[69]. In addition, studies have identified numerous lectin-specific glycoconjugates on the surface of the larvae [61,66–68,70–73], and these have been found to dynamically change during the course of infection in murine [74] and rabbit models [75].

Although the antigenicity and specificity of LES is fairly high, cross-reaction to other parasites, especially nematode parasites, have been observed [76]. To overcome this problem, Yamasaki et al. [77] produced a recombinant antigen that reacted with serum from patients with toxocariasis but not from those with roundworm or hookworm infections.

3.2. Rapid diagnostic test for toxocariasis

For many years, numerous diagnostic measures, such as the double gel diffusion test, immunoelectrophoresis, indirect hemagglutination test, latex agglutination test, plate-based ELISA, membrane-based dot-ELISA, etc., have been employed to detect specific antibodies against LES. However, these tests require 1.5 hours or more to obtain an accurate result. In 1997, a new rapid diagnostic test kit for the detection of anti-LES antibody was introduced by us [78]. The test is based on the antigen-sensitized nitrocellulose membrane-based assay. It is easy to perform, does not require any sophisticated apparatus or expertise and the results can be obtained within 3 min. This test kit can even detect the antibody in intraoccular fluid.

4. Conclusion

In this review article, we present an overview of human toxocariasis in Japan. Due to space limitations, we do not describe in detail the aspects of experimental investigations concerning biology, immunology and molecular biology using animal models. However, we briefly pay special attention to Japanese investigators who contributed to advance the understanding of toxocariasis. In early studies, Oshima established a standard method for the oral inoculation of eggs, in which the albuminoid coat of the egg is first removed in order to prevent the adhesion of eggs onto glassware [79]. Sugane is a longtime co-worker of Oshima, and his colleagues are actively engaged in the field of immunology [80–88]. They demonstrated many examples of cellular immunity to *Toxocara* infection in mice. The late Dr. Tsuji made pioneering efforts to develop immunodiagnostic techniques for toxocariasis [50,89,90]. Recently, Mongolian gerbils, *Meriones unguiculatus* have been established as a suitable animal model for experimental ocular and neurologic toxocariasis [91–94].

Human toxocariasis is a public health hazard not only in children but also in adults, both in developing and developed countries. There are still questions to which we have no answers: How does ocular toxocariasis develop? Why do nearly half of ocular toxocariasis patients not produce detectable antibody to LES? What is the pathogenesis of neurologic toxocariasis? What mechanisms are involved in the reemergence of *Toxocara* larvae during pregnancy both in definitive and undefinitive hosts? In addition, we have not yet established an effective anthelmintic against *Toxocara* parasites in the tissue stage, especially for the ocular toxocariasis. Continuous efforts should be made to address these issues. Finally, toxocariasis is a disease that afflicts two of the very best and oldest friends of humans: dogs and cats. Therefore, we must continue to study this puzzling disease both for the sake of humans, and for that of our animal friends.

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Opinion TRENDS in Parasitology Vol.23 No.2

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.

