120 km from the capital city of Changsha, the village lies northeast of Dongting Lake (Junshan district) where the broad marshland (i.e. a type of wetland, a transition zone between land and water) appears in the dry season. The village is in the vicinity of Dongting Lake (Figure 1). Climatologically, the lake has unique characteristics in that the range of water coverage and water level vary dramatically by season [11]. Human habitation is observed near the dike, where floods were often experienced during the wet season. The village is populated by 1,200 individuals (village leader, personal communication) consisting of three distinct groups. Our study focused on a group of 210 persons. Almost all adults make a living as fishermen, and they are officially allowed to catch shrimp and fresh water fish in the Dongting Lake only between July and October. During other seasons, the villagers find jobs in a distant city and work as migrants or continue fishing under special permission from the government.

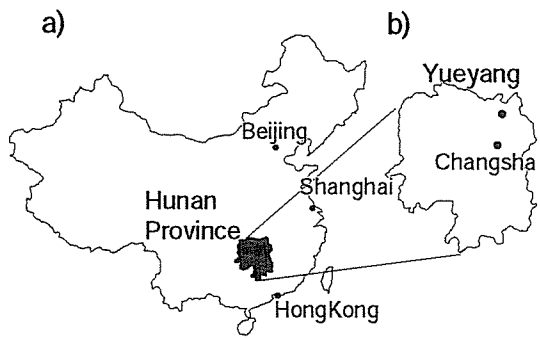

Figure 1. Map of the study area

a) Hunan province is located in the southeast inner area of mainland China.

b) The study area, Junshan district in Yueyang city, is the northern-most part of the province, approximately 120 km from the capital city of Changsha.

2. 2. Methods

2. 2. 1. Study preparation

With the assistance of researchers from the Hunan Institute of Parasitic Diseases, we first identified the climatic and environmental conditions in the study area. According to study members from the local Anti-Schistosomiasis Station, the study area has a higher prevalence of *S. japonicum* infection than other villages, averaging three percent of the population throughout the year. Other areas have succeeded in reducing water contact, mainly as a result of the prevention of floods by dike construction. Since the marshland has been recognized as a particularly high-risk location for potential exposure, we decided to explore behaviors at this location. A previous study also documented that the marshland as well as the lake region were specific areas of risk [6]. In other words, we selected this study area because we could expect characteristic high-risk behaviors to be more obvious here than at other sites.

2. 2. 2. Collection of specimens and ethical considerations

This study was approved by the Ethics Committee at the University of Tokyo and the Hunan Institute of Parasitic Diseases. Before commencement of the study, we informed participants that enrollment was voluntary and gave them the right to withdraw at any time. Each subject was informed as to how the information would be used and assured of the confidentiality of responses. The purpose of the study was explained in Chinese, and written informed consent was obtained from participants.

Subsequently, 137 (65.2%) of the individuals approached agreed to participate in the study and detailed survey. Before conducting the behavioral survey, interviews in Chinese were used to obtain demographic information (i.e. age, sex and occupation). The age of subjects ranged from 6 to 87 years. Single stool specimens were collected from the subjects. The Kato-Katz thick smear technique with 41.7 mg of stools (three slides per participant) was used to measure infestation with *S. japonicum* (eggs per gram of feces; epg). The participants, who clearly remembered having recieved previous treatment, had been given a single oral dose of praziquantel, 40 mg/kg, in 2000 and 2001.

2. 2. 3. Behavioral survey

To explore the behavioral characteristics, time-saving spot-check observations [12] were conducted in October and November 2004. This method records in great detail the time engaged in certain activities and checks the activities of respondents at a scheduled interval in the day. The first author visited the houses of all participants according to a planned time schedule and observed and recorded the activity and location of each participant. The time between 5 am and 7 pm was divided into 14 one-hour intervals and visits were made every other interval on the first day. The intervals not examined on the first day were examined on the second day (Figure 2). Fourteen spot-check records were collected for each individual, or 1708 spot-check observations in total. If a participant was not seen in or around his house when the researcher made a visit, household members or neighbors were asked for his whereabouts. Then, the researcher went to the place to observe his activity there. If the participant had gone to town, no direct observation was made. Continuing this task for two days, almost all activities within the village, including the marshland, had been observed. All observed behaviors were recorded in detail and classified later into 60 categories. In this study, the fol-

lowing 12 behaviors were thought to be particularly high-risk behaviors on the basis of a literature review and discussions in the study location: fishing on the marshland, preparation for fishing on the marshland, repairing ships on the marshland, breeding ducks on the marshland, manual separation of ducks on the marshland, feeding ducks on the marshland, collection of firewood on the marshland, electrical facility fishing, washing, preparation for fishing on dry land, repairing ships on dry land, and working near the fishpond. Electrical facility fishing is defined as an in-river fish sampling method that involves capturing fish using an electric shock technique. As for the last behavior, the fishpond is located outside the dike and has been believed to be safe. The first seven behaviors involve exposure on the marshland, while the latter five are on dry land. Most of the behaviors on the marshland except those related to breeding ducks are conducted when the water level is low and the marshland appears. Therefore most behaviors on the marshland can be observed between October and May, and the villagers had started the behaviors on the marshland about one month before our survey.

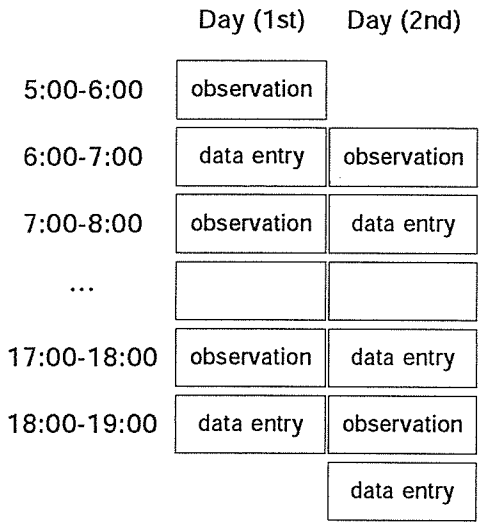

Figure 2. Design of the time-allocation study
Each person was observed for two consecutive days, or 14 hours in total. We repeated observations every other hour. On the second day, we reversed the time for observing and entering the data.

2. 3. Statistical analysis

First, associations between infection and demographic or behavioral variables were examined. Except sex, which is a dichotomous variable, age and time allocations for each of the examined behaviors were measured as continuous variables. Thus, to examine the univariate associations, either the χ^2 test or the non-parametric Mann-Whitney test was used. Second, intensity was used as a dependent variable referring to the geometric mean epg in the population sampled. As the distributions of egg counts were extremely skewed, geometric epg (logarithmic transformation of epg+1) was used instead. To examine univariate association and correlations between the intensity of infection and other explanatory variables, the Mann-Whitney test (for sex) and the Spearman's rank correlation (for other continuous variables) were used, respectively. We used the non-parametric tests because the distributions of both intensity after the logarithmic transformation and the time allocations were skewed to the right. The level of statistical significance was set at α= 0.05. Then, a multiple regression model was used to determine risk factors significantly associated with intensity of *S. japonicum* infection and to eliminate confounding variables. In the multiple regression, we selected the set of variables to be included in the model by the stepwise method. Since there were many potential predictor variables concerning the intensity of *S. japonicum* infection, we selected sex, age, and only variables that were significantly associated or correlated with *S. japonicum* in the univariate analysis.

3. RESULTS

3. 1. Study population and demographic variables

We completed observations for 122 (89.1%) of the 137 individuals who agreed to participate. Nearly one-half were female (n=58; 47.5%). The mean age (and standard deviation; SD) of the individuals investigated was 42.1 (19.5) years. Among these, 18 (14.8%; 95% Confidence Interval (CI): 8.5, 21.0) were positive for *S. japonicum*. Two-thirds (n=12) of those infected were male, although the Mann-Whitney test showed no significant influence of sex on infection (p=0.08). Figure 3 shows the age distributions strati-

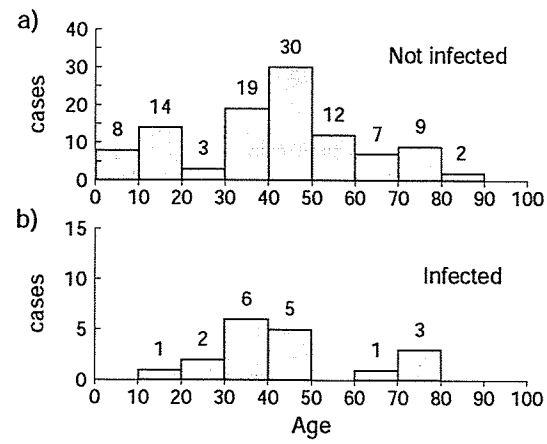

Figure 3. Age distribution by infection with *S. japonicum*
Top: Not infected (n=104; 85.25%). Bottom: Infected (n=18; 14.75%).

fied by infection. Due to the poverty of the community, many young villagers in their twenties had out-migrated to major cities to seek work. As a result, there were only five villagers in their twenties in this village as shown in figure 3. Age was also not associated with infection (p=0.91). Among those diagnosed, the median (25-75% quartile) epg was 8 (8−16). The minimum and maximum epg were 8 and 280, respectively. The geometric epg was neither associated with sex (p=0.49) nor correlated with age (p=0.62).

