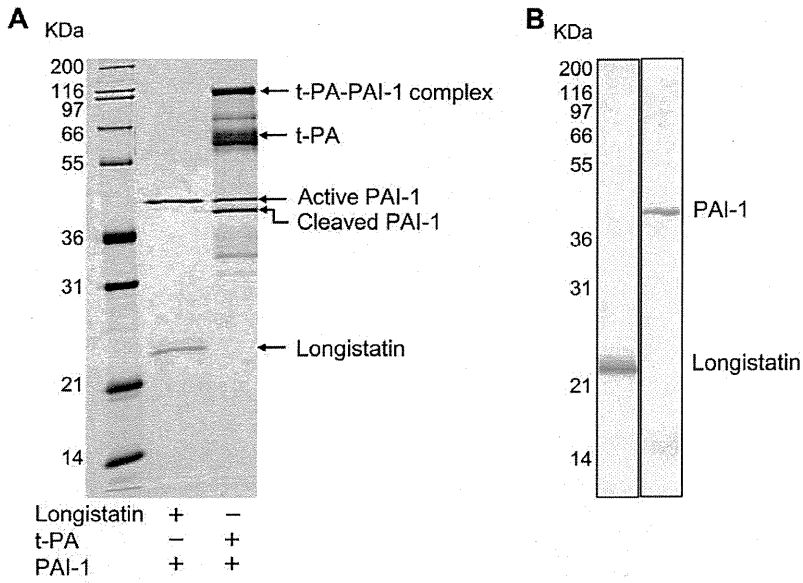

Fig. 4. Detection of enzyme-PAI-1 complex. (A) Enzyme-PAI-1 complex study by silver staining. PAI-1 was incubated with longistatin or t-PA in an equimolar concentration at 37 °C for 15 min and separated by electrophoresis using 12.5% SDS–PAGE gel under reducing conditions. The gel was stained with silver stain. (B) Enzyme-PAI-1 complex study by immunobloting. PAI-1 was incubated with longistatin or t-PA following the same procedures as described above and separated by electrophoresis using 12.5% SDS–PAGE gel under reducing conditions. Proteins were transfer onto nitrocellulose membranes and probed with either mouse anti-longistatin (1:200) or anti-PAI-1 (1:500).

To test our foresaid hypothesis, we treated longistatin directly with activated PAI-1 in different concentration for long period of time. We clearly observed that longistatin exhibits significant resistance to the purified and activated human PAI-1. Generally, PAI-1 level in plasma is 5–20 nM which is sufficient to inhibit physiologic PAs and efficiently prevents the generalized activation of plasminogen [1,6,7]. In fact, physiologic level of t-PA is circulated as PAI-1-t-PA complex in the body; thus, PAI-1 plays crucial roles in the maintenance of homeostasis between haemostasis and fibrinolysis [1,7,20]. During thrombogenesis, since the PAI-1 level is greatly increased, the plasminogen activation capability of exogenous PAs and subsequent thrombolysis is significantly hampered. Our longistatin can tolerate ~90 nM of PAI-1, whereas t-PA and u-PA are almost completely inhibited by 5 nM PAI-1. Furthermore, both t-PA and u-PA are rapidly (within 5–15 min) inhibited by PAI-1 (5 nM) but longistatin can tolerate the upper physiological level of PAI-1 (20 nM) for a significantly longer period of time (>3 h). Notably, longistatin completely hydrolyzed fibrin clot by activating plasminogen in the presence of an amount of active PAI-1 which completely abolished the fibrinolytic capability of t-PA and u-PA. Our results clearly showed that longistatin can evade the inhibitory effects of PAI-1.