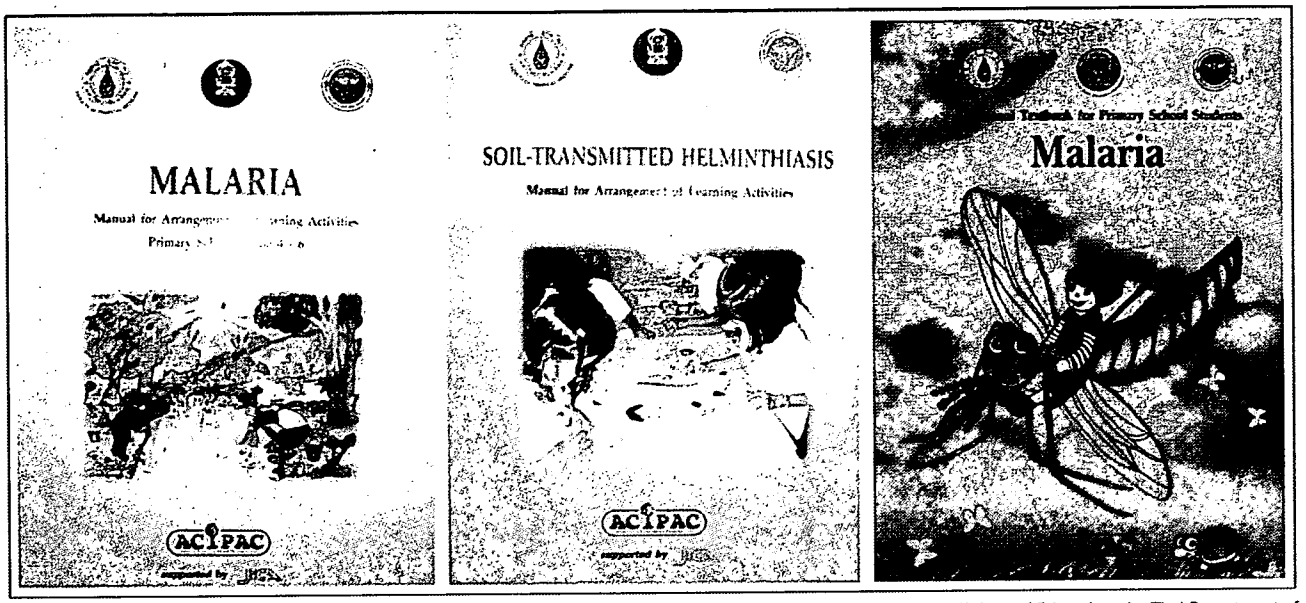

Figure 1. Teachers' manuals and a textbook for schoolchildren developed by ACIPAC in collaboration with OBEC, the Thai Ministry of Education, the Thai Department of Diseases Control, the Thai Ministry of Public Health, and Mahidol University, Thailand.

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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THE EPIDEMIOLOGY OF VISCERAL LEISHMANIASIS AND ASYMPTOMATIC LEISHMANIAL INFECTION IN A HIGHLY ENDEMIC BANGLADESHI VILLAGE

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Abstract. We examined the epidemiology of kala-azar and asymptomatic leishmanial infection measured by serologic and leishmanin skin test results in a Bangladeshi community. In a subset, we measured serum retinol, zinc and C-reactive protein (CRP). Kala-azar and seroconversion incidence were 15.6 and 63.1 per 1,000 person-years, respectively. Proximity to a previous kala-azar case increased the likelihood of both kala-azar and asymptomatic infection. Bed net use protected against kala-azar (rate ratio = 0.35, $P < 0.01$), but not subclinical infection (rate ratio = 1.1, $P = 0.82$). Kala-azar patients were younger ($P < 0.001$) and reported lower red meat consumption ($P < 0.01$) than asymptomatic seropositive individuals. Retinol and zinc levels were lower in current kala-azar patients and those who later developed kala-azar compared with uninfected and asymptomatically infected subjects. The CRP levels were higher in kala-azar patients compared with the other two groups. Low red meat intake and poor zinc and retinol status may characterize a group at higher risk of symptomatic disease.

INTRODUCTION

The intracellular protozoan parasite *Leishmania donovani* is the etiologic agent of kala-azar (visceral leishmaniasis), which is characterized by prolonged fever, weight loss, hepatosplenomegaly, and pancytopenia.[1] In southern Asia, infected humans are the sole reservoir.[1] Kala-azar is a progressive, lethal systemic disease; records of kala-azar outbreaks that occurred before specific drug treatment was available document case-fatality rates > 95%.[2] Nevertheless, most people infected with leishmanial parasites never develop clinical disease.[3,4] In asymptomatic individuals, leishmanial infection is usually detected using serologic tests that measure antileishmanial IgG or the leishmanin skin test (LST) for the delayed hypersensitivity response.[3,5] Positive serologic test results are presumed to reflect a relatively transient increase in serum antibodies caused by recent infection that lasts for months,[6] whereas a positive LST result is thought to indicate durable cell-mediated immunity after asymptomatic infection or clinical cure of kala-azar. A positive LST result may not appear for months to years after successful treatment of kala-azar, but then lasts for decades after exposure.[1,7] The reported ratio of asymptomatic infections to kala-azar cases varies widely from 6:1 in a cohort of children in a community in Brazil endemic for visceral leishmaniasis[5] to 50:1 in an LST survey in Spain.[8] This variation is presumed to reflect differences in parasite virulence and host population characteristics, such as nutritional status and immunogenetic factors,[9,10] and may also depend on the tests used to define asymptomatic infection.[11]