3. 2. Description of high-risk behaviors

Figure 4 shows distributions of the time-allocation for each behavior by *S. japonicum* infection. Five of a total of seven behaviors on the marshland were performed by participants who were either positive or negative for *S. japonicum*. The participants spent the longest time washing (0.33 hours; 95% CI: 0.22, 0.44), followed by electrical facility fishing (0.25 hours; 0.04, 0.47). On the marshland, fishing (0.12 hours; −0.02, 0.26) and preparation for fishing (0.12 hours; −0.05, 0.29) were the behaviors allocated the long-

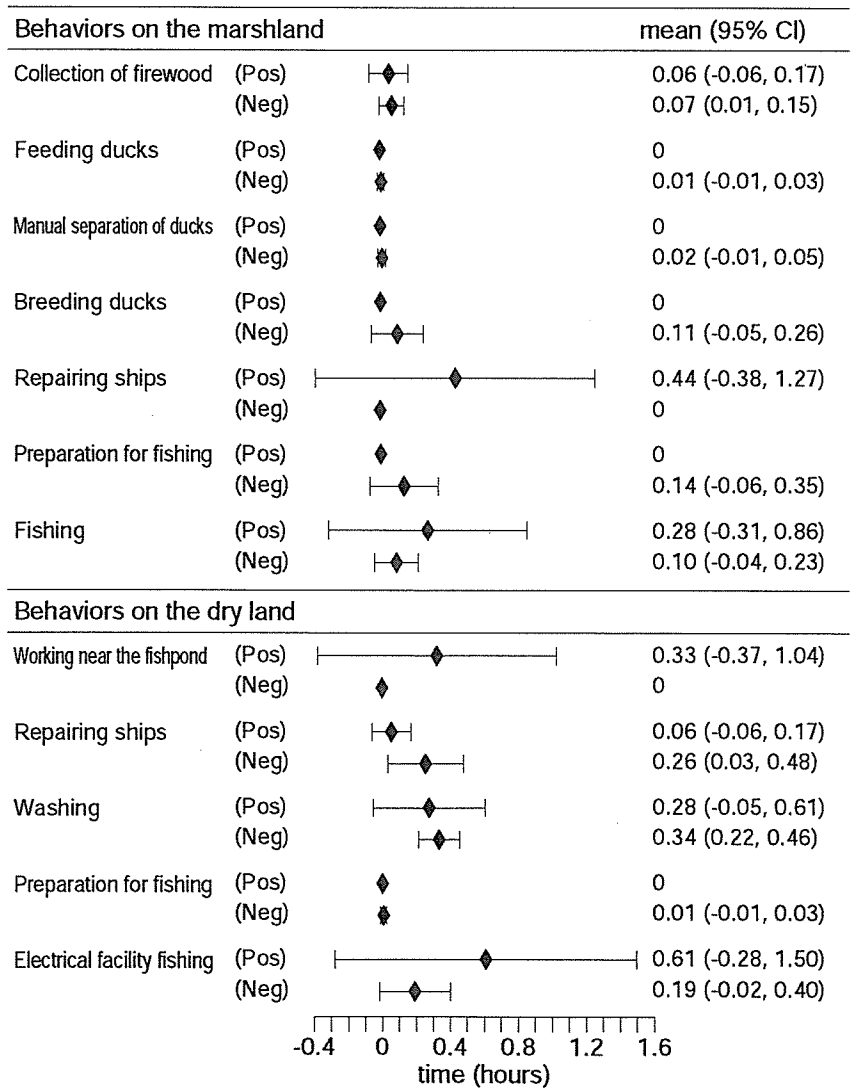

Figure 4. Distribution of time allocated for behaviors on the marshland and dry land
The upper seven behaviors were those observed on the marshland; the lower five were in other areas. Each time-allocation for the observed behaviors was stratified by infection (i.e. 'Pos' and 'Neg' refer to infection and not by means of the single Kato-Katz thick smear method). Diamonds represent the mean time allocated for each behavior. The whisker extends from lower to upper 95% confidence limits.

est times. As a whole, the high-risk behaviors observed were performed for only a few hours per day.

3. 3. Univariate analysis of behavioral factors associated with infection and intensity of *S. japonicum* infection

The Mann-Whitney test revealed that the frequency of infection was significantly higher among those who repaired ships on the marshland ($z=3.40$, $p<0.001$). A similar tendency was seen among those who worked near the fishpond ($z=2.38$, $p=0.02$). Moreover, significant positive correlations with intensity of infection were observed for participants who repaired ships on the marshland (Spearman's $\rho=0.32$, $p<0.001$) and those who worked near the fishpond (Spearman's $\rho=0.20$, $p=0.02$).

3. 4. Multivariate analysis of behavioral factors associated with intensity of *S. japonicum* infection

A multiple regression model (Table 1) shows an overall weak model for predicting *S. japonicum* intensity. This model identified repairing ships on the marshland as the only variable significantly associated with the intensity of *S. japonicum* infection ($p<0.001$). Working near the fishpond and sex were not useful predictors of worm intensity.

Table 1. Multiple regression analysis for *Schistosoma japonicum* worm intensity and behavioral factor-related exposure

Independent variables	Parameter coefficient	S.E.	t	p-value
Intercept constant	0.36	0.09	4.11	<0.001
Sex	0.15	0.09	1.69	0.09
Repairing ships on the marshland	0.75	0.14	5.49	<0.001
Working near the fishpond	0.28	0.16	1.74	0.08

From the reuslts of the univariate analyses, repairing ships on the marshland and working near the fishpond were examined with age and sex by stepwise multiple regression analysis. Then sex, repairing ships on the marshland and working near the fishpond were selected for the final model of the predictor of *S. japonicum* intensity (log [epg+1]). $R^2=0.24$, (F value $=12.7$, $p<0.001$).

4. DISCUSSION

This study investigated specific behaviors associated with water contact and examined the relationship between the behaviors and infection and intensity of *S. japonicum* in a rural village near Dongting Lake. The study location was unique with regard to the variation in water level by season, which could influence the temporal and spatial spread of the disease [13]. The participants in this study were particularly at risk for potential water contact behaviors on the marshland. According to the local Anti-Schistosomiasis Station, the average prevalence in Junshan district was around three percent (personal communication). The prevalence in this village was higher than the average, but the intensity of *S. japonicum* was low as a whole. The prevalence might be largely influenced by different occupations (which can create heterogeneous patterns), the period of our survey (i.e. seasonal changes in vector ecology and exposure) and mass treatment [14, 15]. Statistical analyses overall did not reveal significant correlations between intensity and the potentially risky behaviors examined. The correlation between working near the fishpond (probably no risk of infection) and *S. japonicum* intensity was significant in the univariate analysis but not significant ($p=0.08$) in the multivariate analysis. Villagers who worked near the fishpond during the survey were fishermen who worked on the marshland on other occasions. Therefore, an apparent correlation was observed between working near the fishpond and *S. japonicum* intensity because working near the fishpond distorted the outcome as confounding factor. In both univariate and multivariate analyses, repairing ships on the marshland was identified as a predictor of *S. japonicum* intensity.

In our direct observation of behaviors, we used a time-saving spot-check method for data collection because it seemed difficult to visit the village, which is located in rural Hunan, to perform repeated observations. Time-allocation observations enabled us to quantify the behavioral data in more detail than questionnaires, 24-hour recall methods or activity diaries. Moreover, a simple questionnaire survey may not be suitable for the identification of behavioral characteristics in detail, because over-reporting of 'correct' behavior (what participants thought they should do) has been claimed in a questionnaire survey related to sanitation and hygiene [16]. With regard to precision and validity, there is a trade-off between exactness of the data and the willingness of subjects to participate in the survey. Since the habitation of the villagers was particularly aggregated, and because their overall participation in the survey was sufficiently high, use of the spot-check method was most suitable for data collection within a limited time period. Consequently, quantifications of the time-allocations for each behavior were reasonable, successful and supported by a valid methodology.

Although the significantly higher risk among participants who repair ships on the marshland may be a straightforward result, it is necessary to discuss the reasons why the other behaviors did not reveal particular relationships with infection and intensity. We attribute this to the following four factors. First, although our study examined infection and intensity by means of the single Kato-Katz thick smear method, it is widely accepted that mild and moderate schistosomiasis japonica can be easily missed when multiple Kato-Katz is not employed [14, 17]. Since it is essential to

measure the relationship between intensity and potential factors, and because *Schistosoma* spp. are macroparasites [18], the low sensitivity of single-field evaluation must be kept in mind [19]. Second, repairing ships on the marshland included the removal of matter clinging to the bottom and side of ships. This activity requires a lot of water. Because of this, repairing ships on the marshland was conducted near water. This is a reason why repairing ships on the marshland was related to both the infection and the intensity. Third, this study differs from previous epidemiologic investigations in that the behaviors were measured in terms of time-allocation (continuous variables); i.e., the statistical correlations between the intensity of infection and exposure doses were examined on the basis of a rough assumption that the dose is proportional to the time-allocation. This anthropological measure enables us to evaluate behaviors quantitatively and qualitatively [12]. But it was extremely difficult to obtain further specific answers in the present setting because of statistical conditions (we examined, not 'associations', but 'correlations' with intensity, which are far less efficient in elucidating detailed relationships) and because most individuals had experienced exposure on the marshland. Finally, the intensity of schistosomiasis is heavily influenced by genetic heterogeneity [20] and acquired immunity [21]. Considering the potential confoundings among the parasitological factors, future epidemiologic studies should incorporate more biological variables in addition to socio-cultural and socio-behavioral factors.

In conclusion, this study attempted to evaluate the details of high-risk behaviors associated with water contact, and it demonstrated a consistent correlation between the intensity of *S. japonicum* infection and exposure on the marshland. In future studies, several key factors as described above should be taken into account to improve understanding of the epidemiologic process of this disease. By elaborating the details of our investigations at both parasitological and anthropological levels, our methodology may be useful to explicit identify behaviors at the local level which could be relevant to specific prevention.

ACKNOWLEDGMENTS

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Parasitology in Japan

School-health-based parasite control initiatives: extending successful Japanese policies to Asia and Africa

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Japan controlled its major parasitic diseases by the 1970s. Based on this experience, the Government of Japan proposed the Global Parasite Control Initiative in 1998 and established three research and training centres around the world. The Asian Centre of International Parasite Control (ACIPAC) is the first such centre, and completed five years of activities focused on school-health-based parasite control in the Greater Mekong Subregion in 2005. The lessons learned and experiences gained by ACIPAC should be applied to all health promotion programmes worldwide.

'Wormy world'

Malaria and other parasitic diseases still cause a huge amount of disease and disproportionately affect the poor: in particular, impoverished communities in low-income countries. Highly debilitating, rather than deadly, worm-induced diseases such as schistosomiasis and soil-transmitted helminthiases (STH) remain a major health problem in tropical developing countries. Today, the picture is little better than when Stoll succinctly stated the situation in the title of his article 'This wormy world' in 1947 [1]. At the turn of the millennium, infectious diseases accounted for 32% of mortality and 41% of disease worldwide [2]. Today, ~200–450 million cases of malaria occur in the world annually, causing the death of 1–3 million people, predominantly African children [3]. More than 190 million people are estimated to be infected with schistosomiasis in 76 countries and territories. Although related mortality is lower than it was five decades ago [4], there are locations where 50–70% of the population is affected by geohelminths such as *Ascaris lumbricoides*, *Necator americanus*, *Ancylostoma duodenale* and *Trichuris trichiura*, as recently reported in the Great Mekong Subregion [5].