Additionally, t-PA forms an SDS-stable complex at ~110 kDa level but we could not detect any complex between PAI-1 and longistatin, which further validates our hypothesis that longistatin is resistant to the inhibitory effect of PAI-1. PAI-1 binding to the target physiologic PAs is mediated mainly by the formation of salt bridges between the series of positively charged residues (variable region 1, VR-1) in PAs with the complementary series of negatively charged residues in PAI-1. Deletion of the residues ^{296}KHRRSPG^{302} or ^{179}RHRGGS^{148}, the VR-1 of human t-PA and u-PA, respectively, renders the proteases resistant to PAI-1 inhibition. Furthermore, chicken u-PA (ch-uPA) that does not contain PAI-binding site (VR-1) is refractory to the inhibition by human PAI. VR-1 of PAs is not a general serpin binding site rather it is a specific motif of PAs for the interaction with PAI-1 [22,23]. In fact, one serpin can inhibit several enzymes but each serpin is expected to have a target protease (since a particular phenotype develops due to the deficiency of a particular serpin). The ability of a serpin to neutralize a given target protease more specifically and more rapidly in a complex mixture of numerous proteases (as in plasma) depends on the interaction between one/more secondary binding site(s) of the serpins and complementary region of the protease. For example, α_2-antiplasmin can inhibit many serine proteases but plasmin is recognized as the main target due to a lysine binding site located in the kringle 4 of plasmin to which carboxy-terminal lysines of α_2-antiplasmin interact [1,7,23]. Similarly, the 60-loop of thrombin contributes to the specific interaction with anti-thrombin III [24]. Unlike human t-PA and u-PA, longistatin does not contain a series of such positively charged residues [15]. Therefore, substantial tolerance of longistatin to the rapid and irreversible inhibitory effect of PAI-1 is not unexpected. Collectively, our results suggest that longistatin is resistant to PAI-1 and maybe an interesting tool for the development of modern, PAI-1 resistant therapeutic agent for the efficient medication of occlusive cardiovascular events.

Acknowledgments

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Interleukin-4 (IL-4) and IL-13 Suppress Excessive Neutrophil Infiltration and Hepatocyte Damage during Acute Murine Schistosomiasis Japonica

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Due to the importance of neutrophils and proinflammatory cytokines in schistosomal liver damage, we analyzed the mechanisms underlying neutrophil and proinflammatory responses in murine schistosomiasis japonica. We found that granulomatous inflammation around parasite eggs in the liver was greater in *Schistosoma japonicum*-infected IL-4$^{-/-}$ IL-13$^{-/-}$ (double-knockout [DKO]) mice than in infected wild-type (WT) mice at 6 weeks, but not at 8 weeks, postinfection, suggesting the importance of Th2 responses in these typical hepatic lesions. Infected DKO mice also showed increased neutrophil infiltration accompanying more severe pathology, as shown by the enhanced necrosis of hepatocytes. This was not likely due to a Th1/Th2 imbalance, because there was no detectable increase in gamma interferon (IFN-γ) production in these DKO mice. mRNA expression of interleukin-17A (IL-17A), proinflammatory cytokines, and the neutrophil chemoattractant CXCL2 in liver was higher in infected DKO mice than in WT mice. However, in IL-4$^{-/-}$ IL-13$^{-/-}$ IL-17A$^{-/-}$ (triple-knockout [TKO]) mice, the absence of IL-17A was associated with only marginal differences in schistosomal liver damage, suggesting that IL-17A is only partially responsible for neutrophil-driven hepatic damage. Furthermore, the expression of mRNAs encoding proinflammatory cytokines was not under the control of IL-17A in TKO mice. These findings indicate that IL-4 and IL-13 suppress excessive neutrophil recruitment, proinflammatory cytokine production, and hepatic damage during the acute stage of *S. japonicum* infection, suggesting that neutrophils and proinflammatory cytokines are mainly responsible for hepatocyte damage during acute murine schistosomiasis japonica. However, neutrophil induction and the production of proinflammatory cytokines were not due solely to IL-17A.

Schistosomiasis is a major tropical disease caused by trematode parasites of the genus *Schistosoma*. Currently more than 200 million people are infected, with 280,000 deaths reported annually (28). Although two of the three major species of human schistosomes, *S. japonicum* and *S. mansoni*, cause intestinal forms of schistosomiasis, their pathogenetic mechanisms have not been fully clarified. Although host Th responses to parasite eggs are essential for clinicopathological features (6), the quality and quantity of host responses differ for these two pathogens. For example, necrotic lesions are widespread in the livers of hamsters infected with *S. japonicum* but not in those of hamsters infected with *S. mansoni* (45). This may be due to differences in cells composing the cicrum oval lesions in the liver, with a higher ratio of neutrophils observed in animals infected with *S. japonicum* (23, 45) and a higher ratio of eosinophils in granulomas of animals infected with *S. mansoni* (23). Hepatic pathology is more severe in schistosomiasis japonica than in schistosomiasis mansoni, a difference related to necrosis (45). The accumulation of neutrophils may therefore be responsible for necrotic lesions in the liver (21), making it necessary to analyze mechanisms of neutrophil regulation during infection with *S. japonicum*.