Data on risk factors for asymptomatic leishmanial infection are scarce, and its epidemiology is not well understood. Such information is essential for visceral leishmaniasis prevention and control efforts, such as the elimination program currently being mounted in South Asia,[12] because asymptomatically infected individuals harbor latent parasite and may become ill if immunosuppression occurs[13,14] and may act as leishmanial infection reservoirs. In one study in India, *Leishmania* parasites were visualized in peripheral blood smears of persons with asymptomatic infection.[15] In a Brazilian study, 25% of sand flies fed on kala-azar patients became infected, but none of the sand flies fed on asymptomatically infected humans were infected.[16] Nonetheless, dogs with asymptomatic or presymptomatic *L. infantum* infection have been shown to infect sand flies, albeit less frequently than dogs with symptomatic disease.[17–20] In one canine study, the more severe the symptoms, the higher the proportion of fed flies that became infected.[20] These findings suggest that humans with asymptomatic leishmanial infection may have the potential to transmit leishmaniasis, but are likely to be significantly less infectious to sand flies than patients with active kala-azar. Nevertheless, in many leishmaniasis-endemic communities, ≥ 30% of the population is infected based on skin testing.[3,21–23] Even limited infectiousness of a portion of these people could represent a large reservoir of infection and jeopardize efforts to control or eliminate the disease.

We recently characterized the epidemiology of kala-azar in a highly endemic Bangladeshi village.[24] We found that the strongest determinant of an individual's risk of kala-azar was living with or near a person with recent kala-azar. Living in the same house with a kala-azar patient was associated with a 26-fold increased risk, and living within 50 meters increased risk by 3-fold compared with those living ≥ 50 meters away. Consistent use of locally available untreated bed nets in the summer months and increased density of cattle around the individual's house were associated with significant protection from kala-azar. In the course of our investigation, we also surveyed the population with the recombinant rK39 enzyme-linked immunosorbent assay (ELISA) and LST and measured serum retinol and zinc levels in a subset of participants. In this analysis, our objectives are to describe the incidence and determinants of asymptomatic leishmanial infection compared with those of kala-azar, and to assess the correlation between micronutrient status and the clinical outcome of leishmanial infection.

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METHODS

The study site was a village in Fulbaria subdistrict, Mymensingh district, Bangladesh chosen based on a high incidence of kala-azar reported in government surveillance data in the two years prior to initiation of the study.[24,25] From January to April of 2002, 2003, and 2004, we performed house-to-house surveys to enumerate all residents who had spent at least six cumulative months in the village in the three years prior to data collection. The data collected included demographic information, ascertainment of past and current kala-azar cases, risk factor data, and in participants ≥ 3 years of age collection of a capillary blood specimen and intradermal application of the LST. The protocol was reviewed and approved by the International Centre for Diarrhoeal Disease Research, Bangladesh, Research and Ethical Review Committees and the Institutional Review Board of the Centers for Disease Control and Prevention (CDC). Informed consent was obtained from all adult participants and the parent/guardian of all participating children. Assent was also obtained from children ≥ 7 years of age.

The skin test antigen consisted of a suspension of 5×10^6 *L. infantum* (World Health Organization reference strain MHOM/TN/80/IPT1) promastigotes/mL and was provided by the Istituto Superiore de Sanità (Rome, Italy). The test was applied and read following standard methods: 0.1 mL of antigen was injected intradermally; 48–72 hours later, the induration was measured in two perpendicular directions using the ball-point pen method.[26] The LST result was considered positive if the mean of the two measurements was ≥ 5 mm. Because of substantial loss of leishmanin antigen quality in 2003 and 2004, the current analysis includes only the LST results from 2002.[7]

Serum specimens were tested by ELISA using recombinant K39 antigen (Corixa Corporation, Seattle WA) and a modified protocol that included a standard curve based on a pool of known positive sera on each plate.[6,27] The positive ELISA cut-off was placed at 20 standard curve concentration units (the 99th percentile of specimen readings from 38 people living in a region of Bangladesh not endemic for visceral leishmaniasis), while a cut-off of 61 concentration units demonstrated the best performance for diagnosis of kala-azar.[27]

We defined a past case of kala-azar as an illness with two or more weeks of fever plus at least one of the following: weight loss, abdominal fullness or skin darkening, with clinical improvement after 20 days of intramuscular injections (corresponding to the sodium stibogluconate regimen prescribed by the Bangladesh national kala-azar treatment guidelines at the time). We defined a current case of kala-azar as illness meeting the above definition plus physical examination consistent with kala-azar (splenomegaly and/or hepatomegaly with or without measured fever, evidence of weight loss, skin darkening, or jaundice), and a positive rK39 ELISA result and/or rK39 immunochromatographic strip test (Kalazar Detect; Inbios International, Seattle, WA) result. Among participants with no symptoms suggestive of past or current kala-azar, remote asymptomatic infection was defined by a positive LST result, and recent asymptomatic infection by a positive ELISA result and negative LST result. Participants with a negative ELISA result, a negative LST result, and no history of kala-azar were considered uninfected.

