Table 2. Experimental infection of rats with metacercariae of *P. ohirai*

Rat No.	Source of mc[a)b)]	Dose of mc	Duration of infection (days)	Rate of mc recovered as adult flukes (%)	No. of adult flukes recovered		
					Total	Pleural cavity	Lungs
1	Yahiro	20	42	15	3	0	3
2	Yahiro	20	70	75	15	1	14
3	Yotsugi	20	42	25	5	0	5
4	Nishi-Kasai	20	47	45	9	1	8

a) Location of Arakawa River site where metacercariae-infected crabs were isolated.
b) Metacercariae.

```
001:TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAGCA: TKO
001:-------------------------------------------------------------: TNE

061:TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG: TKO
001:---------------.....................................................: TNE

                   *
121:TCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT: TKO
045:.....................................................................: TNE

181:GATTTCCCCAATCTGACCATGTGTTGGTGGGGTGCCAGATCTATGGCGTTTCCCTAACCT: TKO
105:.....................................................................: TNE

241:ATCCGGGCGTACCCATGTTGTGGCTGAAGGCCTTGGTGGGGATGTGGCAACGGAATCGTG: TKO
165:.....................................................................: TNE

301:GCTCAGTGGATTATTTATGTGCGCGTTCCGTTGTCCTATCATCATCTATGGTTGATGCTG: TKO
225:.....................................................................: TNE

                                                          *
361:CGCATGGTGTGCGTCCGACGCCAACCTACGTATGGGCCGTGTGGCTCATTCTCCTGACCT: TKO
285:.....................................................................: TNE

421:CGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA: 463: TKO
345:.....................----------------------: 363: TNE
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Fig. 4. Sequence of the ITS2 region. The nucleotide sequence of the ITS2 (plus flanking region) obtained from the metacercariae in this study (TKO) was aligned with the ITS2 sequence from *P. ohirai* isolated from the Tanegashima Island of Kagoshima Prefecture, Japan (TNE, accession number: U96911). Identical bases are represented by dots. A hyphen indicates no sequence data. An asterisk denotes the putative ITS2 origin and terminus, thus the ITS2 5' and 3' sequence termini are within the 5.8S and 28S rDNAs, respectively. The numbers refer to the alignment positions.

282 μm, respectively, with an average of 302 × 232 μm. All larvae had a stylet in the oral sucker and red granules in the body (Fig. 2B).

Infection of rats with metacercariae and morphology of adult worms: We necropsied 4 rats 42 to 70 days after inoculation with *Paragonimus* metacercariae collected from 3 locations along the Arakawa River (Table 2). The lungs and pleural cavities of infected rats contained from 3 to 15 adult worms with an average of 8.0. The average size of 20 mounted adult worms from the 4 rats was 7.4 × 3.7 mm, ranging from 6.4 to 8.8 mm in length and from 2.9 to 5.0 mm in width. The ratio of body length to width ranged from 1.5 to 2.4, with an average of 2.0. The average transverse diameters of the oral and ventral suckers were 616 and 792 μm, respectively. The ventral sucker was always larger than the oral sucker and was situated somewhat anterior to the middle of the worm body. The ovary was intricately ramified with a coral-like appearance (Fig. 3A). The seminal receptacle was small but filled with spermatozoa. The uterus was situated on the opposite side of the ovary. The testes, situated on both sides of the posterior part of the body, were larger than the ovary. The cuticular spines were arranged in groups (Fig. 3B).

ITS2 sequence analysis: We sequenced the ITS2 region of the ribosomal DNA from 12 metacercariae. Alignment of these data revealed that all of the ITS2 regions were 463 bp, with no variation in length and composition among the specimens (Fig. 4). Searches of nucleotide databases showed that the ITS2 (plus flanking region) sequence was identical to that of ITS2 from *P. ohirai*, which was isolated from the Tanegashima Island in Kagoshima Prefecture, Japan (GenBank/EMBL/DDBJ accession number: U96911) [1].

DISCUSSION

In Japan, the lung flukes, *P. westermani, P. miyazakii* and *P. ohirai*, have caused symptomatic illness in humans and/or animals. These species can be differentiated by morphological differences seen in fresh metacercariae and mounted adult worm specimens [7, 8].

The *Paragonimus* metacercariae obtained in this study had thin outer and thick inner cyst walls. The stylet in the oral sucker and red granules in the body were identified in all larvae examined. The adult worms from infected rats had intricately ramified ovaries and cuticular spines arranged in groups. These morphological features are consistent only with those of the metacercarial and adult stages of *P. ohirai* [2, 11, 12]. Thus, the *Paragonimus* metacercariae found in this investigation were all identified as *P. ohirai*. The ITS2 sequence data support this conclusion.

About 50 years ago, sporadic *P. ohirai*-infection was identified in a stray dog that lived for 8 months and died in Bunkyo Ward, Tokyo [4] (Fig. 1). However, the source of this canine infection was not determined and since then, *P. ohirai* infection of its second intermediate crab host in Tokyo has not been documented. The data reported here imply that this dog might have become infected with *P. ohirai* by eating crabs from the banks of the Arakawa River.

Though the Arakawa River flows through the densely populated Tokyo metropolitan area, the natural features and abundant greenery of its lower part are protected and promoted. The riverside provides an ideal site for recreation and people often walk their dogs along its banks. In fact, while collecting crabs, we observed dogs with collars running off leash at or near the water's edge. Since we found a high prevalence of *P. ohirai* metacercariae in crabs at many locations along the Arakawa River, dogs in this area might become infected through consuming these crabs. Therefore, dog owners must be persistently educated about the benefits of obeying local regulations when using riverbanks and put dogs on a leash to prevent canine exposure to *P. ohirai*.

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Laboratory and Epidemiology Communications

Changing Epidemiology of Angiostrongyliasis Cantonensis in Okinawa Prefecture, Japan

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Okinawa Prefecture experienced an outbreak of angiostrongyliasis in January of 2000 (1). The origin of the infection's outbreak could not be identified. We examined the past records of *Angiostrongylus cantonensis* (*Ac*) infection outbreaks and investigated the current distribution of *Ac*'s intermediate and paratenic hosts with infective third-stage larvae in Okinawa. In order to find the infective larvae of *Ac* in the giant African snail, *Achatina fulica*, the pallial organ (lung) of the snail was compressed between two glass plates and examined under a microscope (2) (Figs. 1A, 1B). In other host animals, the whole body was digested in artificial gastric juice (1% pepsin/1% HCl), and the digested material was allowed to sediment; the sediment thus formed was then examined microscopically. In particular, albino rats were given larvae from *Platydemus manokwari* (Fig. 2) and *Parmarion martensi* (Fig. 3) orally with the specimen, and identification was made based on the morphology of the adult *Ac* recovered at 59 days post-inoculation.