After the end of World War II, >70% of the Japanese population was infected with intestinal parasites, with conditions in many rural parts of the country resembling those in some developing countries today. However, in the space of two to three decades, Japan eliminated most major parasitic diseases, including malaria, filariasis, schistosomiasis and STH. This achievement was facilitated by using a school-health-based approach to gain access to the community; this approach was implemented through triangular cooperation among government agencies, community-based non-governmental organizations (NGOs) and scientific experts. The enactment of the School Health Law (1958), which included mass examination and selective mass treatment targeted at schoolchildren, and the foundation in 1955 of a specialized non-profit organization, the Japan Association of Parasite Control (JAPC), greatly contributed to successful control measures [6]. The causative parasite of schistosomiasis japonica was discovered as a result of people's awareness of the disease and their request to the local government to clarify its aetiology [7]. Interventions to help control the disease included active case detection and mass chemotherapy, periodic distribution of molluscicides to kill the snail host, storage of night soil (which causes parasite egg degradation), environmental management such as cement lining of irrigation ditches, land reclamation and control of animal reservoirs (e.g. cows, stray dogs and wild rodents) [8–10]. The achievement in Japan shows that, to achieve the goal of parasite control, a comprehensive and coordinated programme of activities is required. The organization of voluntary associations in cooperation with national and local governments is essential to educate, motivate and engage communities in nationwide self-help efforts. The scientific community also has to be fully involved to ensure the production of and the best and most cost-effective use of diagnostics, therapeutics and preventive technologies and products. In addition, the private sector has an important role [11].

At the Group of Eight (G8) summits in Denver (USA; 1997) and Birmingham (UK; 1998), the late R. Hashimoto, Prime Minister of Japan at the time, emphasized the importance of parasitic-disease control as a means of improving public health, and stated the necessity for strengthening international cooperation towards global parasite control. Based on a report [12], the Government

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Box 1. Relevant websites

ACIPAC: http://www.tmd.ac.jp/med/mzoo/acipac/index.html
European Commission: http://www.europa.eu
Japanese Society of Parasitology: http://jsp.tm.nagasaki-u.ac.jp/~parasite/
JICA: http://www.jica.go.jp
Kenan Institute: http://www.kiasia.org
Kenya Medical Research Institute: http://www.kemri.org
Mahidol University: http://www.mahidol.ac.th
Ministry of Foreign Affairs, Japan, Health and Development Initiative (2005): http://www.mofa.go.jp/policy/health_c/forum0506/hdi/pdf
Noguchi Memorial Institute for Medical Research: http://www.noguchimedres.org
Partnership for Child Development: http://www.child-development.org
UNESCO: http://www.unesco.org
UNICEF: http://www.unicef.org
WHO: http://www.who.int

of Japan proposed to establish three centres for research and training, one in Asia and two in Africa. This was known as the Hashimoto Initiative (HI).

The Asian Centre of International Parasite Control (ACIPAC) (Box 1) was established in 2000 as a bilateral technical cooperation project in connection with the region-wide work of the Japan International Cooperation Agency (JICA) and in collaboration with Mahidol University and the Ministry of Public Health, Thailand. A further two centres were established in Africa, one in the Kenya Medical Research Institute, Kenya, and the second in the Noguchi Memorial Institute for Medical Research, University of Ghana, Ghana.

The ACIPAC approach

Mortality due to malaria is concentrated in sub-Saharan Africa, and the majority of deaths occur in children under five years of age. At the moment, treatment of children above this age is considered to be of secondary importance. However, in malaria-endemic areas, most children are infected with malaria parasites and, because they might not develop disease or because they exhibit only mild symptoms, continue to attend school; therefore, these children contribute to disease transmission. Thus, control measures aimed at school-age children should be effective at helping to prevent malaria transmission across the community.

In the south of Thailand, ~20–30% of schoolchildren are infected with STH (primarily ancylostomiasis) [13], whereas ~60% of schoolchildren in the mountainous region of the northern provinces are infected [14]. In neighbouring countries, morbidity is also high: for example, 70% for ascariasis and 86% for ancylostomiasis in Cambodia. A similar situation is observed worldwide. This demonstrates the impact of STH, not only on the health of schoolchildren but also on their education. In addition, these children might be a source of infection in the community. A cross-sectional study conducted in an area of southern Thailand revealed that schoolchildren with less knowledge of STH are likely to be infected more quickly and that boys, who dislike wearing shoes, have a higher intensity of hookworm infection than do girls [15].

Various health education programmes (including the prevention of malaria and other infectious diseases) that combine visible and easy-to-understand control measures are required as part of a successful strategy for parasite control. Although health education is not a tool with an immediate impact, it can have long-term benefits. Besides strengthening manpower in the health sector by mobilizing schoolteachers (e.g. to improve health education and to help administer anthelmintics), it is also useful to develop cooperative relationships between different ministries and sectors.

The ACIPAC mission and activities

ACIPAC started operations in 2000 with the overall goal of creating parasite control programmes, strengthened by human health resource development, in Southeast Asia. The outline of ACIPAC activities carried out during the past five years is summarized next.

The school-based approach advocated by ACIPAC is effective for parasite control in the region

ACIPAC advocated and promoted the school-based approach through international training courses, symposia and workshops. ACIPAC put an emphasis on the concept that schoolchildren should be considered as active health partners rather than simple recipients of health services (e.g. deworming, food and nutrient supply and health checks). Health messages conveyed through teachers would be relayed to the children and then to their siblings and friends. Schoolchildren would also make information and education communication (IEC) tools, with the idea that such hand-made tools would have a greater impact on parents than would those printed and distributed in large volumes by the authorities. To motivate the children to think and learn by themselves, health education *per se* must be changed from a top-down system.

Within the framework of the ACIPAC advocacy, the Office of Basic Education Commission (OBEC) of the Ministry of Education in Thailand developed model schools for malaria and STH prevention. OBEC, in collaboration with local teachers, also prepared user-friendly textbooks for children and manuals for teachers for the prevention and control of parasitic diseases. The English versions (Figure 1) were distributed to partner countries, international organizations and, upon request, NGOs.

In these model areas, schoolchildren developed IEC materials (e.g. posters and advocacy books) by themselves and brought them to the community for a demonstration. The children worked with teachers to identify mosquito breeding sites and to develop activities that have a positive impact on sanitation in communities. In addition, children were taught the proper use of bednets (to prevent malaria) [16], which was expected to lead to better care of siblings under five years of age who would otherwise be at greatest risk of death. The benefits of health education in the prevention of malaria has been shown in Thailand [17] and was achieved through behavioural changes in schoolchildren, using improved teacher training, interactive education and good teaching materials.

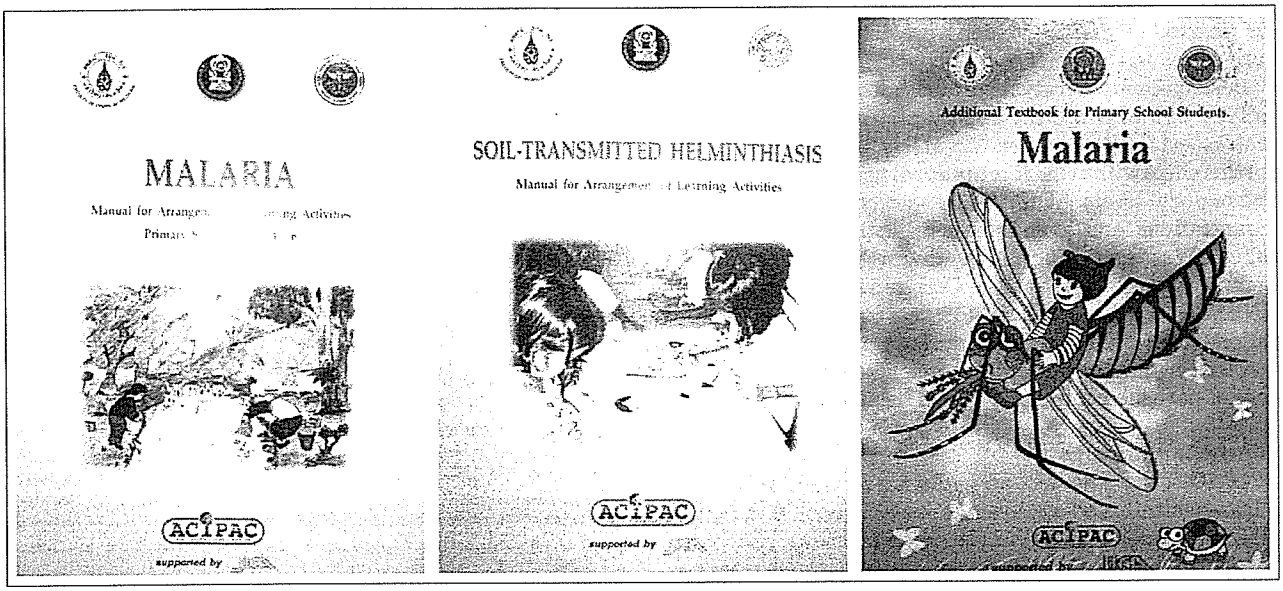

Figure 1. Teachers' manuals and a textbook for schoolchildren developed by ACIPAC in collaboration with OBEC, the Thai Ministry of Education, the Thai Department of Diseases Control, the Thai Ministry of Public Health, and Mahidol University, Thailand.

In response to the ACIPAC advocacy, the Ministry of Health in Laos stated its National Intestinal Helminth Prevention and Control Policies in 2003. In addition, ACIPAC ex-trainees started school-based control activities for malaria and dengue fever in Laos, thus showing the possibility of expanding the school-based approach to control other infectious diseases. The ex-trainees also increased coordination between the Ministries of Health and Education towards preparation of a national school health policy with support from the World Health Organization (WHO) and United Nations Educational, Scientific and Cultural Organization (UNESCO) [18]. In 2004, the Cambodian Government announced the establishment of the National Task Force for the Control of STH and Schistosomiasis, the Elimination Programme of Lymphatic Filariasis and a Helminthiasis Prevention and Control Policy.

These actions in partner countries indicate that the school-based approach advocated by ACIPAC has been accepted as an effective component of parasite control in the region.

Human resources for parasite control

ACIPAC has held international training courses for managers of school-based control programmes for malaria and STH four times during the past five years. In addition to trainees from Thailand and neighbouring countries, trainees from Kenya, Ghana and East Timor were enrolled. After finishing the course, trainees were requested to start small-scale pilot projects (SSPPs) in their respective countries, and the SSPPs were expected to be used for further development of human resources through in-country training for health personnel.

These training courses are considered unique in terms of the 'follow-up' of trainees because, by implementing SSPPs in each partner country, ACIPAC trainees also had important roles as trainers.

Small scale pilot projects

SSPPs were carried out in each of the partner countries. Other activities such as the provision of clean water and latrines were combined for some pilot schools, and teachers' manuals and comic books for schoolchildren were developed to facilitate health education.