The Th2 response is characteristic of allergies and helminth infections, including schistosomiasis (1, 6, 14, 39). In murine schistosomiasis mansoni, the interleukin-4 (IL-4)/IL-13-mediated pathway, which acts via the IL-4 receptor α chain (IL-4Rα) and STAT6 (31, 50), is important for the development of hepatic pathology, since granuloma formation was impaired in *S. mansoni*-infected IL-4$^{-/-}$ IL-13$^{-/-}$ (double-knockout [DKO]), IL-4Rα$^{-/-}$, and STAT6$^{-/-}$ mice but not in IL-4$^{-/-}$ or IL-13$^{-/-}$ mice (11, 25, 27), and mortality rates were higher in DKO than in wild-type (WT) mice (11). IL-4/IL-13 induces alternatively activated macrophages (AAMs), which protect against hepatic damage through egg-induced inflammation, with a mortality rate of 100% in mice without AAMs (LysM^{cre} IL-4Rα$^{-/flox}$ mice) following acute infection with *S. mansoni* (13, 19). Therefore, IL-4/IL-13 is essential for granuloma formation and host survival in acute murine schistosomiasis mansoni. IL-4 and IL-13 were recently shown to suppress excessive airway inflammation and neutrophil accumulation in ovalbumin-induced airway inflammation, as well as to downregulate excessive production of the proinflammatory cytokine IL-17A (18). Further, IL-4 has been found to suppress Th17 differentiation *in vivo* and *in vitro* (17, 32).

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TABLE 1 Primers used in this study

Primer	Sequence	
	Forward	Reverse
β-Actin	GCTCTAGACTTCGAGCAGGAGA	AGGCAGCTCATAGCCCTTCT
IL-4	CATCGGCATTTTGAACGAG	CGAGCTCACTCTCTGTGGTG
IL-5	ACATTGACCGCCAAAAAGAG	ATCCAGGAACTGCCTCGTC
IL-13	GGTCCACACAGGGCAACT	AATAAGATCAAGAAGAAATG TGCTCAA
IFN-γ	GGAGGAACTGGCAAAAGGAT	TTCAAGACTTCAAAGAGTCT GAGG
IL-10	CAGAGCCACATGCTCCTAGA	TGTCCAGCTGGTCCTTTGTT
IL-17A	CAGGGAGAGCTTCATCTGTGT	GCTGAGCTTTGAGGGATGAT
IL-17F	CAAGAAATCCTGGTCCTTCG	GAGCATCTTCTCCAACCTGAA
IL-22	TTTCCTGACCAAACTCAGCA	TCTGGATGTTCTGGTCGTCA
IL-6	GCTACCAAACTGGATATAATC AGGA	CCAGGTAGCTATGGTACTCC AGAA
TNF-α	TGCCTATGTCTCAGCCTCTTC	GAGGCCATTTGGGAACTTCT
G-CSF	GCTGCTGGAGCAGTTGTG	GGGATCCCCAGAGAGTGG
CXCL2	AAAATCATCCAAAAGATACTG AACAA	CTTTGGTTCTTCCGTTGAGG
S100A8	TGCGATGGTGATAAAAGTGG	GGCCAGAAGCTCTGCTACTC
S100A9	CACCCTGAGCAAGAAGGAAT	TGTCATTTATGAGGGCTTCA TTT

Although IL-4 and IL-13 are necessary for granuloma formation and host survival in murine schistosomiasis mansoni, the function of this pathway in murine schistosomiasis japonica remains unresolved. We hypothesized that IL-4 and IL-13 suppress hepatic granulomatous inflammation by downregulating excessive neutrophil accumulation associated with the production of proinflammatory cytokines during *S. japonicum* infection. We have therefore focused on mechanisms regulating neutrophil infiltration and the production of proinflammatory cytokines that are directly or indirectly related to IL-4/IL-13.