As shown in Table 1, the *Ac* epidemic showed different features before and after 1990. Before 1990, 17 in 21 (80%) of the infections were traced to their infection sources, while after 1990 only 2 in 14 could be traced to its source. The infection was more frequent (15/21) in April-November before 1990, although it was more frequent (11/14) in December-

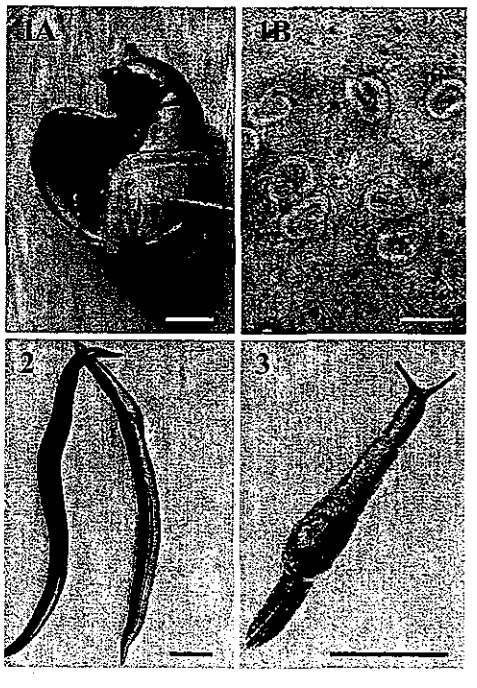

Fig. 1A. *Achatina fulica* (the giant African snail), the pallial organ (lung) is shown. Scale: 1 cm.
Fig. 1B. I nfective third-stage larvae of *Ac* in the tissue. Scale: 100 μm.
Fig. 2. *Platydemus manokwari*; newly discovered paratenic host of *Ac* in Okinawa. Scale: 1 cm.
Fig. 3. *Parmarion martensi*; newly discovered intermediate host of *Ac* in Okinawa. Scale: 1 cm.

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March after 1990.

Table 2 shows the *Ac*-positives among the intermediate and paratenic hosts collected in the present and in past field surveys. In the 2000s, among six intermediate host species that were positive in the 1970s, *Satuma mercatoria*, *Acusta despecta*, and *Bradybaena circulus* became negative, and the positive rate of *A. fulica*, the prevailing host, that was 39% in the 1970s decreased to 10% in the 2000s. Meanwhile, *P. martensi* whose presence was not recognized in the 1970s became prevalent in the 2000s, particularly in the northern part of the mainland of Okinawa, and its infection rate was as high as 20.3%. *P. manokwari*, whose presence in Okinawa was noticed recently

Table 1. Epidemiologic characteristics of angiostrongyliasis cantonensis in Okinawa, 1969-1989 and 1990-2000

Characteristics	1969-1989 (20 years)	1990-2000 (10 years)
Human cases	21	14
Age range (mean) (y)	1-68 (32.4)	11-62 (26.0)
Ratio, Male to Female	1:1.25	1:1.8
Suspected place of infection (%)		
Okinawa Island	14 (66.7)	14 (100)
Miyako Island	6 (28.6)	0 (0.0)
Unknown	1 (4.8)	0 (0.0)
Suspected source of infection (%)		
Eating host with the infective larva	12 (57.1)	0 (0.0)
Achatina fulica	6 (28.6)	0 (0.0)
Veronicella alte	3 (14.3)	0 (0.0)
Bufo asiaticus	3 (14.3)	0 (0.0)
Handling of *Achatina fulica*	5 (23.8)	1 (0.7)
Crushing with the bare hand	1 (4.8)	1 (0.7)
Handling with the bare hand	3 (14.3)	0 (0.0)
Swallowing by the play	1 (4.8)	0 (0.0)
Ingestion of a fresh vegetable	0 (0.0)	1 (0.7)
Unknown	4 (19.0)	12 (85.7)
Occurrence time (%)		
Apr.–Nov. (Active period of *A. fulica*)	15 (71.4)	3 (21.4)
Dec.–Mar. (Non-active period of *A. fulica*)	3 (14.3)	11 (78.6)
Unknown	3 (14.3)	0 (0.0)

(Ref. 6)

Table 2. Surveys of the host animals of *Angiostrongylus cantonensis* infective larvae in Okinawa, the 1970s and the 2000s

Species	The 1970s[1)] Infected/Examined (%)	The 2000s Infected/Examined (%)
Intermediate hosts		
Terrestrial snails		
Achatina fulica	1,049/2,683 (39.1)	222/2,189 (10.1)
Satsuma mercatoria	36/240 (15.0)	0/139 (0.0)
Acusta despecta	3/427 (0.7)	0/904 (0.0)
Bradybaena circulus	3/448 (0.7)	0/53 (0.0)
Cyclophorus turgidus		0/529 (0.0)
Parmarion martensi	**	153/753 (20.3)
Aquatic snail		
Ampullarium sp.	**	0/3,764 (0.0)
Slugs		
Veronicella alte	76/347 (21.9)	108/783 (13.8)
Limax valentianus	9/50 (18.0)	3/78 (3.8)
Meghimatinum confusum	0/24 (0.0)	0/95 (0.0)
Paratenic hosts		
Land planarian		
Platydemus manokwari	**	227/1,613 (14.1)
Amphibians		
Bufo asiaticus	37/108 (34.0)	1/18 (5.6)
Bufo marinus	0/37 (0.0)	0/6 (0.0)
Rana catesbeiana	7/44 (15.9)	*
Rhacophorus leucomystax	1/4 (25.0)	*
Rana limnocharis	1/8 (12.5)	*

*: Not examined, **: Not discovered from the field.
1): Based on references 7, 8.

in 1993 (3), also showed a high infection rate of 14.1%.

Ac infection is mediated not only by ingestion of the *Ac*-carrier intermediate or paratenic hosts but also through the ingestion of vegetables, drinking water, and by contact with fingers that are contaminated by the infective larvae of *Ac* (4).

In the outbreak in January 2000 in Okinawa that involved seven patients, the clinical symptoms and immunological reactions were too weak for typical angiostrongyliasis and were somewhat similar to those of infection by low doses of *Ac* larvae. In the epidemic, larvae-contaminated fresh vegetables such as lettuce and cabbage were suspected as the source of infection. The first *Ac* infection case via a fresh vegetable salad had been reported for a patient who developed symptoms 7 days after a short trip to Okinawa in December 1999 (5). Among the 52 *Ac* cases reported so far in Japan, 35 were from Okinawa Prefecture (6).

As already indicated, the peak season of infection was displaced from April-November to December-March, and tracing the infection to its source has become more difficult in recent years. The displacement of the outbreak season coincided with the decline in the infection rate in *A. fulica* that is more active in April-November and the appearance of new hosts *P. martensi* that is more active in the winter season, while *P. manokwari* is active throughout the year. In relation to the displacement of the outbreak season, it should be noted that the harvest season for lettuce is November-May and that for cabbage is November-July.

Ac-infected *P. martensi* and *Veronicella alte* were examined histologically. *Ac* larvae were present in the muscular layer just beneath the body surface of these snails. However, the former species appeared to be more easily infected because of the less dense muscular layer and tended to contain a greater number of larvae. *P. manokwari* is frequently observed adhering to the lower side of cabbage leaves. It is quite possible that the larvae are released from the hosts when sliced together with the cabbage leaves in the preparation of fresh salad.