Typical examples of activities performed were a 'model children' activity in Cambodia, cost sharing by the community for the construction of a water supply system in Laos and broadcasting (using loudspeakers) radio programmes about health education to the community in Vietnam. Model children were selected from upper forms (10–12 years old), and received two days of training on hygiene, the life cycles of the malaria parasite and of the soil-transmitted helminths and communication methods. These children then taught personal hygiene and prevention-of-infection methods to other children and kept detailed records of their activities. Children also developed IEC materials such as pictures and stories related to STH and malaria, which were used for delivering health messages to the community. In Laos, the communities of pilot project areas contributed 43.6% of the total budget for construction of water supply systems in schools. In these countries, KAP (knowledge, attitude and practice) surveys among schoolchildren showed changes in the children's behaviour after SSPP implementation compared with results in baseline surveys carried out before starting SSPPs.

Human and information networking

By implementing several activities and having meetings with those people enrolled in school health and parasite control, ACIPAC has made efforts to establish and strengthen the human and information network. For example, the homepage of the ACIPAC project was linked to the website of the Japanese Society of Parasitology. ACIPAC issued newsletters (Mekong Parasite News)

and printed magazines, which were distributed by related authorities in the partner countries.

Besides communication with both health and education sectors, one of the most important factors for the three centres established under the HI is the coordination of partnerships with other international agencies at the global and regional levels. ACIPAC also worked closely with other organizations and agencies such as the WHO Regional Offices (Western Pacific and Southeast Asia), the European Commission, United Nations Children's Fund (UNICEF), UNESCO, Southeast Asian Ministers of Education Organization (SEAMEO) and NGOs such as the Kenan Institute and the Partnership for Child Development to share experience of parasite control and school health activities.

Concluding remarks

ACIPAC has made an effort to establish the school-health-based approach to malaria and STH control mainly through human resource development, which can be applied to all health promotion programmes. Implementation of SSPPs resulted in the establishment of national policies on parasite control and/or school health in some partner countries, in addition to providing a good opportunity for the formulation of partnerships among health and education sectors and international partners. The lessons learned and experiences gained have helped shape the comprehensive approach encapsulated in the Japanese Health and Development Initiative, which, although global in scope, will focus strongly on Africa [19]. To achieve the Millennium Development Goals related to health issues, the Government of Japan has declared that Japan will provide assistance for education focusing on sanitation and prevention of infectious diseases such as HIV/AIDS and a range of parasitic diseases. This will be achieved by addressing local health issues at primary and secondary schools, at non-formal schools for out-of-school children, to those who have left school early and street children, and by providing literacy classes for adults. The two Africa-based centres will continue and will increase their efforts towards human resources development, orchestration of effective parasite control and improving living standards on the continent. Further details of the achievements by ACIPAC can be found in other publications [20–23]. Although the five-year achievements by ACIPAC were rather limited, the school-health-approach should be considered as an effective entry point to solve various issues related to providing comprehensive health care for children and their community.

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TEMPORARY SHIFT OF MICROFILARIAE OF *BRUGIA PAHANGI* FROM THE LUNGS TO MUSCLES IN MONGOLIAN JIRDS, *MERIONES UNGUICULATUS*, AFTER A SINGLE INJECTION OF DIETHYLCARBAMAZINE

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ABSTRACT: A single-dose treatment with diethylcarbamazine (DEC) reduced microfilaria (mf) counts of *Brugia pahangi* by >90% at 30 min post-treatment in Mongolian jirds (*Meriones unguiculatus*). The reduction was followed by a rapid increase in microfilaremia, with the count reaching pretreatment level in 3 hr. The mechanisms behind this temporary reduction of mf were investigated. Without treatment, mf accumulated in the lungs. At 30 min post-treatment, they had moved from the lungs and accumulated in the muscle. At the same time, electron microscopy revealed many mf in the muscle interstitium. DEC concentrations at 30 min were much lower in the muscle (12.2 μg/g of tissue) than in the lungs, liver, and kidneys (19.8–40.7 μg/g), all of which declined to <0.6 μg/g by 3 hr. The presence of mf in the muscle would be advantageous for avoiding high DEC concentrations, and their extravascular location could prevent attack by host effector cells.

Diethylcarbamazine (DEC) is a powerful microfilaricidal drug (Hawking, 1979; Mackenzie and Kron, 1985). However, the efficacy of a single-dose treatment is variable in different combinations of host and filarial parasite species. Microfilariae (mf) of *Litomosoides carinii* in a cotton rat (*Sigmodon hispidus*) and *Mastomys natalensis* disappeared quickly from circulation after DEC administration. They were found trapped in capillaries of the liver, surrounded by phagocytes, and degenerated subsequently (Hawking et al., 1950; Taylor, 1960; Zahner et al., 1978). In *L. carinii*–jird (*Meriones unguiculatus*), *Brugia pahangi*–cat, and *B. malayi*–jird combinations, DEC was inactive or only slightly active against mf. In these combinations, however, DEC induced an interesting mobilization of mf in the hosts, i.e., they almost disappeared from the host circulation within an hour after DEC administration, but they returned to the original level as soon as 6–7 hr postexposure (Matsuda et al., 1976; Denham et al., 1978; Maeda et al., 1991). To explain this phenomenon, temporary sequestration of mf in a host was suggested (Denham et al., 1978). Later, Horii and Aoki (1997) injected *B. pahangi* mf intravenously into Wistar rats and established a microfilaremia model without adult worms. DEC treatment with this model resulted in a rapid decrease of mf (95% reduction at 30 min), which was followed by complete recovery of microfilaremia by day 3, confirming that sequestration alone could cause the phenomenon, without replenishment with new and/or already accumulated mf. More recently, mf of *B. malayi* were injected intravenously into BALB/c mice. DEC treatment in this model resulted in a rapid and profound reduction in mf counts, which recovered almost completely in 2 wk (McGarry et al., 2005).

Meanwhile, in a jird infected with *L. carinii*, electron microscopy revealed apparently viable mf within hepatic cells after DEC treatment (Schardein et al., 1968). The authors mentioned a possibility that these mf escaped phagocytosis and could re-enter the blood circulation. *Dipetalonema viteae* and *L. carinii* mf also were shown to be in liver and muscle cells after treatment (Mehlhorn et al., 1981), and it was suggested that they might escape the activity of the drug. Thus, it was of interest to investigate exactly where mf move after DEC treatment, or to where they escape drug and/or host effector cells. In this regard, it is important to know the quantitative distribution of mf in the whole animal body before and after DEC treatment; these sorts of studies are few. In the present investigation, we report a temporary shift of mf from the lungs to muscle tissues after DEC treatment. The mf in muscles were examined via transmission electron microscopy (TEM) for their exact location. DEC concentrations in various organs and blood also were measured to determine the relationship between drug concentrations and mf distribution.

MATERIALS AND METHODS

Parasite and animals

Brugia pahangi used in this study has been maintained for years in our laboratory, with Mongolian jirds (*M. unguiculatus*) as a host and *Aedes aegypti* (Liverpool strain) as a mosquito vector. In all experiments, 10- to 15-wk-old male jirds were infected subcutaneously with 100 infective larvae that were obtained from *Ae. aegypti* fed on microfilaremic jirds 14 days previously. The animals were used for experiments 6 or more mo after infection, when the mf count had been stabilized.

Effect of DEC treatment on microfilaremia in a *B. pahangi*–jird model

To confirm temporary decrease of peripheral mf counts after DEC treatment, and then their subsequent increase, 5 microfilaremic jirds were treated intraperitoneally with a single dose of DEC citrate (Supatonin®, Tanabe Ltd., Osaka, Japan) at 100 mg/kg body weight. Before and 15, 30, 60, 120, and 180 min after treatment, 20 μl of blood was taken into a heparinized capillary tube, under anesthesia with ether, from the retro-orbital sinus of the jird and examined for mf counts by Knott's concentration method.

Counting the number of mf in host organs and tissues

Nine infected jirds were used. Six were treated with intraperitoneal DEC at 100 mg/kg and necropsied 30 min (3 jirds) and 3–4 hr (3 jirds) after treatment. The remaining 3 were treated with physiological saline as a control group, and necropsied 30 min after treatment. Before each treatment and necropsy, 20 μl of retro-orbital sinus blood was examined for mf counts.

Thirty minutes after treatment, the DEC- or saline-treated animals were anesthetized with intraperitoneal injection of pentobarbital sodium

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(Nembutal) at 60 mg/kg, and, after tracheotomy, they were given artificial respiration by using a respirator (model KN56100, Natsume Seisakusho, Tokyo, Japan). The thorax and abdomen were then opened, and the blood vessels connecting to the heart, lungs, kidneys, liver, and spleen were ligated simultaneously, before the organs were removed. Muscles, excluding those on the head and paws, were removed/scraped from bones. The pelt was separated, except the areas covering the head and paws, which were discarded. The testes, digestive tract, large vessels, and so on, were combined as "other". The organs and tissues were weighed and then cut into small pieces, at which time visible adult worms were removed. The organ/tissue pieces were minced with a homogenizer (Nissel AM-3, Nihonseiki Seisakusho, Tokyo, Japan) at 7,000 rpm for 5 min on ice, suspended in phosphate-buffered saline (PBS), and digested with 0.1% actinase E (Kaken Seiyaku, Tokyo, Japan) at 37 C for 2 hr. The digested homogenates were washed repeatedly with PBS, and, finally, the sediments were fixed in 10% formalin, and a total number of mf per organ or tissue was determined. Three to 4 hr after treatment, 3 DEC-treated jirds were processed in the same way.

Histological study

For a histological study to clarify the location of mf in host organs, 8 infected jirds, 4 DEC-treated at 100 mg/kg and 4 saline-treated, were used. Thirty minutes after treatment, the animals were processed as described above, and the heart, lungs, kidneys, liver, and 1 side of femoral muscles were removed. They were fixed in 10% formalin and processed for histological examinations. Tissue blocks were embedded in paraffin, sectioned at 4 μm in thickness, and stained with hematoxylin and eosin. For each organ, 3 to 6 nonoverlapping square fields (640 by 640 μm/field) were examined at ×100 magnification to count the number of mf with the aid of a tetragonal latticed ocular micrometer, which comprises 100 square compartments. Only mf that had been sectioned longitudinally or obliquely were identified and counted, because it was difficult to detect a cross section of mf in capillaries/tissues. Also, in each square field, the numbers of mf in the capillaries/tissues and mf lying in the blood vessels (other than minute vessels) were counted separately (Hawking et al., 1950). The total size of the capillary/tissue area or the blood vessel area in each square field was estimated by counting the number of micrometer compartments. Finally, the number of mf in each organ was expressed as per square millimeter of the capillary/tissue area, or blood vessel area.