Venous blood specimens for micronutrient assays were collected in 2002 from currently ill kala-azar patients, participants found to be positive by the rK39 ELISA on capillary specimens, and a sample of seronegative participants. Participants were classified by their findings throughout the study period up to the end of follow-up in 2004: those with negative ELISA results and LST results were considered uninfected, and participants with positive ELISA results and/or LST results without clinical findings or history of kala-azar were considered to have an asymptomatic infection. Participants who developed kala-azar ≥ 1 month after specimen collection were considered to have subsequent kala-azar. For the micronutrient analysis, the groups of participants without infection and with asymptomatic infection were frequency-matched by age group to current and subsequent kala-azar patients. The blood specimens for zinc assay were collected in trace element-free tubes from a lot shown to be zinc-free by the CDC National Center for Environmental Health laboratory prior to use. Venous blood specimens were placed immediately after collection in a cold box, protected from light exposure, and transported on ice packs to Dhaka. The serum was separated within six hours of collection and stored at −20°C until processing. Serum retinol levels were measured by high-performance liquid chromatography (Millipore Corp., Bedford, MA).[28] The C-reactive protein levels were determined by the immune turbidimetry method,[29] and were used to assess the presence of inflammation caused by kala-azar, or to other intercurrent infections, which are common in this community, and may affect levels of micronutrients. Serum zinc levels were measured by atomic absorption spectrophotometry (Shimadzu AA 65015; Shimadzu, Nakagyoku, Japan).[30] The generally accepted threshold for defining zinc deficiency based on serum measurements is 60 μg/dL (9.2 μmol/L).[31] Retinol levels less than 30 mg/dL (105 μmol/L) are considered low or possibly responsive to greater intake, and those less than 20 mg/dL (70 μmol/L) are considered as vitamin A deficiency.[32]

Data were analyzed using SAS version 9.0 (SAS Institute Inc., Cary, NC). We examined logistic regression models of risk factors for asymptomatic leishmanial infection in the 2002 survey. The eligible population consisted of those study participants with defined leishmanial status in 2002 (kala-azar or no kala-azar and results available for both ELISA and LST). All study households were mapped by global positioning system and data were uploaded into ArcView Geographic Information System (GIS) version 3.3 (Environmental Systems Research Institute Inc., Redlands, CA). Using GIS data, we calculated the distance from each household to the closest kala-azar case in any of the preceding years. To evaluate the impact of cattle (cows, oxen, or calves) on kala-azar risk for nearby residents, kernel density estimation was used to estimate cattle per 1,000 meters^2.[24] The current analysis focused particularly on the risk factors identified in the published analyses of kala-azar cases with onset from 2000 to 2003: proximity to previous kala-azar cases, use of untreated bed nets in the summer months, and the kernel density of cattle around the subject's house.[24] We tested the following additional variables: household income, materials used in the walls, roof and floor of the house, animal ownership, and dietary intake of fish, goat, beef, and chicken. For comparisons within the current analysis, we also modeled risk factors

TABLE 1

Multivariable logistic regression models for determinants of kala-azar, positive rK39 ELISA results, and positive leishmanin skin test result in 2002*

Outcome	Kala-azar (onset 2001 or 2002)		rK39 ELISA positive in 2002‡§		Leishmanin skin test positive in 2002‡	
	OR (95% CI)†	P	OR (95/5 CI)	P	OR (95% CI)	P
Distance from closest previous kala-azar case						
Same household	6.37 (3.30–12.28)	< 0.0001	1.85 (1.09–3.16)	0.03	2.86 (1.98–4.14)	< 0.0001
Outside household but within 50 meters	1.85 (0.95–3.60)	0.07	1.37 (0.88–2.11)	0.16	1.72 (1.24–2.39)	0.002
> 50 meters away	1.0	–	1.0	–	1.0	–
Use of bed net in summer months						
Always	0.48 (0.31–0.74)	0.001	0.82 (0.51–1.33	0.43	0.98 (0.70–1.38)	0.91
Sometimes or never	1.0	–	1.0	–	1.0	–
Cattle density (additional cow per 1,000 meters^2)	0.75 (0.58–0.96)	0.02	0.97 (0.81–1.16)	0.74	1.17 (1.00–1.36)	0.05
Each 10-year increase in age	0.83 (0.74–0.93)	0.002	1.12 (1.01–1.23)	0.03	1.48 (1.38–1.59)	< 0.0001