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喀痰から虫卵が検出され形態と塩基配列から種同定したウェステルマン肺吸虫症の1例

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Key Words：肺吸虫症，ウェステルマン肺吸虫，種同定，塩基配列

緒言

喀痰から肺吸虫卵が検出された症例について虫卵の形態を精査し，また虫卵を出発材料として塩基配列を解読したところ，2倍体型のウェステルマン肺吸虫（以下，ウ肺吸虫）と同定できた。排卵を認める肺吸虫症例は3倍体型のウ肺吸虫を原因とすることが多く[1]，本例は貴重な症例と考えられた。

症例（臨床的事項等は他の肺吸虫症自験例とともに既報[2]）

和歌山県に14年間在住する韓国人女性，44歳。半年間持続した血痰を主訴に受診した。胸部X線とCT検査で右肺上葉に硬化性病変（直径1-2cm，一部で空洞形成を伴う）が認められたため肺吸虫症を疑い，喀痰と糞便を調べて虫卵（図1）が検出されたので本症と確定した。マルチドットELISAで，患者血清はウ肺吸虫抗原と宮崎肺吸虫抗原とに反応したが，発色の程度は前者との方が強かった。プラジカンテルが体重1kgあたり75mgで3日間連続して経口投与された結果，排卵，画像所見および症状は消失・軽快し，治癒と判定された。患者に食歴を尋ねると，発症の約1年前より(モクズガニらしき)淡水産のカニを地元で採集したり，

A Case of Paragonimiasis westermani : Identification of the Causative Parasite as the Diploid Type of *P. westermani* from the Egg Morphology and DNA Sequence

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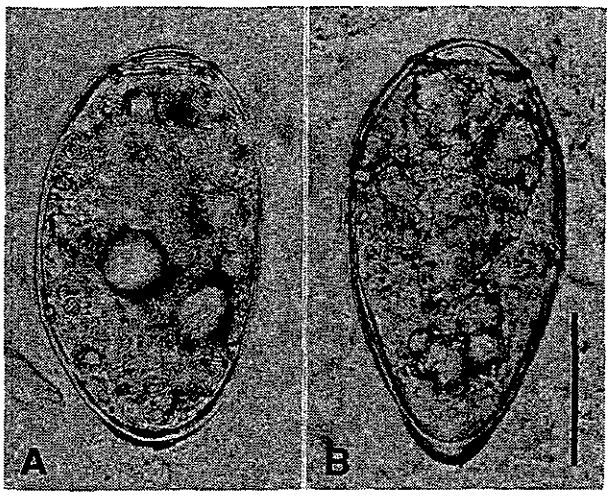

図1　症例の喀痰中に認められた虫卵，強拡大像。最大幅部が中央にあり，かつ無蓋端部の卵殻肥厚が明らかでない虫卵（A）が60%を占め，最大幅部が蓋端側で，かつ無蓋端部に卵殻肥厚を認めた虫卵は20%（B）に過ぎなかった。Bar=30 μm。

あるいは地元の市場で購入し，塩ゆでにして頻繁に摂食したと述べたことから，不完全加熱の淡水産のカニが感染源になったと考えられた。

虫卵の形態学的特徴

虫卵の形態学的特徴から原因虫種を推定するため，YM式喀痰固定液（50%エタノール他，武藤化学）に保存されていた喀痰中の虫卵のうち，形態が良く保持されたもの（20個）について計測・観察した（図1）。まず虫卵の大きさは，長径が77-88 μm（平均83 μm），短径は43-50 μm（平均46 μm）であった。次に最大幅部の位置（卵の幅が最も広い箇所）は，中央が14個（70%），蓋端側（蓋のある側）は6個（30%）であった。また無蓋端部（蓋と反対側）における明らかな卵殻の肥厚（卵殻側部の2.5倍以上とした）は，「認めない」が16個（80%），「認める」は4個（20%）にとどまった。

わが国に分布する人体寄生性肺吸虫は，ウ肺吸虫と宮崎肺吸虫の2種類である。このうちウ肺吸虫は，染色体構成により2倍体型と3倍体型の2タイプに分けることができる[1]。各々の虫卵には種（あるいはタイプ）に特有の形態学的特徴[3]がある。まず3倍体型ウ肺吸虫は，長径が時に90 μmを超え，最大幅部が蓋端側にあって，ほぼ総ての虫卵の無蓋端部に卵殻の肥厚を認め，その程度も顕著なものが多い。これに対して，2倍体型ウ肺吸虫と宮崎肺吸虫とは，虫卵の形態が相互に類似し，長径が3倍体型より10 μm以上も短く，最大幅部は中央にあるものが多い。しかしながら両者は無蓋端部の卵殻肥厚の程度が異なり，宮崎肺吸虫では無蓋端部の卵殻肥厚がないのに対し，2倍体型ではある程度の肥厚を半数以上の虫卵に認める。

本症例の虫卵は，最大幅部が概ね中央部にあり，長径も余り長くなかった。このような虫卵の特徴は，宮崎肺吸虫と2倍体型ウ肺吸虫とに共通するものである。そこで3倍体型ウ肺吸虫である可能性をまず除外した。一方で，無蓋端部における明らかな卵殻肥厚を20%の虫卵に認めた。しかしながらこの結果からは，本症例の原因虫が2倍体型ウ肺吸虫か，宮崎肺吸虫かを判定することが困難であった。そこで分子生物学的手法を用い，検討を加えることにした。

塩基配列の解読と比較検索

虫卵からのDNA抽出に先立ち，実体顕微鏡下に有柄針で虫卵を圧し，卵殻を破綻させた。処理した虫卵は1個ずつ，マイクロピペットを用いて，極少量の精製水（0.5 μl）とともに速やかにエッペンドルフチューブに移した。以降の手技，すなわちDNA抽出とPCR，塩基配列の解読・解析に関しては，概ね既報に準じた[4]。なお今回は，吸虫類のITS2領域に対してコンセンサスなプライマー（3SおよびA28）を用い，PCRと配列解読を行った。

卵殻を破綻させてDNA抽出を行うと，虫卵1個ずつからでも，電気泳動で明瞭な充分量の産物がPCR増幅された。ゲルからバンドを切り出して，塩基配列を解読・解析したところ，PCR産物は463 bpで，しかも2倍体型ウ肺吸虫の配列（U96907）と完全に一致した。一方，宮崎肺吸虫（U96912）とは92.6%の相同性に留まった。以上の結果から，本症例の原因虫を2倍体型ウ肺吸虫と判定した。

考察

わが国に分布する人体寄生性肺吸虫のうち，3倍

体型ウ肺吸虫は肺内に虫嚢を形成して産卵するのに対し，2倍体型ウ肺吸虫と宮崎肺吸虫とは肺に定着せず，胸腔内を移行して胸膜炎を引き起こすと説明されてきた[3]。しかしながら，宮崎肺吸虫が肺の虫嚢（画像では結節状陰影）に定着し，排卵を認める症例も報告されるようになってきた[5)6)]。相当数の虫体が寄生し，しかも長期間駆虫されない場合に，宮崎肺吸虫の感染でもこのような病態が発現すると考察されている[5]。ステロイド投与も関与すると推察されている[6]。本症例では，カニを繰り返し食べることで相当数のメタセルカリアが取り込まれたと考えられ，しかも血痰から半年後にようやく通院して投薬治療が開始されている。このような場合に，2倍体型ウ肺吸虫でも，宮崎肺吸虫と同様に，虫体が肺の虫嚢で同棲して成熟し，排卵を認めるようになると考えられた。わが国に分布する人体寄生性肺吸虫は，いずれも喀痰や糞便に虫卵を排出させることが示唆されたので，症例から得られた肺吸虫卵は，形態を精査し，また遺伝子配列を解析して，種（およびタイプ）を判定することが望まれる。

寄生虫卵を用いた塩基配列解読に関しては，線虫卵1個を出発材料としても可能であることが報告され[7]，また条虫卵でも可能であると示唆された[7]。一方，吸虫卵に関しては，肺吸虫卵を用いた成績がその後に報告された[8]。これらの方法を実験動物由来の宮崎肺吸虫卵を用いて追試したが，時にPCR増幅が上手くいかないことを経験した。そこで卵殻を破綻させてDNA抽出を行ったところ，配列解読にも充分な量の産物が，より確実にPCR増幅されることが分かった（杉山，他，未発表）。今回はこの方法をエタノール等で処理された人体症例由来の虫卵に適用した。その結果同様に，充分量の産物がPCR増幅され，さらに塩基配列も解読できた。肺吸虫卵を材料としてPCRを行う場合，増幅をより確実とするために試みるべき方法と考えられた。

本邦産人体寄生性肺吸虫に対する淡水産カニの感受性[1]については，サワガニはいずれにも感受性があり，モクズガニはウ肺吸虫（2倍体型・3倍体型）に感受性があることが記されている[1]。実際に和歌山県では，モクズガニでの肺吸虫寄生は見出されていないが，サワガニからは宮崎肺吸虫のメタセルカリアが検出されている（隣県の三重および奈良のサワガニからは2倍体型ウ肺吸虫も検出されている）[1]。和歌山県在住者の肺吸虫症例[2]の中には，県内のサワガニで宮崎肺吸虫に感染したものも含まれていた[9]が，今回報告した症例は，上に述べた疫学的な背景から，県外に由来する淡水産のカニで，恐らく市販されていたものが感染源になったのではないかと考えられた。

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Immunogenetic analysis of post-schistosomal liver fibrosis

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Abstract

Schistosomiasis is a major endemic parasitic disease in the world. In China, we have identified two major genes related to the severity of liver fibrosis, one an HLA class II gene, and the other the IL-13 gene. The frequency of the HLA-DRB5*0101 allele and that of the IL-13 promoter A/A (IL-13P- A/A) genotype were elevated in fibrotic patients, although the two genes are located on different chromosomes, chromosomes 6p and 5q, respectively. Subjects with both genotypes had odds ratios (OR=24.5) much higher than the sum of the ratios for each individual genotype (OR=5.1, 95% confidence interval 1.3–24.7 for HLA-DRB5*0101, OR=3.1 95% CI 1.5–6.5 for IL-13P- A/A). Although we have not yet characterized the functional difference between HLA-DRB5*0101 and other alleles, peripheral blood mononuclear cells from IL-13PA/A donors produced much higher amount of mRNA than IL-13PA/B 24 h after the stimulation with PHA. Those findings strongly suggest that the pathogenic Th2 response directly influences the prognosis of post-schistosomal liver fibrosis.