TEM study

To determine the exact location of mf, femoral muscles from 2 control and 3 DEC-treated (at 100 mg/kg) jirds were processed 30 min after treatment for TEM. The specimens were fixed in 2.5% glutaraldehyde, transferred into 0.1 M sodium cacodylate buffer, pH 7.4, rinsed several times, and then postfixed in 1% osmium tetroxide for 1 hr. After aqueous rinses, they were dehydrated in graded ethanol solutions and embedded in Epon 820/850. Thick sections (1 μm) were then made and stained with toluidine blue for light microscopy. After confirming the presence of an mf, ultrathin sections were made, stained with uranyl acetate and lead citrate, and examined with the JEOL-100 CT transmission electron microscope (Jeol Ltd., Tokyo, Japan).

Measurement of DEC concentrations in host organs and blood

The competitive enzyme-linked immunosorbent assay (ELISA) developed by Mitsui et al. (1996) was used with slight modification. Eight jirds were treated with DEC at 100 mg/kg, and 4 each were killed at 30 min and 3 hr post-treatment. Blood was drawn from the retro-orbital sinus, and the heart, lungs, kidneys, liver, and femoral muscle were removed. After blotting adhered blood with a filter paper, the organs, together with blood samples, were kept at −30 C until use. DEC was extracted as follows: 50 mg of tissue of each organ was homogenized in 400 μl of 1 N NaOH at 4 C for 3 min. Two milliliters of ether was added to the homogenate in a glass-stoppered tube, which was vortexed for 2 min and then centrifuged at 1,500 rpm for 5 min. The lower aqueous layer was frozen at −30 C for 1 hr, and the upper organic layer, which contains DEC, was transferred to another test tube. DEC extraction with ether was repeated twice. The ether was dried under nitrogen gas at room temperature. The residue was redissolved in 300 μl of 0.01 M PBS containing 0.1% bovine serum albumin and measured for DEC concentrations by ELISA. Because DEC recovery from different organs might vary significantly, a separate experiment was carried out in which known amounts of DEC (0.1–1.0 μg/g tissue or /ml blood) were added to tissues and then extracted as described above. The recovery of DEC obtained was applied to adjust concentrations for different organs.

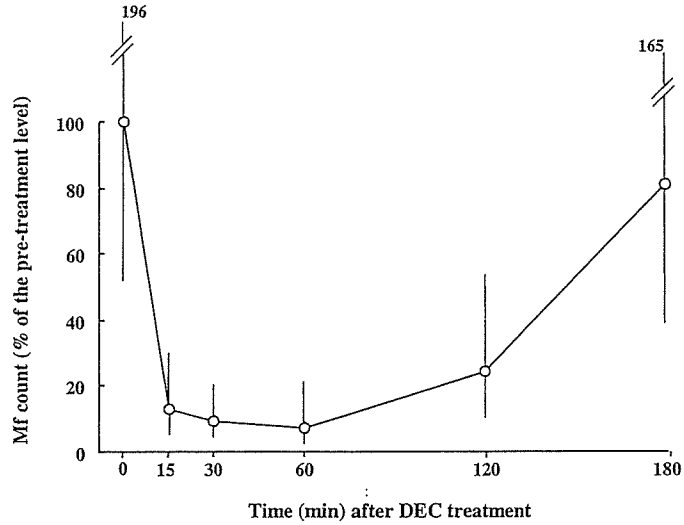

FIGURE 1. Change in peripheral microfilaria counts in Mongolian jirds after a single injection of diethylcarbamazine (DEC) at 100 mg/kg. Note: The vertical bar at each time point indicates a variation based on a geometric standard deviation.

RESULTS

Reduction and increase in peripheral mf after DEC treatment

Pretreatment mf counts in 5 jirds ranged from 126 to 741/20 μl of blood with the geometric mean of 244.6. Mean mf counts at different times after treatment are shown in Figure 1 as percentages of the pretreatment mean. The mf count declined sharply to 12.7% at 15 min after DEC injection, to 9.1% at 30 min, and to 7.0% at 60 min. The means at 15, 30, 60, and 120 min were significantly lower than the original level (paired t-test, $P < 0.003$ at all time points). The count started increasing thereafter and reached 80.9% of the original level at 180 min, which was not different from the original level (paired t-test, $P > 0.1$).

Distribution of mf in the organs and tissues

In 3 control jirds, pretreatment counts of mf in peripheral blood varied from 82 to 270/20 μl with the geometric mean of 161.1 (Table Ia). Thirty minutes after saline treatment, the counts changed considerably (88–148/20 μl; mean = 114.4) but remained at 71.0% of the pretreatment level on average (paired t-test, $P > 0.2$). The total number of mf per animal as recovered in all organs/tissues examined varied from 138,100 to 378,080. Relative abundance of mf in each organ/tissue (% of the total mf recovered/animal) was similar among the animals. The largest portion of mf (44.9–53.0%) was recovered from the lungs, which was followed by the liver (12.3–16.3%) and the muscle (11.2–15.7%). When the number of mf per gram of tissue was estimated based on organ/tissue weights of individual animals, the lungs had exceptionally high figures (91,414–260,481), whereas the muscle had only 435–1,268.

TABLE I. Distribution of *Brugia pahangi* microfilariae (mf) in the organs and tissues of jirds treated with saline or diethylcarbamazine (DEC).

Animal no. (pretreatment mf count)	No. of mf recovered								
	Heart (%)*	Lungs (%)	Kidneys (%)	Liver (%)	Muscle (%)	Skin (%)	Spleen (%)	Other (%)	Total (%)
(a) 30 min after saline treatment (control group)									
1	37,700	200,570	18,860	61,320	48,040	8,610	130	2,850	378,080
(270)	(10.0)	(53.0)	(5.0)	(16.2)	(12.7)	(2.3)	(0.0)	(0.8)	(100)
	[53,857]†	[260,481]	[18,311]	[13,627]	[1,268]	[273]	[283]	[157]	
2	19,680	103,320	6,540	32,720	31,540	830	230	5,760	200,620
(189)	(9.8)	(51.5)	(3.3)	(16.3)	(15.7)	(0.4)	(0.1)	(2.9)	(100)
	[31,742]	[147,600]	[7,976]	[8,819]	[885]	[70]	[821]	[331]	
3	11,820	62,070	19,710	16,990	15,530	2,040	2,330	7,610	138,100
(82)	(8.6)	(44.9)	(14.3)	(12.3)	(11.2)	(1.5)	(1.7)	(5.5)	(100)
	[19,377]	[91,414]	[28,565]	[5,276]	[435]	[175]	[10,591]	[457]	
(b) 30 min after DEC treatment									
4	12,830	20,070	3,100	17,140	95,810	5,840	260	4,540	159,590
(173)	(8.0)	(12.6)	(1.9)	(10.7)	(60.0)	(3.7)	(0.2)	(2.8)	(100)
	[22,430]	[31,507]	[3,781]	[5,390]	[2,969]	[534]	[1,083]	[234]	
5	4,660	7,300	5,000	12,310	29,920	640	80	4,370	64,280
(118)	(7.3)	(11.4)	(7.8)	(19.2)	(46.6)	(1.0)	(0.1)	(6.8)	(100)
	[9,915]	[14,039]	[6,757]	[3,621]	[918]	[56]	[250]	[243]	
6	16,240	25,400	29,530	121,770	330,810	1,810	6,610	4,930	537,100
(883)	(3.0)	(4.7)	(5.5)	(22.7)	(61.6)	(0.3)	(1.2)	(0.9)	(100)
	[22,745]	[31,950]	[18,456]	[17,829]	[8,146]	[93]	[25,423]	[237]	
(c) 3–4 hr after DEC treatment									
7	10,450	31,940	11,430	17,280	36,860	6,210	3,610	7,850	125,630
(78)	(8.3)	(25.4)	(9.1)	(13.8)	(29.3)	(4.9)	(2.9)	(6.3)	(100)
	[16,747]	[45,957]	[13,447]	[4,184]	[945]	[384]	[12,893]	[393]	
8	100,210	306,490	61,500	253,900	135,100	1,800	6,800	34,100	899,900
(438)	(11.1)	(34.1)	(6.8)	(28.2)	(15.0)	(0.2)	(0.8)	(3.8)	(100)
	[149,567]	[409,415]	[38,438]	[51,816]	[3,860]	[154]	[40,000]	[1,788]	
9	12,270	37,530	10,200	44,500	29,100	1,700	ND‡	9,500	144,800
(130)	(8.5)	(25.9)	(7.0)	(30.7)	(20.1)	(1.2)		(6.6)	(100)
	ND	ND	ND	ND	ND	ND		ND	

* Relative abundance (% of the total).
† Number of mf per gram of tissue.
‡ Not done.

In 3 DEC-treated animals (Table Ib), the pretreatment counts of blood mf varied from 118 to 883/20 μl (mean = 262.2). Thirty minutes after treatment, the mf counts declined significantly to 11–23/20 μl (mean = 14.5) or 5.5% of the pretreatment level on average (paired *t*-test, $P < 0.01$). The total number of mf recovered from each animal varied from 64,280 to 537,100. The muscle harbored the largest proportion of mf (46.6–61.6%), and the liver followed with 10.7–22.7%. The mf in the lungs declined sharply, possessing only 4.7–12.6% of the total. Despite a large difference in pretreatment mf counts, it was apparent that the numbers of mf per gram of lung tissue (14,039–31,950) were reduced considerably from the control level (91,414–260,481).

The mf distribution 3–4 hr after DEC treatment is shown in Table Ic. The pretreatment counts of blood mf varied between 78 and 438/20 μl (mean = 164.4). After treatment, it changed to 68 to 673/20 μl with the mean of 161.5, which is 98.2% of the initial level (paired *t*-test, $P > 0.4$). The total number of mf recovered from each animal ranged from 125,630 to 899,900. Relatively high proportions of mf were recovered from the lungs (25.4–34.1%), liver (13.8–30.7%), and muscle (15.0–29.3%).

The relative abundance of mf in each organ/tissue is summarized in Figure 2 according to DEC treatment status. A clear contrast can be seen between the lungs and the muscle. At 30 min, the abundance increased significantly from the pretreatment level in the muscle (Mann–Whitney *U*-test, $P < 0.05$), and decreased in the lungs ($P < 0.05$). At 3–4 hr, the abundance decreased from the 30-min level in the muscle ($P < 0.05$) and increased in the lungs ($P < 0.05$).