MATERIALS AND METHODS

Animals and parasite infection. BALB/c (WT) mice were purchased from CLEA Japan (Tokyo, Japan). BALB/c IL-4$^{-/-}$ IL-13$^{-/-}$ (DKO) mice (35) and BALB/c IL-17A$^{-/-}$ mice have been previously described (37). IL-4$^{-/-}$ IL-13$^{-/-}$ IL-17A$^{-/-}$ (triple-knockout [TKO]) mice were produced by crossing DKO and IL-17A$^{-/-}$ mice. Six- to 9-week-old mice were percutaneously infected with 30 *S. japonicum* cercariae (Japanese Yamanashi strain, maintained in our laboratory using *Onchomelania hupensis nosophora*). At 6 and 8 weeks later, the mice were sacrificed and perfused from the portal vain, and recovered worms were counted. Liver tissue and spleens were dissected and used for analysis. Samples of liver and intestine were digested in 4% KOH at 37°C, and the numbers of eggs were quantified by microscopy, followed by calculation of the egg burden in tissues and egg production per female adult worm.

Animal care was in accordance with the guidelines of and approved by the Committee of Animal Ethics of Tokyo Medical and Dental University (0110038A).

Preparation of liver and spleen cells. Spleen cells were prepared as described previously (3). Isolated liver tissue was briefly minced, passed through 200-μm steel mesh, and digested in 0.5 mg/ml collagenase (Wako

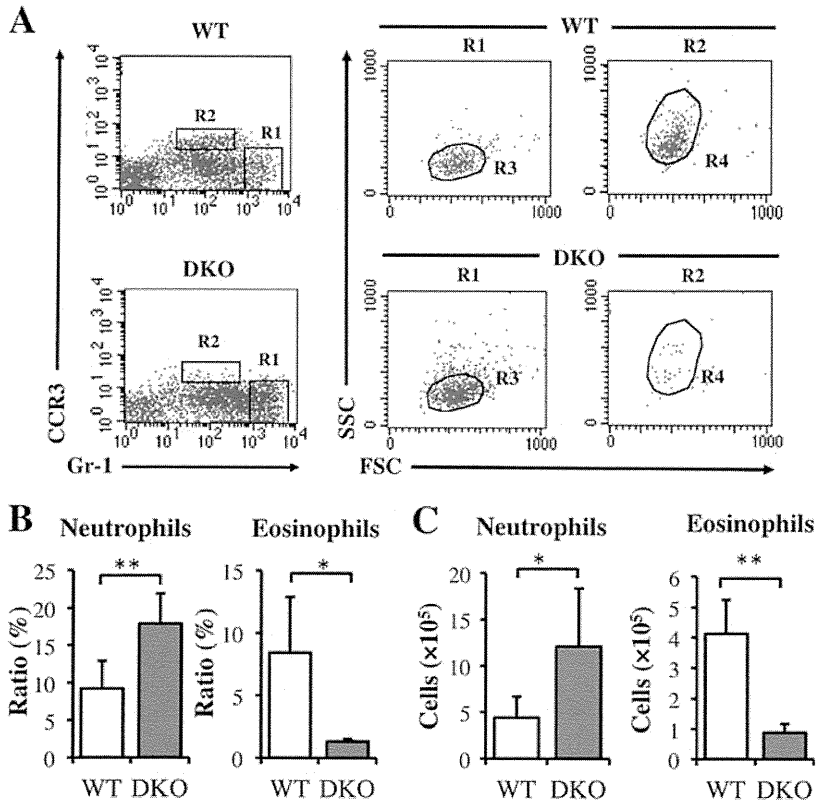

FIG 1 Upregulation of neutrophil recruitment and downregulation of eosinophil recruitment in DKO mice at 6 weeks after infection with *S. japonicum*. (A) Liver cells isolated from WT and DKO mice at 6 weeks after infection were stained with anti-Gr-1 and anti-CCR3 MAbs, followed by gating on FSC/SSC parameters. R1 shows Gr-1^{high} CCR3^{-}, and R2 shows Gr-1^{+} CCR3^{+}. Gr-1^{high} CCR3^{-} SSC^{int} (R3) cells represent neutrophils, and Gr-1^{+} CCR3^{+} SSC^{high} (R4) cells represent eosinophils. (B and C) Average populations (B) and numbers (C) of neutrophils or eosinophils from infected WT and DKO mice. Means and standard deviations (SD) are shown (n = 4 to 6). Naïve, noninfected mice; infection, infected mice. *, $P < 0.05$; **, $P < 0.01$.