Keywords: *Schistosoma japonicum*; Cytokine; HLA; Single nucleotide polymorphism; Microsatellite; Liver fibrosis

1. Introduction

One of the important subjects in the biomedical research field is the analysis of human reactivity to exogenous stress. Exogenous stress in the infectious diseases is usually one species of microorganism that is called pathogen. The reactivity of the human host is so variable that wide range of clinical spectrum spanning from asymptomatic to grave is shown in almost all infectious diseases as shown in SARS. The reason why such a wide range occurred is that many factors are involved in the pathogenesis during the infection including host and parasite genetic factors (multifactorial diseases). Usually this multifactorial disease is very difficult to analyse, because to identify several responsible genes to develop the disease, statistical approach is necessary using linkage study or population based case–control study which needs a number of subjects. After you get an appropriate quality and quantity of subjects or family members, two different genetic approaches are generally undertaken to identify the responsible genes: targeted gene analysis and genome-wide survey. We adopted the former method, focusing especially on immunity-related genes such as those for HLA, cytokines and adhesion molecules.

HLA genes are highly polymorphic and their alleles are well characterized at the DNA sequence level. For example, the HLA-DRB1 gene has over 100 alleles in the human population. In particular,

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HLA-DR, -DQ, -DP, -A, -B, and -C are believed to function as immune-response genes that are known to control host immune reactivity to specific antigen of T cells and NK (T) cells. Cytokines are also believed to play important roles in controlling the intensity and duration of the immune response. Recently, single nucleotide polymorphisms (SNPs) have been observed very commonly in the promoter regions of some cytokines, including tumour necrosis factor (TNF), interleukin (IL)-4, IL-13, and interferon-γ. Several studies have demonstrated that these polymorphisms directly affect promoter activity. Therefore, we focused on those supposed to be very strong candidate genes for the search to pathogenic or resistant genes to post-schistosomal liver fibrosis in humans.

2. HLA class II genes and the IL-13 promoter alleles genetically interacted to enhance the development of post-schistosomal liver fibrosis

Schistosomiasis japonica is a chronic helminthic infectious disease that affected at least 860 000 individuals in China in 1995. Morbidity and mortality are dependent on its chronic sequelae, post-schistosomal hepatosplenic disease, which is characterized by liver fibrosis, portal hypertension, ascites accumulation, oesophageal varices, and eventual death. The liver fibrosis seen in these patients is induced by a granulomatous immune response against the eggs that are deposited in the periportal area [1]. Schistosomal egg antigen-specific $CD4^+$ T cells play a major role in the formation of granuloma through Th2-type cytokine production in experimental schistosomiasis mansoni [2,3]. However, in humans, little is known about the immunological response during the chronic phase of hepatosplenic disease [4]. Because only 5–10% of patients with chronic schistosomiasis japonica develop hepatosplenic disease, and because the granulomatous response is initiated by $CD4^+$ T cells reactive to schistosomal antigen, polymorphisms of the HLA class II antigens, which control the reactivity of the $CD4^+$ T cells, may be associated with a susceptibility to hepatosplenic disease. Indeed, associations between schistosomal hepatosplenic disease and HLA alleles have been reported for schistosomiasis mansoni [5,6] and for schistosomiasis japonica [7,8]. Recently, more objective diagnostic methods using ultrasonography have become popular and have been standardized to measure changes in liver morphology [9]. Therefore, we used this method to categorize the patients into a 'fibrotic' group and a 'non-fibrotic' group, and examined their genetic characteristics by analysing the polymorphisms of candidate genes encoding HLA class II and class I antigens, TNF-α and cytokines.

A total of 230 current or former patients with chronic schistosomiasis japonica were examined for clinical changes. All patients were from the agricultural village of Beishan, in Yushan County, China, and had their first episode of infection and treatment at least 10 years before the initiation of this study in 1994. The mean age of the subjects was 52.6 ± 10.5 years and the mean duration between 1994 and their initial treatment year was 27.4 ± 8.8 years. Ultrasonographic diagnosis was carried out according to the WHO standard for the diagnosis of liver fibrosis due to schistosomiasis japonica [9–11]. Ultrasonographic diagnosis determined that there were 44 persons with grade 0 fibrosis, 81 with grade I fibrosis, 99 with grade II fibrosis, and six with grade III fibrosis. The presence of hepatitis B virus (HBV) was not assessed in these patients, but the prevalence of HBV is approximately 15% in Jiangxi Province [12]. Most of the men in the village smoke tobacco and drink alcoholic beverages, but the women generally do not. The patients had all been treated after each positive faecal examination throughout their lives, but it was not possible to estimate the precise total worm burden of each patient during the clinical course of the disease. Therefore, we tentatively defined an appropriately exposed person as a repeatedly treated for schistosomiasis japonica over a 10-year period [13].