Histological location of mf in the organs

Thirty minutes after DEC or saline treatment, histological location, i.e., the blood vessel area or the capillary/tissue area and the number of mf in each area, were determined based on hematoxylin and eosin-stained sections. In the control animals (Table IIa), the mean count of blood mf was 597.2/20 μl (range 250–956) before saline treatment, which became 444.8/20 μl (range 216–642) or 74.5% of the pretreatment level (paired *t*-

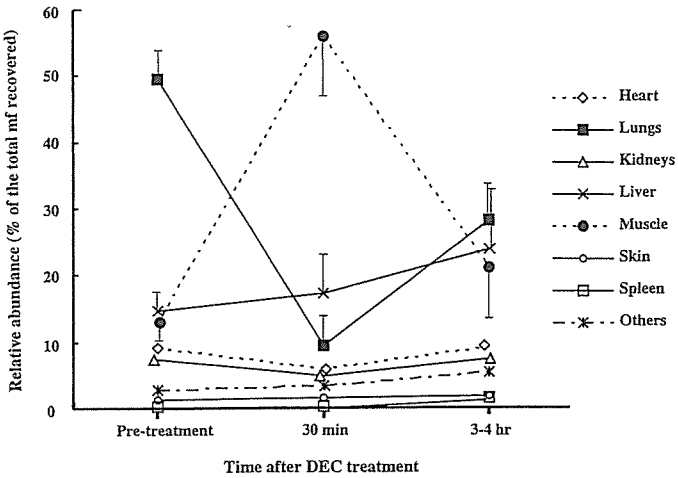

FIGURE 2. Change in the relative abundance of microfilariae (mf) in the organs/tissues after diethylcarbamazine (DEC) treatment. Note: Standard deviations are shown for the lungs, muscle, and liver only for clarity.

test, $P > 0.2$). The lung and the heart had relatively more mf than the other sites, with the muscle having the fewest mf. In the DEC-treated animals (Table IIb), the mf count changed from 347.3/20 μl (range 269–477) to 26.5/20 μl (range 12–45), which is a 92.4% reduction (paired t-test, $P < 0.001$). Thus, the number of mf in each examined area was small as a whole. A clear difference with or without DEC treatment was seen; in control organs, the blood vessel areas (Vessel in Table II) had more mf than in the capillary/tissue areas (Tissue in Table II), whereas in treated organs, the tissue had clearly more worms than the vessel. To determine the statistical difference between the control (n = 4) and DEC-treated (n = 4) animals, the organs in all animals were divided into 2 categories, one category having more mf in tissue than in vessel (tissue > vessel), and the other category with tissue ≤ vessel; the frequencies of the 2 categories were compared for each organ between the control and DEC-treated groups. A significant difference was observed for the hearts, kidneys and muscles (Fisher's exact test, $P = 0.014$ in all tests), indicating that mf shifted from the blood vessel area to the capillary/tissue area after DEC treatment. The lungs and livers showed a similar tendency ($P = 0.071$).

Electron microscope observation

To determine whether mf in muscles at 30 min were inside minute blood vessels or outside, 23 mf sections were randomly selected from control jirds (n = 2) and 33 sections from DEC-treated animals (n = 3). When examined by TEM, 18 worms (78.3%) were inside blood vessels and 5 (21.7%) were outside in controls. In DEC-treated animals, 24 (72.7%) of 33 mf were outside blood vessels (χ^2 test, $P < 0.001$). It was concluded that some mf were outside a blood vessel without treatment and that DEC treatment (Fig. 3A, B) induced a shift of mf from inside to outside of a blood vessel. No mf were found inside muscle cells.

DEC concentrations in the organs and blood

The recovery of DEC from different organs and blood was determined in a separate experiment. The concentrations ranged from 38.4 to 54.2% of the total DEC added to various tissues (Table III). Thirty minutes after injection, the highest levels of DEC, after adjustment based on recovery rates, were recorded in the kidneys (40.7 μg/g) and the liver (36.4 μg/g), whereas the lowest was in the blood (4.8 μg/ml). The muscle and the heart showed relatively low concentrations of approximately 12 μg/g. At 3 hr, the residual DEC diminished sharply in all organs and blood to <0.6 μg/g (Table III).

TABLE II. Number of *Brugia pahangi* microfilaria found in 1 mm² of the blood vessel area or of the capillary/tissue area according to organ.

(a) 30 min after saline treatment (control group)

Organ	Area*	Animal no. 1	2	3	4
Heart	Vessel	15.6	37.2	13.4	14.2
	Tissue	7.3	29.4	2.0	14.2
Lungs	Vessel	75.7	53.7	35.6	12.6
	Tissue	38.6	17.1	2.0	1.6
Kidneys	Vessel	12.7	15.6	3.4	4.1
	Tissue	0.0	6.8	2.4	4.1
Liver	Vessel	10.3	34.2	7.8	4.1
	Tissue	1.5	0.0	0.0	3.3
Muscle	Vessel	7.3	3.9	0.0	0.0
	Tissue	0.8	0.0	0.0	2.8

(b) 30 min after DEC† treatment

		5	6	7	8
Heart	Vessel	0.0	2.0	0.0	0.8
	Tissue	5.9	23.4	2.0	3.7
Lungs	Vessel	1.5	0.5	1.0	2.0
	Tissue	7.3	1.5	3.4	2.0
Kidneys	Vessel	1.0	0.0	0.5	0.8
	Tissue	2.9	4.4	2.0	1.6
Liver	Vessel	0.0	0.5	0.0	1.6
	Tissue	0.0	4.9	0.5	6.1
Muscle	Vessel	0.0	0.0	0.0	0.0
	Tissue	5.5	8.8	3.4	7.7

* Vessel, the blood vessel area; Tissue, the capillary/tissue area.
† Diethylcarbamazine.

DISCUSSION

The present study was designed to elucidate the mechanisms of a rapid decrease and increase of *B. pahangi* mf in the peripheral blood after a single injection of DEC in jirds. In the first experiment, 5 microfilaremic jirds were treated with DEC, and mf counts were determined at different time points. The experiment confirmed a rapid decrease between 30–60 min after treatment (>90% reduction) and an increase after 3 or more hr (81% of the initial level); accordingly, the times for necropsy in the following experiments were fixed at 30 min and 3–4 hr post-treatment.

In the second experiment, the numbers of mf in different organs/tissues were studied. To minimize the effects of agonal blood flow on the distribution of mf, artificial respiration was given before thoracotomy, and the blood vessels connecting to main organs were ligated simultaneously before the organs were removed. Also, adult worms present in the organs/tissues

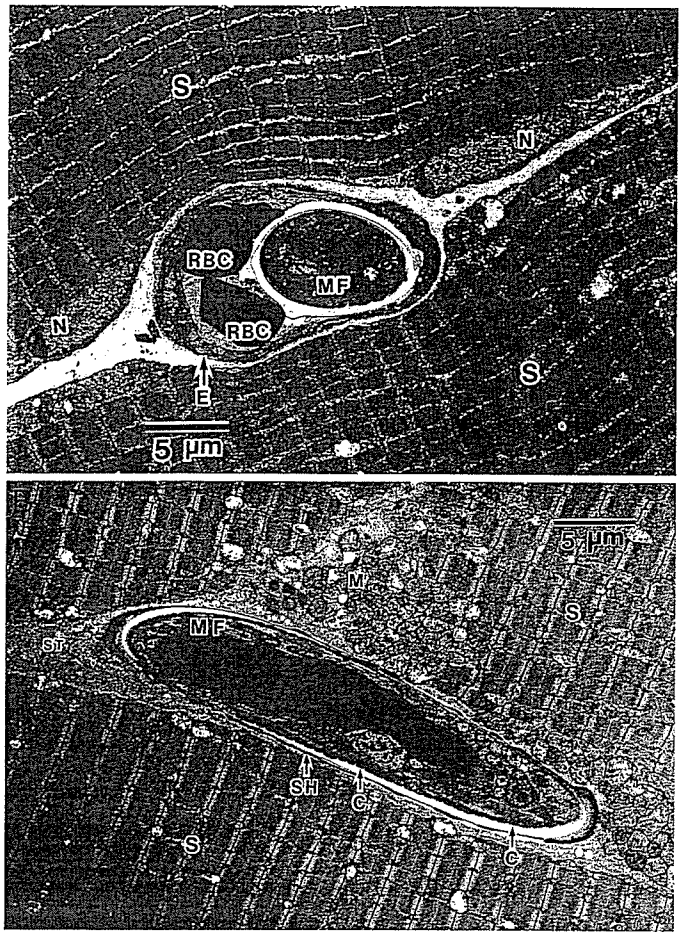

FIGURE 3. Transmission electron micrographs of a microfilaria in the femoral muscle. (above) Cross section of a microfilaria inside a capillary. (below) Oblique section of a microfilaria located in the muscle interstitium. No tissue reaction is observed. C, cuticle; E, endothelium; M, mitochondria; MF, microfilaria; N, nucleus of muscle; RBC, red blood cell; S, striated muscle; SH, sheath; and ST, stroma.

were removed as much as possible to minimize the number of mf that could be released from the uterus. The study revealed that 30 min after saline treatment in control animals, a far greater proportion of mf (44.9–53.0%) was in the lungs, with the highest number of mf per gram of tissue (Table Ia). The accumulation of mf has been well documented by several workers and explained by the difference of pre- and postcapillary oxygen tension in the lung (Hawking and Thurston, 1951; Kawasaki, 1958; Hawking, 1967). A remarkable finding of our experiment was that DEC treatment reduced the relative abundance of mf in the lungs by 80% at 30 min, whereas the abundance in the muscle increased 4.3 times from the pretreatment level (Fig. 2). This finding suggests a possible massive movement of mf into the latter site. This movement can be confirmed more clearly by comparing the numbers of mf per gram of lung and muscle tissues before and 30 min after treatment. Thus, using animal nos. 4–6 (Table Ib), their pretreatment numbers of mf per gram were estimated as follows. First, because relative abundances of mf in the lungs and the muscle were similar among the control animals (Table Ia), and averaged 51.1 and 13.3%, respectively; the total numbers of mf recovered in animal nos. 4–6 were multiplied by 0.511 to obtain the pretreatment numbers of mf in the lungs, and by 0.133 to obtain numbers in the muscle. Second, these numbers were divided by weights (grams) of the lungs or the muscle of individual animals. The estimates obtained were 128,022, 63,167, and 345,230 for the lungs and 658, 262, and 1,759 for the muscle, respectively, in animal nos. 4, 5, and 6. Comparing these numbers with the corresponding figures in Table Ib, the DEC treatment reduced the pretreatment counts/g of lung tissue by 75.4–90.8% and increased those of muscle tissue 3.5–4.6 times. To our knowledge, this is the first report on the DEC-induced mass mobilization of mf from the lungs to muscles. Three to 4 hr after DEC treatment, when peripheral mf counts returned to the pretreatment level, mf in the muscle decreased to 15.0–29.3%, and those in the lungs increased to 25.4–34.1% (Table Ic), suggesting that mf were returning from the muscle to the lungs. A similar movement of mf was not as clear in the other organs/tissues.