* ELISA = enzyme-linked immunosorbent assay.
† Odds ratio (95% confidence interval) adjusted for intra-household correlation by generalized estimating equations.
‡ Past and current kala-azar cases excluded from analysis.
§ Leishmanin skin test–positive subjects excluded from analysis.

for kala-azar, limiting the analysis to those with onset in 2001 and 2002 to approximate the time period in which the ELISA-positive individuals in 2002 were likely to have become infected. All models incorporated generalized estimating equations to account for within-household correlation.

We modeled the incidence of and risk factors for kala-azar and seroconversion for the study period of 2002–2004 using a Poisson regression analysis limited to participants who were ELISA and LST negative at their time of study entry in either 2002 or 2003. The prospective analysis included additional subjects who entered the study population in 2003. New kala-azar cases were defined as those that occurred in this subset of the study population. New subclinical infection was defined as a positive ELISA result in 2003 or 2004 in participants not meeting the case definition for kala-azar. Follow-up time was defined as the period of time from the first negative ELISA test result to the time of seroconversion, onset of kala-azar symptoms, or the date of the last ELISA test result. We also used Poisson regression to model incidence and predictors of negative seroconversion (conversion from a positive ELISA result in one year's survey to negative result in a later survey) in the subset of participants who had at least one positive ELISA result in 2002 or 2003, and at least one ELISA result subsequent to the positive reading. We compared retinol, zinc, and C-reactive protein values between clinical groups (no evidence of leishmanial infection, asymptomatic infections, current kala-azar, subsequent kala-azar) using the Kruskal-Wallis test.

RESULTS

At the time of the 2002 survey, the study population comprised 1,379 people with no history of kala-azar, 151 treated for kala-azar before 2002, and 16 people with current untreated kala-azar. A total of 579 people listed in the 2002 household census data were excluded from this analysis: 229 were < 3 years of age, 114 were unavailable for blood and skin testing, 135 had moved out of the village, 12 had died, 53 had a kala-azar status that could not be determined, and 36 refused to participate. Of those with no history of kala-azar, 788 (57%) were negative by the rK39 ELISA and LST, 396 (29%) positive by the LST only, 138 (10%) positive by the ELISA only, and 57 (4%) positive by the ELISA and LST in 2002.

We attempted to isolate the factors associated with kala-azar and asymptomatic infection by constructing separate logistic regression models using the 2002 survey data (Table 1). As shown in our previous analyses,[24] living in proximity to a recent case of kala-azar carried an increased risk of kala-azar in 2001–2002, and sleeping under a bed net in the summer months, higher density of cattle near the house, and increasing age were protective (Table 1, model 1). When those with past or current kala-azar were excluded from analysis, the likelihood of asymptomatic infection as measured by either ELISA or LST increased with proximity to a previous kala-azar patient and with increasing age (Table 1, models 2 and 3). In contrast to the model for kala-azar, higher cattle density was associated with an increased likelihood of a positive LST result, but had no significant effect on the likelihood of a positive ELISA result. A model that compared kala-azar cases to recent asymptomatic infection demonstrated that living in the same house as a previous kala-azar patient was associated with significantly increased risk, and increasing age and higher consumption of beef or goat meat with protection from symptomatic disease (Table 2). None of the other examined variables (listed in the methods) demonstrated any significant association with risk of asymptomatic infection as measured by ELISA or LST.

In 2003, 396 people with negative ELISA results, LST results, and kala-azar history entered the study, yielding 1,184

TABLE 2

Multivariable logistic regression model for factors associated with risk of kala-azar in 2001–2002 compared with recent asymptomatic infection*

Factor	OR (95% CI)†	P
Distance from closest previous case of kala-azar		
Same household	2.85 (1.45–5.61)	0.003
Outside household	1.0	–
Frequency of beef or goat consumption		
At least twice per month	0.49 (0.26–0.91)	0.03
Less than twice per month	1.0	–
Each 10-year increase in age	0.74 (0.62–0.88)	0.0007

* Recent infection defined by rK39 enzyme-linked immunosorbent assay–positive specimen in 2002.
† Odds ratio (95% confidence interval) adjusted for intra-household correlation by generalized estimating equations.