The frequencies of several HLA class II alleles [14] were significantly increased or decreased in the fibrotic groups. When we compared the frequencies of alleles between grade 0 and grades I, II, and III, we found that HLA-DRB1*1101 ($P < 0.001$), DQA1*0501 ($P < 0.02$), and DQB1*0301 ($P < 0.03$), which are closely linked, were significantly elevated in the grade 0 group, and that

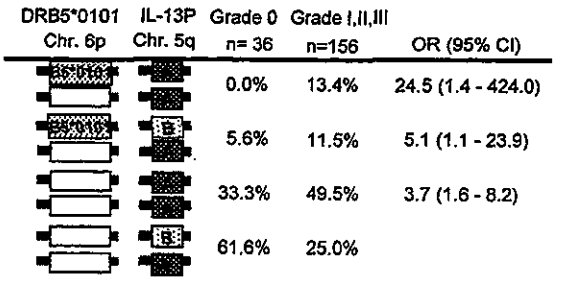

Fig. 1. Synergistic effect of the two susceptibility markers, HLA-DRB5*0101 and IL-13P- A/A. OR and 95% CI were calculated relative to individuals negative for both DRB5*0101 and IL-13P- A/A.

HLA-DRB5*0101 was significantly elevated in grade I, II, and III fibrotic patients ($P<0.03$). This suggests that the HLA-DRB1*1101–DQB1*0301–DQA1*0501 haplotype ($P<0.02$) decreases susceptibility to grades I, II, and III fibrosis, whereas the HLA-DRB1*1501–DRB5*0101 haplotype ($P<0.02$) increases this susceptibility. If we assume that these genetic associations arise from the functions of the HLA molecules themselves, then the critical question is: how do these molecules present antigens to $CD4^+$ T cells to initiate the immunological processes leading to fibrosis? We have not yet identified any pathogenic or protective T cells via such HLA molecules in the exposed donors.

We further analysed the polymorphisms of the Th2 cytokine genes in the same subjects. There was a significant association between IL-13 gene promoter polymorphism and the liver fibrosis group. Because the IL-13 gene is localized on the long arm of chromosome 5, the IL-13P allele must be inherited independently of the HLA class II allele that occurs on the short arm of chromosome 6. Therefore, the next question is whether there is any interaction between these two genetic markers, HLA and IL-13. As shown in Fig. 1, both HLA-DRB5*0101- and IL-13P- A/A-positive subjects had much higher odds ratios (OD=24.5) than subjects positive for only one of these polymorphisms (OD=5.1 for HLA, OD=3.7 for IL-13P-A/A), indicating these two genetic markers synergistically enhance the development of fibrosis after infection. This synergy made us propose this hypothetical story that the pathogenic IL-13- high producer $CD4^+$ T cells are preferentially stimulated by the antigen-presenting cells expressing HLA-DRB5*0101. Actually the in vitro experiment using many healthy volunteers showed that IL-13 mRNA levels of the PHA stimulated T cells from IL-13P A/A genotype donors showed significantly higher than those from IL-13P A/B donors. This strongly supports our hypothesis that IL-13 producing CD4 T cells were pathogenic.

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The miniature pig: a unique experimental model for *Schistosoma japonicum* infection

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Abstract

As part of a search for good animal models for human schistosomiasis, two miniature pigs of the CLAWN strain (C-1, C-2) were inoculated percutaneously with 200 *Schistosoma japonicum* cercariae of the Chinese strain, and the subsequent infection was monitored parasitologically, pathologically and serologically. Egg excretion into feces began at 5 weeks post-infection (p.i.) and became pronounced from 8 weeks to 17–20 weeks p.i. The average number of eggs in 1 g feces of each pig at the peak period between 8 and 20 weeks were 288 and 277, respectively. C-1 and C-2 were killed and perfused at 27 and 47 weeks p.i. and adult worm numbers recovered were 35 and 15, respectively. C-2 had at least four pairs of viable mature worms but no detectable fecal eggs for a month before perfusion, suggesting that any produced eggs were not excreted into the feces during this period. Egg deposits associated with inflammatory reactions were observed by histological examination of the liver, spleen, pancreas, mesenteric lymph nodes, lung, and small intestine. This suggests that reduced fecal excretion of eggs into the feces did not correlate to reduced parasite numbers in the chronic phase of schistosomiasis. This is the first report showing the miniature pig to be a potential model for human *S. japonicum* infection.

Keywords: Miniature pig; *Schistosoma japonicum*; Animal model

1. Introduction

Schistosomiasis is one of the major communicable diseases to endanger public health and is of socio-economic importance worldwide. In spite of various efforts to control it, an estimated 200 million people are still infected [1]. In China, the Philippines, and Indonesia, *Schistosoma japonicum* is endemic. Extensive control programs such as snail control, mass chemotherapy, and education have been carried out for over 40 years in these areas and have brought about the control of *S. japonicum* infections in some endemic regions, but large endemic areas still remain in China and the Philippines. In these areas, the presence of reservoir hosts, such as water buffalo and cattle, has made the control more difficult [2,3]. Studies of *S. japonicum* infection in these animals, as well as in man, have been required to overcome this difficulty.

Most of our knowledge about schistosomiasis is drawn from experiments in primates and rodents. Although primates are good hosts for experimental infection [4], the high cost and ethical concerns make them a difficult model for researchers to maintain. The use of rodents has several problems as a model for schistosomiasis. A single worm

Abbreviations: SEA (schistosome soluble egg antigens); SWA (schistosome adult worm antigens); EPG (eggs number per 1 gram feces); MHC (major histocompatibility complex); SLA (swine leukocyte antigen).

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pair in the mouse model affects hepatic blood flow and is said to be equivalent to a burden of approximately 4000 worms in man. The average life span of the schistosomes in humans is longer than that of mice, thereby eliminating their use in studies for long-term effects [5].

Although water buffalo and cattle are the most important reservoir hosts in China, the pig is also known to be another reservoir [2,9]. As an experimental model, it is impractical to use water buffalo and cattle, and, therefore the pig has gained more attention from researchers who are involved in pathology or protective immunity [6–11]. However, the major drawback of standard pigs is the large body mass at 3–4 months old, which reaches more than 100 kg. The schistosome infection needs at least 3 months for researchers to be able to observe its clinical course, and the standard pig's large body size makes handling more difficult compared with other animal models, like rodents. Therefore, smaller pigs, such as the miniature pig, were expected to be ideal. However, Reid and Lichtenberg [12] had already reported that the miniature pig could not serve as an adequate substitute for primates. Following this initial report, miniature pigs have not been used as an animal model. Recently, a new miniature pig strain established in Japan (the CLAWN strain) has become available. Therefore, we evaluated the use of this strain of miniature pigs as a model for human schistosomiasis.

2. Materials and methods

2.1. Experimental animals

The CLAWN strain miniature pigs (Fig. 1) were originally established by Y. Nakanishi during the 1980s and bred in the Japan Farm Clawn Institute (Kagoshima, Japan). Briefly, this pig was developed by crossbreeding two kinds of F1: one is the F1 crossbreed of the Göttingen strain miniature pig and the Ohmini strain miniature pig; and the other is the F1 crossbreed of the Landrace strain pig and the Great Yorkshire strain pig. Generally the weight of the CLAWN miniature pig is approximately 30 kg in the 8-months old and approximately 80 kg in the 24-months old. Two 6-week-old CLAWN strain pigs (C-1 and C-2, 3 kg and 2.5 kg, respectively) were used in this study. The pigs were fed standard nutrient chow based on their body weights, with water ad libitum. The experimental protocol was approved by the Animal Ethical Committee of Nagasaki University.

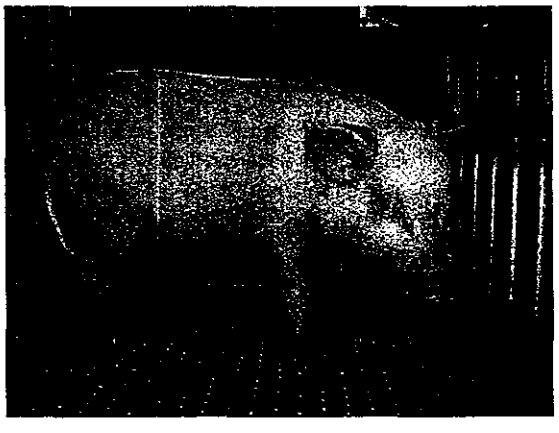

Fig. 1. The CLWAN miniature pig.