TABLE III. Diethylcarbamazine (DEC) concentrations in the organs and blood of jirds 30 min and 3 hr after treatment.

Organ/blood	Recovery*	DEC concentration (μg/g tissue) ± SE	
		30 min	3 hr
Liver	0.410	36.4 ± 9.3	0.354 ± 0.075
Kidneys	0.432	40.7 ± 7.6	0.579 ± 0.149
Lungs	0.418	19.8 ± 3.5	0.225 ± 0.049
Heart	0.542	12.0 ± 3.3	0.247 ± 0.086
Muscle	0.479	12.2 ± 5.4	0.150 ± 0.025
Blood	0.384	4.8 ± 0.9 (μg/ml)	0.134 ± 0.022 (μg/ml)

* Recovery rate of known amount of DEC added to different tissues at 0.1–10.0 μg/g.

The histological study using hematoxylin and eosin-stained tissue sections revealed that, in the control animals, more mf were in the blood vessels than in the capillary/tissue areas and that DEC treatment at 30 min reversed the distribution almost completely. This finding would imply movement of mf away from circulating blood and corresponded directly to the significant reduction of mf counts in the peripheral blood. Release of a large number of mf from the lungs would increase the circulating mf, but this increase was not observed in the present study. The released mf might be "absorbed" very quickly into muscles, or the increase of mf might occur immediately after treatment with a very short duration. In humans, *Wuchereria bancrofti* microfilaremia was induced in the daytime within 5 min after DEC treatment at 2 mg/kg and reached its peak in about 15 min (Katamine, 1972).

To identify exactly where mf are in the muscle, a TEM study was conducted. The results revealed that some morphologically intact mf (21.7%) were located outside blood vessels in control animals and that DEC treatment at 30 min increased the number of extravascular mf to 72.7%. It seems that mf pass through the thin vascular wall relatively easily. However, it is not certain how an object as large as an mf can migrate through a capillary wall, and whether the migration causes damage to capillaries. Dreyer et al. (1992) reported DEC-induced hematuria in *W. bancrofti*-infected cases and speculated direct glomerular damage by mf, although more importance of immunological pro-

cesses was mentioned as a cause of hematuria. Meanwhile, it was reported that DEC did not affect mf that were outside the circulation, e.g., mf of *L. carinii* in the pleural cavity and those of *W. bancrofti* in hydrocoele (Hawking, 1950). A report that platelets play a key role in killing mf in DEC treatment (Cesbron et al., 1987) seems to be in accordance with the above-mentioned report. Considering the mass movement of mf shown in the present study, the extravascular localization of mf could be a mechanism for escaping host effector mechanisms.

It is of interest to consider the reason why muscles are the site of mf accumulation. One important factor would be DEC concentrations in various organs. The concentrations at 30 min were high in the kidneys (40.7 μg/ml) and the liver (36.4 μg/g), whereas they were much lower in the muscle (12.2 μg/g), heart (12.0 μg/g), and blood (4.8 μg/g). Phenomenally, it was as if mf preferred the tissue with a low DEC concentration. Blood showed the lowest DEC level, but it could carry mf to the organs with high DEC concentrations. Although DEC concentration would be one of many factors relating to the accumulation, to our knowledge, the present data are the first to show the quantitative correlation.

In the present study, only a single dose of DEC was given. Because multiple doses resulted in continuous suppression of peripheral mf in the same *B. pahangi*–jird model (Yamashita et al., 1983), it would be interesting to determine whether the muscle could be a site of mf destruction. In human lymphatic filariasis, muscle pain is one of the common adverse reactions of DEC treatment, but only in microfilaremic patients (Dreyer et al., 1994). Based on our results, it is suggested that accumulated, and probably damaged, mf in human muscles could induce inflammatory reactions and cause such a reaction.

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Altered expression of goblet cell- and mucin glycosylation-related genes in the intestinal epithelium during infection with the nematode *Nippostrongylus brasiliensis* in rat

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Yamauchi J, Kawai Y, Yamada M, Uchikawa R, Tegoshi T, Arizono N. Altered expression of goblet cell- and mucin glycosylation-related genes in the intestinal epithelium during infection with the nematode *Nippostrongylus brasiliensis* in rat. APMIS 2006;114:270–8.

Intestinal nematode infection induces marked goblet cell hyperplasia and mucus secretion, but the mechanisms of regulation of the changes still remain to be elucidated. In the present study, epithelial cells were isolated from the rat small intestine at various times after *Nippostrongylus brasiliensis* infection, and the levels of expression of goblet cell- and mucin glycosylation-related genes were estimated by semi-quantitative reverse transcription (RT)-PCR. Among the genes investigated, mucin core peptide (MUC) 2, sialyltransferase (Siat) 4c and trefoil factor family (TFF) 3 were upregulated as early as 2–4 days post-infection, suggesting that they are associated with an early innate protective response. Seven days post-infection and thereafter, when the nematodes reached maturity, significant upregulation of MUC3, MUC4, resistin-like molecule β (Relmβ) and 3O-sulfotransferase (3ST)1 was observed, while 3ST2 expression levels increased after the majority of the worms were expelled from the intestine. Similar alterations of glycosylation-related gene expression were also observed in mast-cell-deficient *Ws/Ws* rats, suggesting that mast cells in the epithelium are not relevant to the upregulation of these genes. The present finding that the expression level of each goblet cell- or glycosylation-related gene was altered differently during the time course of infection indicates the progression of sequential qualitative changes in the mucus layer after infection.

Key words: Mucin; glycosylation; goblet cell; intestine; *Nippostrongylus*.

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Intestinal nematode parasites such as Ascaris and hookworms infect more than 1.3 billion people in the world. Although they cause relatively little mortality, infections result in high levels of morbidity that can result in developmental retardation in infected children (1). Although it has been clarified that Th2 cytokines and/or Th2 cytokine-dependent responses such as mastocytosis and eosinophilia might have crucial roles in protective immunity against certain nematode infections (2), further clarification of factors or mechanisms that are responsible for colonization of worms in and/or rejection of worms from the intestine is an issue of importance for developing more effective measures to combat intestinal parasites. Among factors of potential importance, particularly in-

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teresting factors are produced in and secreted from goblet cells, including mucins and other secretory peptides. In fact, intestinal nematode infection induces marked goblet cell hyperplasia and mucus secretion, which may be induced directly via the local release of bioactive factors or indirectly via the activation of host immune cells (3, 4).

The nematode *Nippostrongylus brasiliensis*, a lumen-dwelling parasite, is a suitable model for studying human and other clinically relevant hookworms because of the similarities of habitat and life cycle (5). Interestingly, the mucus response which is induced by infection with *N. brasiliensis* has been suggested to be responsible for the rejection of nematodes from the intestine, whereas mast cells are not essential for expulsion of this parasite (3, 4). It has been shown that alterations in the terminal sugars of goblet cell mucins are associated with *N. brasiliensis* worm expulsion from the intestine by studies of lectin-binding as well as by biochemical analyses (6–10). Recently, Knight et al. (11) and Pemberton et al. (12) reported that the expression levels of a variety of goblet cell- and/or mucin-related genes were altered during infection with *Trichinella spiralis* in mice. The results indicated that molecular changes that are induced in the epithelial and/or mucus layer during nematode infection are a complicated series of changes whose mechanisms of regulation are virtually unknown, as are the roles of each molecule in mucosal protection.

We therefore attempted to determine by semi-quantitative reverse transcription (RT)-PCR whether there were alterations of the expression of some goblet cell-related genes in the small intestine during the time-course of *N. brasiliensis* infection in rats. The genes examined included those of mucin core peptides MUC2, MUC3 and MUC4 (13–16) and goblet cell-specific secretory peptides such as Relmβ (17) and intestinal trefoil factor (TFF3) (18, 19). Because of the possible importance of the terminal structure of oligosaccharide chains of mucins and/or membrane glycoproteins for colonization and/or rejection of worms, we also examined the gene expression of sialyltransferase, sulfotransferase, fucosyltransferase and some histo-blood group transferases, which might modulate the terminal sugar chains of mucin and/or membrane glycoproteins (20–26).

MATERIALS AND METHODS

Animals, nematode infection and autopsy

Specific-pathogen-free male Brown Norway/Sea (BN) rats and male Fischer (F)-344 rats were purchased from SLC Inc. (Shizuoka, Japan). SPF male and female mast cell-deficient *Ws/Ws* rats were produced in our laboratory as described previously (27). Animals at 8 weeks of age were injected subcutaneously with 2,000 *N. brasiliensis* infective-stage (L3) larvae as described elsewhere (27). The animals were allowed to feed *ad libitum* throughout the experiment.

Preparation of intestinal epithelial cells

The animals were sacrificed with an overdose of ether after overnight fasting with free access to water. The separation of intestinal epithelial cells was carried out at 4°C in EDTA-Hanks' solution (Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution supplemented with 10 mM HEPES, pH 7.3, 1 mM DTT and 1.0 mM EDTA) as described elsewhere (28) with slight modifications. In brief, a piece of jejunum 18–22 cm from the pyloric ring and a piece of ileum 10–14 cm from the ileocecal junction, or a 4-cm-long segment of the proximal colon, were removed, opened longitudinally and cut into segments 1 cm in length. After a brief wash in PBS, 4 pieces of tissue were put into a 15-ml tube containing 4 ml of EDTA-Hanks' solution, and debris attached to the mucosal surface was removed by vigorously shaking the tubes 15 times by hand. The tissues were then transferred into another tube containing EDTA-Hanks' solution. After 75 min on ice with occasional agitation of the tissues by inverting the tubes, the epithelial cells were separated by 60 strokes of vigorous shaking of the tube by hand. After discarding the tissue, detached epithelial cells were collected by centrifugation at 600×*g* for 3 min at 4°C, washed once with EDTA-Hanks' solution, and the cell pellets were stored at −80°C until use. Giemsa staining of the separated epithelial fractions showed not only epithelial cells, but also a small number of mononuclear cells, the majority of which we considered might have been intraepithelial lymphocytes. Histological examination of the tissue after collection of the epithelia showed that villus epithelium was separated completely, whereas the epithelial lining cells in the lower part of crypts were still attached to the tissue in approximately half of the crypts. In uninfected rats, the basal lamina of the epithelium was intact and lamina propria cells were retained in the tissue, but in animals after 10 days of infection, the basal lamina was partly obscured, indicating that some propria mucosal cells contaminated the epithelial fractions. Concerning the stomach, a mucosal scrape specimen of the glandular stomach was prepared by scraping the mucosa with the edge of a glass slide and stored at −80°C until use.