2.2. Parasite and parasitological technique

Pigs were percutaneously inoculated with 200 cercariae using a coverslip. The cercariae were shed from infected snails (*Oncomelania hupensis*) with a Chinese strain of *S. japonicum* maintained in the Jiangsu Provincial Institute of Parasitic Diseases Control, Wuxi, Jiangsu Province, People's Republic of China.

Feces were collected every week and the number of eggs excreted into the feces was counted using a method described by Willingham et al. [7]. Briefly, approximately 10 g of feces was homogenized and suspended in 500 ml of 1.2% sodium chloride solution. The feces suspension was poured into a series of three sieves with mesh sizes of 400 μm, 100 μm, and 45 μm, respectively. The residue left on the 45-μm mesh was recovered with 1.2% sodium chloride solution and then centrifuged. One-tenth of the sediment was examined for *S. japonicum* eggs by light microscopy. Eggs per gram of feces (EPG) were calculated from the counts.

2.3. Blood collection

Blood was collected from the auricular vein once a week. Before taking blood, pigs were anesthetized by intramuscular injection with 0.2 mg/kg midazolam (Yamanouchi Pharmaceutical Co. Ltd. Tokyo, Japan) and 40 μg/kg medetomidine (Orion Corp., Espoo, Finland). The ratio of eosinophils number against total white blood cells number were determined on the peripheral blood smear after May-Grünwald Gimsa staining.

2.4. Enzyme-linked immunosorbent assay (ELISA)

An ELISA was performed as described previously [8]. To block the non-specific binding, phosphate buffered saline containing 0.1% blocking agent (Blocking Reagent, 1096176, Roche Diagnostics, Mannheim, Germany) was used.

2.5. Perfusion

S. japonicum adult worms were recovered from the liver and mesenteric veins using a previously described perfusion technique [8]. Pigs were killed by overdose intravenous injection of pentobarbital (30 mg/kg). Heparin sulfate (5000

IU) was also injected intravenously. The thorax and abdomen were cut open by one central longitudinal section from neck to anus. A plastic tube, 1 cm in diameter, was inserted into the descending aorta just above the diaphragm and ligated by silk string just under the renal arteries. Another tube for the flush-out was inserted into the portal vein at the entry to the liver and ligated. Twenty liters of saline containing sodium citrate (15 g/l) was then flushed through the peritoneal vessels. The fluid flushed out of the tube inserted into the portal vein contained the adult worms, and these were captured by the stainless steel mesh. After perfusion, the portal vein was re-examined for residual worms by careful observation.

After recovering the adult worms, the organs were also perfused with periodate–lysine–paraformaldehyde (PLP) solution for fixation. Organs were then excised and immersed in the PLP solution. They were conventionally processed, embedded in paraffin, and sectioned at 3 μm. Sections were stained with hematoxylin–eosin (HE), Masson's trichrome, and the periodic acid Schiff reaction.

A part of liver (left hepatic lobe) was digested in 3% KOH at 37 °C for 24 h. The eggs number in one tenth of the digested fluid was counted to determine the number of eggs per gram of each organ.

3. Results

3.1. S. japonicum infection in miniature pigs

To confirm the establishment of *S. japonicum* infection, fecal egg excretion (EPG) was monitored every week (Fig. 2). Fecal eggs were first detected at five weeks post-infection (p.i.). The kinetics of EPG showed a biphasic pattern. By 8 weeks p.i. in both pigs, EPG increased dramatically to approximately 400. In C-2, EPG increased further to approximately 700 at 10 weeks p.i. The EPG then gradually decreased to approximately 50 at 14–15 weeks p.i. before increasing again. Afterwards, egg excretion persisted at a relatively low level. The eggs excreted at 25 weeks p.i. were still able to hatch in an artificial water pond under light (data not shown). During the infection, the eosinophilia was observed in C-1 at 26 weeks p.i. (9.5%), in C-2 at 13 weeks p.i. (22%).

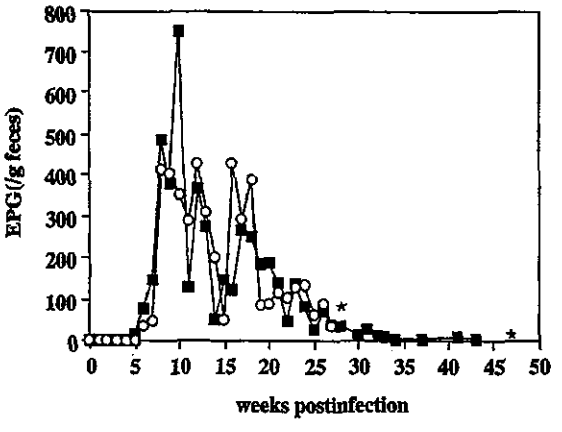

Fig. 2. Fecal egg excretion expressed in eggs per gram of feces of two miniature pigs infected with 200 cercariae of a Chinese strain of *Schistosoma japonicum*. Open circle, data from the C-1 pig. Closed square, data from the C-2 pig. *sacrificed for the perfusion experiment.

C-1 was killed at 27 weeks p.i. to examine the worm recovery. The weight of the pig at the time of killing was 22 kg. The recovered number of worms was 35 (male, 20; female, 13; sex undetermined, 2; 11 paired worms were among these): Thirty-three worms were viable, two worms were dead. The number of eggs in the liver was 361/g liver.

C-2 was killed at 47 weeks p.i. The weight at that time was 42 kg. The adult worms were recovered after portal vein perfusion. The number of worms recovered was 15 (male, 10; female, 5; 4 paired worms were among these). All these worms were alive and all paired female worms had eggs in their uteri. The number of eggs in the liver was 119/g liver.

3.2. Histology

In both pigs, organs (lungs, heart, kidneys, liver, spleen, pancreas, mesenteric lymph nodes, small intestine, large intestine, and brain) were removed and processed for pathological analysis. In C-1 (killed at 27 weeks p.i.), no marked change was macroscopically observed except for moderate enlargement of the mesenteric and portal lymph nodes. Neither apparent fibrosis nor cirrhotic changes were observed in the liver. Histologically, *S. japonicum* eggs were detected in the liver, spleen, pancreas, small intestine, mesenteric lymph nodes, and lungs (Fig. 3a, b, f–h). Deposited eggs were associated with a granulomatous reaction consisting of multinucleated giant cells of foreign body type, epithelioid cells, macrophages, neutrophils, and eosinophils. The granulomatous reaction was surrounded with mild fibrosis and lymphocytic infiltration. The granulomas were localized in and near the portal tract of the liver (Fig. 3a), in the submucosal layer of the small intestine (Fig. 3b), within the peripheral sinus of the mesenteric lymph nodes, and within the vessels of the pancreas and lungs (Fig. 3f, g). Portal–portal bridging fibrosis was occasionally observed in the liver, but lobular disorganization was not apparent.

In C-2 (killed at 47 weeks p.i.), an enlarged spleen and mesenteric lymph nodes, and petechial hemorrhage in the large intestine were observed macroscopically. Histologically, granulomas were found in the liver, pancreas, small intestine, mesenteric lymph nodes, and lungs. The granulomas were relatively small and mainly consisted of epithelioid cells and fibroblasts without being surrounded by eosinophils and lymphocytes (Fig. 3d).