Extraction of total RNA, cDNA synthesis, RT-PCR and relative quantification

Total RNA was extracted using TRIZOL Reagent (Life Technologies, Rockville, MD). Two-microgram aliquots of RNA were reverse transcribed in 20 μl of reverse transcription buffer containing 5 mM $MgCl_2$, 1 mM dNTP mixture, 1U/μl RNase inhibitor, 0.25 U/μl AMV reverse transcriptase and 0.125 μM oligo dT-adaptor primer (Takara RNA LA PCR kit, Takara Biomedicals, Osaka, Japan) at 42°C for 50 min. One-microliter aliquots of the synthesized cDNA were mixed with Sybr Green PCR master mix (Applied Biosystems, Foster City, CA) with appropriate primers and amplified using a real-time PCR system 7300 (Applied Biosystems, Foster, CA, USA). The sense and antisense primers used were:

5′-CGGATCCAATGGAACAGTGG-3′ and 5′-TGCCACTGGTAGGATGATTG-3′ for MUC2; 5′-GTTTCAACTCGACTGCCACC-3′ and 5′-ATAGCTGCAGTTCTTGGAGG-3′ for MUC3; 5′-GCGGAAGAGGAGTGGAGAAG-3′ and 5′-AGATGGCCAGTAGCAAGAGG-3′ for MUC4; 5′-TTCCTTCTCTCGCTGATGGT-3′ and 5′-GCAGTGGCAAGTAGTTCCAT-3′ for Relmβ; 5′-ATGGAGACCAGAGCCTTCTG-3′ and 5′-TGGGATGCTGGAGTCAAAACA-3′ for Tff3; 5′-CTACACCTCTGCGACTTGGT-3′ and 5′-GGTTCTTGACAGCTCCCATC-3′ for Siat4c; 5′-CCCTTTCCCTGAGATCCAGA-3′ and 5′-CCGGCCTTTGGACTCATGTA-3′ for 3ST1; 5′-CCCAGATCCACTTCGTCAGT-3′ and 5′-AAAATTCCCGGAGCTGGTCT-3′ for 3ST2; 5′-AGCAATGGCATGAGATGGTG-3′ and 5′-TCTGGAAGGGTGAAGTTAGC-3′ for FUT1; 5′-GGTGCCGGGAGAACATTAAT-3′ and 5′-GAGAATCCGGAAGGGTGTAG-3′ for FUT2; 5′-GATTTCCCTAGTGCTGCCTC-3′ and 5′-GTTGTGGATACTCTTGGGCT-3′ for FUT4; 5′-ATGTACAAGTGGCCAGCCTA-3′ and 5′-GAATCTTCCCTTCCCCAGAG-3′ for Lew 1. Abbreviated terms for each gene are listed in Table 1. The specificity of each amplified product was confirmed by dissociation analyses giving a single sharp dissociation peak, the absence of the amplified product without reverse transcription, and the appearance of a band of the expected size on electrophoresis of the amplified product. For the amplification of β-actin, Actb primers (Rn00667869, Applied Biosystems) and TaqMan PCR master mix (Applied Biosystems) were used. For relative quantification, standard curves of the threshold cycle (Ct) of amplification of each target against log ng total RNA were created using cDNA samples which showed the lowest Ct value in preliminary runs, and relative quantification was performed for each sample. All quantified values were normalized to those of β-actin (quantified value for a certain target/quantified value for β-actin).

Tissue preparation for histology and goblet cell count

A segment of the jejunum 22–26 cm distal to the pyloric ring and a segment of ileum 6–10 cm from the ileocecal junction were removed, opened longitudinally, fixed in 4% buffered formalin overnight and embedded in paraffin in such a position that histological sections could be cut perpendicular to the luminar surface. Five-micrometer sections were cut and the periodic acid-Schiff (PAS) reaction with hematoxylin nuclear staining was carried out. Ten villi, which were cut as nearly perpendicularly as possible, were selected per animal, and the numbers of goblet cells and numbers of epithelial nuclei in each villus were counted under a microscope. Goblet cell number/100 epithelial cells was calculated as [number of goblet cells/number of epithelial nuclei]×100. The average number of goblet cells/100 epithelial cells in 10

TABLE 1. *Relative expression levels of goblet cell- and mucin glycosylation-related genes in the gut mucosa of normal BN rats. Total RNA was extracted from epithelium separated from the jejunum, ileum or proximal colon, or was extracted from the glandular stomach mucosal scrape, and RT-PCR was performed. Levels of each gene expression were normalized to that of β-actin. In the table, levels in the jejunum were arbitrarily expressed as 1.00*

	Stomach	Jejunum	Ileum	Colon
MUC2 (mucin core peptide 2)	UD	1.0±0.2	4.5±1.8	153.2±49.8[a]
MUC3 (mucin core peptide 3)	UD	1.0±0.4	1.5±0.3	7.5±3.0[a]
MUC4 (mucin core peptide 4)	UD	1.0±0.3	0.7±0.1	41.0±13.1[a]
Relmβ (resistin-like molecule β)	2.8±1.7	1.0±0.4	0.4±0.1	47.3±21.1[a]
TFF3 (intestinal trefoil factor)	0.2±0.1	1.0±0.4	2.7±0.5[a]	3.9±0.4[a]
Siat 4c (α-2,3-sialyltransferase IV)	3.6±1.0[a]	1.0±0.2	11.7±4.1[a]	7.0±3.0[a]
3ST1 (3-0 sulfotransferase-1)	12.7±2.2[a]	1.0±0.2	1.3±0.2	15.4±3.0[a]
3ST2 (3-0 sulfotransferase-2)	1.9±0.7	1.0±0.4	1.1±0.3	3.3±1.6
FUT1 (α-1,2-fucosyltransferase 1)	1.0±0.2	1.0±0.2	1.5±0.1	13.8±0.5[a]
FUT2 (α-1,2-fucosyltransferase 2)	3.6±0.6[a]	1.0±0.1	1.5±0.3	14.3±2.8[a]
FUT4 (α-1,3-fucosyltransferase 4)	4.7±0.9[a]	1.0±0.1	0.7±0.1[a]	3.0±0.2[a]
Lew 1 (Lewis type 1 antigen synthase: β1,3-N-acetylglucosaminyltransferase 5)	0.1±0.0[a]	1.0±0.3	0.3±0.0	2.9±0.9

Data shown are mean±SE of four rats. [a]Significantly different from the levels in the jejunum ($p<0.05$). UD: undetectable.

villi was used as the representative value in a given animal, and means and SE of 4 animals were calculated.

Worm counts

After removing the jejunal and ileal segments for separation of the epithelium and tissue section preparation, the numbers of worms in other parts of the small intestine were determined by the saline incubation method.

Statistical analysis

Student's *t*-test (two-tailed) was employed for statistical analysis; a p-value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Expression of goblet cell- and glycosylation-related genes along the gastrointestinal tract

The relative expression levels of goblet cell- and mucin glycosylation-related genes in the normal BN rat gastrointestinal tract were examined by semi-quantitative RT-PCR (Table 1). Except for Lewl and 3ST2, the goblet cell- and mucin synthesis-related genes examined were expressed at significantly higher levels in the colon than in the jejunum. This may partly reflect the greater abundance of goblet cells in the colon than in the small intestine, in which absorptive cells are the major epithelial constituent, and is consistent with previous reports that MUC2 and MUC4 gene/protein expression levels were higher in the colon than in the small intestine (14, 16). In the stomach, MUC2, MUC3 and MUC4 expression was undetectable, while abundant expression of glycosylation-related genes was observed. This is consistent with the fact that MUC5AC and MUC6 are the major mucin core peptides in the stomach (29).

Goblet cell hyperplasia during the course of Nippostrongylus brasiliensis *infection in the small intestine*

Nippostrongylus brasiliensis larvae reach the small intestine as early as 2–3 days after cutaneous infection, develop to sexual maturity, and begin to lay eggs by 7 days post-infection (PI). However, infection does not continue for a long time: in the normal rat, the majority of adult worms are rejected from the small intestine around 14 days PI by a T-cell-dependent mechanism, leaving only a small number of residual worms in the intestine (5, 30). To examine the kinetics of goblet cell response during the time course of infection, BN rats were infected with 2,000 L3 larvae of *N. brasiliensis* and autopsied 7, 14 and 21 days PI. The numbers of worms recovered from the intestine, excluding the intestinal segments used for tissue preparation and epithelial separation, were 345.8±180.4, 40.0±17.1 and 42.2±32.9 (average±SD) after 7, 14 and 21 days of infection, respectively, showing that the majority of worms were rejected from the intestine by 14 days PI, but a small number of worms escaped the rejection and continued to parasitize the rat at least until 21 days PI. PAS staining of the jejunal and ileal tissue sections revealed goblet-cell hyperplasia 7 and 14 days PI, while goblet cell numbers decreased to preinfection levels by 21 days PI (Table 2).

Alterations of goblet cell- and glycosylation-related gene expression during the course of Nippostrongylus brasiliensis *infection in the small intestine*

At least three types of mucins are expressed in the small intestine: MUC2, which is restricted to goblet cells, MUC3, which is expressed in both columnar and goblet cells, and MUC4, which is expressed in columnar cells (13–16). RT-PCR analyses of gene expression in isolated epithelial cells of BN rats showed upregulation of MUC2, MUC3 and MUC4 in the jejunum, but not in the ileum after infection (Fig. 1), indicating that upregulation of mucin core peptide genes occurred mainly in the local mucosa, which was parasitized by large numbers of worms. In F-344 rats, similar kinetics of gene

TABLE 2. *Number of goblet cells in the jejunum and ileum after* N. brasiliensis *infection*

Days after infection	Jejunum	Ileum
0	13.1±0.7	12.3±0.5
7	19.1±1.2[a]	14.9±1.2
14	21.5±1.2[a]	20.0±0.9[a]
21	11.3±1.0	11.3±1.3

Each measurement was performed on paraffin-embedded tissue sections. Figures in the table represent numbers of goblet cells/100 villus epithelial cells. All data are mean±SE of four rats. [a]Significantly different from day 0 ($p<0.05$).