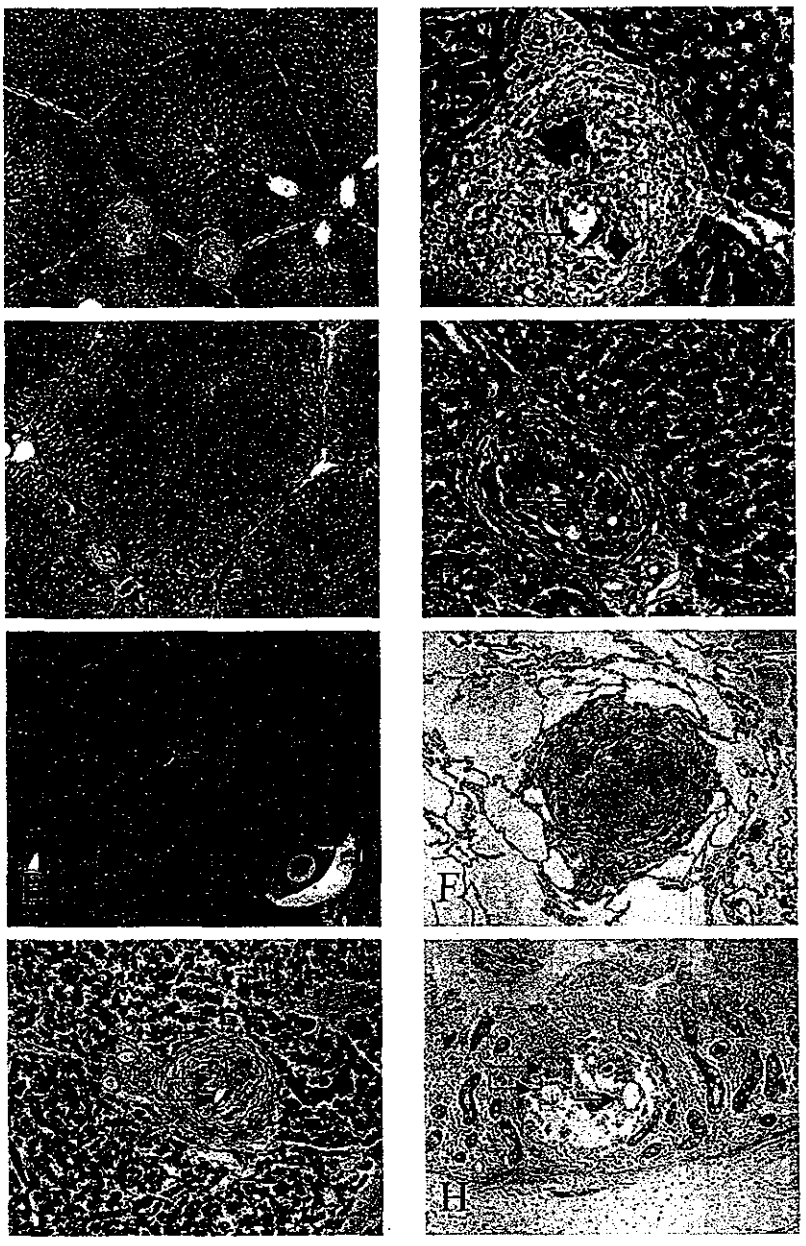

Fig. 3. (a) Liver (C-1), Masson's trichrome stain, × 100 magnification. There were two granulomas in the portal areas (arrowed). The eggs are associated with a granulomatous reaction and fibrotic changes. (b) Liver (C-1), Hematoxylin–eosin stain, × 200 magnification. The eggs (arrowed) were surrounded by epithelial cells, fibers and eosinophils. The eggshells still remained visible. (c) Liver (C-2), Masson's trichrome stain, × 100 magnification. There was a granuloma in the portal areas (arrowed). (d) Liver (C-2), Hematoxylin–eosin stain, × 200 magnification. The eggs (arrowed) were surrounded by mainly epithelioid cells and fibers. There was no infiltration of eosinophils and lymphocytes. The size of the granuloma was relatively small compared to C-1 (Fig. 2a). (e) Liver (naïve, non-infected miniature pig liver) Masson's trichrome stain, × 100 magnification. (f) Lung (C-1), Hematoxylin–eosin stain, × 200 magnification. Foreign body granuloma formation in the interstitium. An egg might be in the center of the granuloma. (g) Pancreas (C-1), Hematoxylin–eosin stain × 100 magnification. The eggs (arrowed) were surrounded by epithelial cells and fibers. (h) Intestine (C-1), Hematoxylin–eosin stain, × 100 magnification. Foreign body granulomas within the lamina propria. Arrows show the eggshells.

3.3. Antibody responses against schistosome adult worm antigens (SWA) and schistosome soluble egg antigens (SEA)

In order to examine the serological response of the host against *S. japonicum* infection, the antibody levels against egg and adult worm antigens were measured (Fig. 4). Specific IgG against SWA (Fig. 4a) began to increase significantly at 4 weeks p.i. In C-2, the overall IgG response against SWA and SEA, shown in Fig. 4, was lower than that of C-1. Specific IgG against SEA (Fig. 4b)

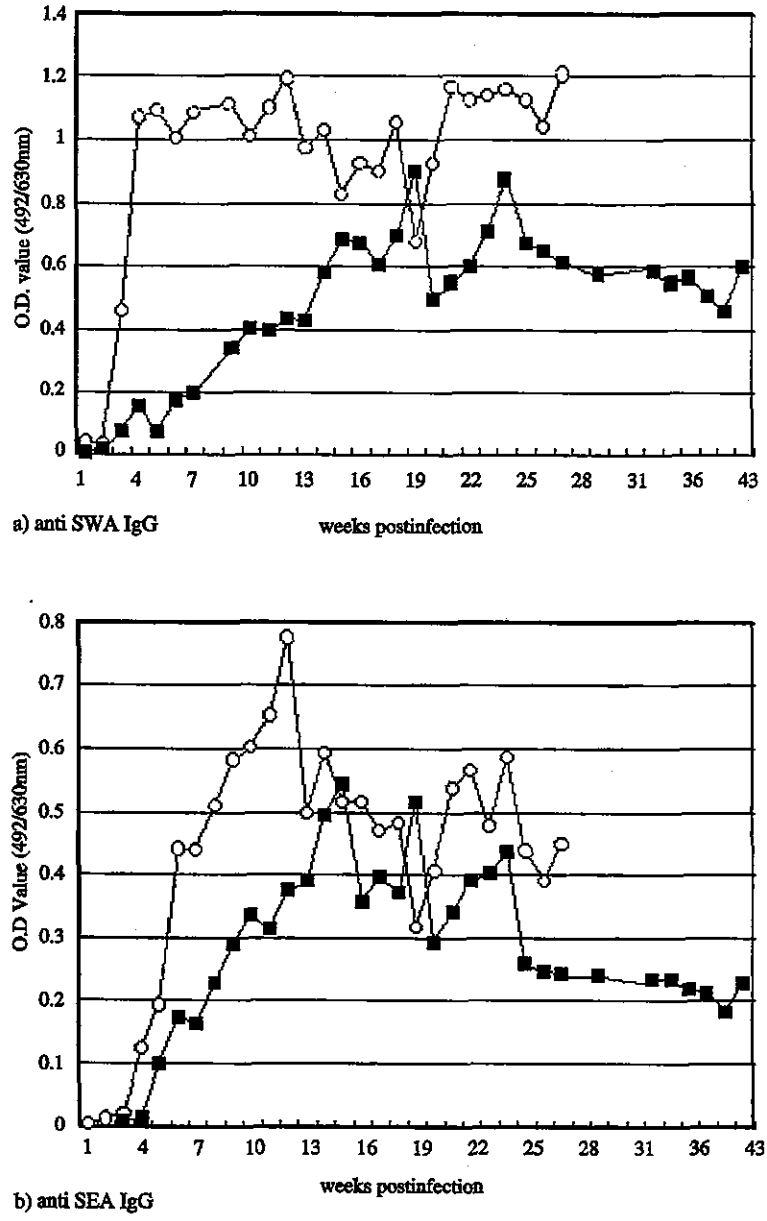

Fig. 4. IgG antibody response against adult worm antigen (SWA) and egg antigen (SEA). The serum diluted with blocking buffer (400-fold) was applied to an ELISA. Open circle, data from C-1; and closed square, data from C-2.

began to increase at 5 weeks p.i. prior to eggs being excreted into the feces. These responses persisted during the course of the experiment.

4. Discussion

In this study, we demonstrated that the CLAWN miniature pig is highly susceptible to *S. japonicum*. The EPG values were higher than those reported in experiments that used domestic pigs [7]. Marked egg excretion persisted until approximately 20 weeks p.i., with many adult worms able to be recovered by portal perfusion even at 27 and 47 weeks p.i.

Eleven paired worms were recovered at 27 weeks p.i. At that time, the EPG was approximately 100. As the weight of the feces produced per day was approximately 200 g, approximately 20 000 eggs were excreted by the 11 pairs of worms. Therefore, each worm pair produced over 1800 eggs per day. This number is similar to that found in a previous report [5]. Assuming the productivity of eggs per pair is constant during the course of infection, the number of

paired adult worms at 8 weeks p.i., when the EPG was approximately 400, was estimated to be approximately 44. Therefore, after the 200 cercarial challenge infections, half of the inoculated cercariae were assumed to establish infection in this pig. However, because we did not perform the perfusion when they showed the peak EPG, it is not clear how many worms were present. After 35 weeks p.i., EPG became almost nil, but at least four pairs of mature worms were present in the portal vein. Logically, these worms should have produced 7000–8000 eggs per day, resulting in an EPG of at least 30–40. There are at least three possible mechanisms to explain why the EPG declined after 18 weeks p.i.: (1) immunity killed the worms, (2) the worms decreased their egg production, and (3) excretion of eggs through gut mucosa was inhibited. Our observation using two pigs cannot exclude any of those possibilities. Willingham et al. [7] reported that pigs inoculated with a single dose of 2000 cercariae of *S. japonicum* showed markedly reduced EPG from 17 weeks p.i. and had eliminated most of the worms by 24 weeks p.i. Thus, this indicated that the first possible explanation, i.e. that immunity killed the worms, was viable.

Reid and Lichtenberg in 1977 [12] concluded that miniature pigs could not be used as an adequate model of human *S. japonicum* infection, because of the difficulty in establishing infection with extremely low egg numbers (EPG≤4), short duration (up to 14 weeks p.i.) of fecal egg excretion, and mild or moderate pathology in the liver. Although only two pigs were used in this study, the EPG values and recovered worm numbers were higher than those observed in their study. Additionally, there were some differences in experimental conditions between our study and theirs. Because our CLAWN strain is newly established in Japan, the miniature pig itself was different from the strain used in the other study. The size and age of their pigs at the time of inoculation also differed from ours. The miniature pigs they used were 10-week-old pigs, ranging in weight from 8 to 11 kg. The origin of the *S. japonicum* also differed as they used a Japanese strain.

Histological examination disclosed the deposition of *S. japonicum* eggs had a widespread distribution, and were found in the liver, spleen, pancreas, lungs, small intestine, and mesenteric lymph nodes. The deposited eggs were surrounded by foreign body granulomas as a result of host responses. It was rather difficult to recognize where the eggs were deposited at first in these tissues, but the presence of eggs in lymph nodes suggests that the eggs were also spread through lymphatic pathways in addition to the peripheral blood circulation. Although Yason et al. [13] reported that the adult worms were present in blood vessels such as pancreatic and splenic veins and pulmonary arteries, no adult worms were observed macroscopically or microscopically in those organs that were positive for egg deposition in this study. However, further detailed examination of blood vessels might show their presence.

In the liver, occasional bridging–bridging fibrasis were observed. Kardoff et al. [14] reported that the dilation of portal vein in infected pigs were observed in ultrasonography. These facts indicated that egg deposition in the liver affected the portal flow even in pigs. The experiments of repeated infection may reveal the role of egg deposition in the portal hypertension and fibrosis observed in the human case.

An antibody response was detected against both eggs and adult worms. The antibody response against adult worms began at 4 weeks p.i. It may indicate that the cercariae inoculated in the miniature pigs became adult worms before 4 weeks and laid eggs before 5 weeks p.i.

Hurst et al. [15] reported that there were CD4 and/or CD8 positive T cells, B cells, γδ T cells around the deposited eggs in the liver. Taking it into consideration, the adaptive immunity really occurred against eggs deposition in the pigs liver.

In the present study, we could not reveal any individual variations in the population due to the limited number of available pigs. The closed colony of the CLAWN strain has already reached approximately 75% consanguinity after 27 years of closed matings from the original two kinds of F1. Regarding the SLA (swine MHC), there are several alleles detected and some of those are the majorities [16]. Although we had no data on our pig's SLA, these alleles might have influenced the outcomes of their course of infection.

As far as we know, this is the first report that miniature pigs are highly susceptible to Chinese strain of *S. japonicum* and that it may be a useful animal model for human schistosomiasis.

Acknowledgements

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Development of membrane-based tests for the detection of urinary antigens and antibodies in human toxoplasmosis: preliminary studies in Ghanaian patients

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Abstract

Two membrane-based ELISA systems were used in detecting *Toxoplasma* antigens and anti-*Toxoplasma* antibodies in urine samples collected from 54 ophthalmology (22 suggestive active and 32 suggestive past infection) patients and 26 pregnant women attending obstetrics/gynaecology clinic (OGP), suspected of toxoplasmosis by eye examination, past medical records and questionnaire, respectively, in Ghana from mid-February to April 2002. The antigen detecting ELISA was able to demonstrate antigen in 100% (22/22) ophthalmology (active infection) and 62.5% (20/32) ophthalmology (past infection) patients, and 42% (11/26) of OGP which included 3 that were sero-negative prior to and during this study, giving an overall prevalence of 66.3% (53/80). The urinary antigen positive samples also included 6 that were negative for both the Dye Test (DT) and latex agglutination test (LAT). Antigen was not detected in the urine of 22 normal (sero-negative for antibodies to *Toxoplasma*) individuals. The membrane-based urinary antibody detecting sandwich ELISA also detected anti-*Toxoplasma* antibodies in 100% (22/22) of ophthalmology (active infection) and 81.3% (26/32) of ophthalmology (past infection) patients, a total of 89% (48/54); and 80.8% (21/26) of OGP with an overall prevalence of 86.3% (69/80), including 7 ophthalmology patients' samples that were sero-negative for both DT and LAT. Antibody sero-positivity of the samples was determined by DT as 87% (47/54) in ophthalmology patients and 73.1% (19/26) in pregnant women, LAT as 85.2% (46/54) and 65.4% (17/26), and an overall prevalence as 82.5%

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