TABLE 3

Mean serum zinc, retinol, and C-reactive protein (CRP) levels among subjects without leishmanial infection, subclinical infection, current kala-azar, and kala-azar with onset one or more months after specimen collection

Serum micronutrient	Group 1, no infection (n = 68)	Group 2, asymptomatic infection (n = 120)	Group 3, active kala-azar (n = 16)	P* for comparison to Group 1	P* for comparison to Group 2	Group 4, subsequent kala-azar (n = 13)	P for comparison to Group 1	P for comparison to Group 2
Zinc (μg/dL)	59.5	59.6	56.8	0.08	0.06	57.0	0.11	0.11
Retinol (mg/dL)	31.8	34.7	22.9†	0.009	0.002	29.1	0.61	0.19
CRP (mg/L)	4.5	3.2	12.2†	< 0.001	< 0.001	4.8	0.06	< 0.001

* By Kruskal-Wallis test.
† Retinol and CRP data missing for one subject.

individuals who met the inclusion criteria for the Poisson regression analysis of follow-up data through mid-2004. The incidence density of kala-azar and seroconversion were 15.6 and 63.1 per 1,000 person-years, respectively. In Poisson regression models adjusted for age, the consistent use of a bed net in the summer months was protective against incident kala-azar (rate ratio [RR] = 0.35, 95% confidence interval [CI] = 0.16–0.73, $P < 0.01$), but not against new subclinical infection (RR = 1.1, 95% CI = 0.58–2.0, $P = 0.82$).

Among 432 participants with positive ELISA result in 2002 or 2003, 299 converted to seronegative in a subsequent survey. The incidence of negative seroconversion was 502.1 per 1,000 person-years of follow-up. The magnitude of the positive ELISA result affected the incidence of conversion to negative serologic results: the incidence was 706.0 per 1,000 person-years if the reading was 20–60 CU compared with 104.1 per 1,000 person-years for ≥ 61 CU. In a Poisson regression analysis, those with a history of kala-azar and higher ELISA results were significantly less likely to convert to negative serologic results (RR = 0.94 for each increase of 10 ELISA concentration units, 95% CI = 0.89–0.99, $P < 0.05$; RR = 0.40 for history of kala-azar, 95% CI = 0.26–0.61, $P < 0.001$).

Micronutrient data were available for 16 patients with active kala-azar, 13 individuals who developed kala-azar a median of 5.3 months (range = 1–22 months) after the specimens were collected, 120 participants with positive ELISA and/or LST results (asymptomatic infection) by the end of follow-up in 2004, and 68 participants with no evidence of leishmanial infection throughout the study. Currently ill kala-azar patients had lower zinc and retinol levels and higher C-reactive protein levels than those without infection or with asymptomatic infection (Table 3). There was also a trend for lower zinc and retinol levels among the 13 participants who subsequently developed kala-azar, but these differences did not reach statistical significance. The prevalence of low zinc and retinol values was high among all groups (Table 4).

DISCUSSION

Visceral leishmaniasis has a predominantly bimodal pattern of clinical manifestations. Although mild, self-resolving disease has been reported in cohort studies in Brazil,[33] these cases appear to be rare.[4] Most overtly symptomatic visceral leishmaniasis patients have progressive disease that eventually results in death without treatment,[1,2] and those with infection detected by serologic analysis or LST usually report no symptoms.[4] Our data document a ratio of four cases of seroconversion for each incident case of kala-azar, and confirm the finding of many other researchers that asymptomatic leishmanial infection is substantially more common than kala-azar.[5,34,35] However, there are few previous data describing risk factors for the condition and published results are inconsistent, identifying bathing outdoors, playing outdoors, and reported presence of sand flies as risk factors in some models, and reported presence of sand flies as a protective factor in other models.[36]

In our analyses, not surprisingly, proximity to a previous case of kala-azar increased the likelihood not only of kala-azar, but also of asymptomatic infection. The lack of a protective association between bed net use and asymptomatic infection is somewhat surprising in light of our earlier finding that bed net use was strongly protective against kala-azar.[24] Possibly these locally available, untreated bed nets are only partially protective against sand fly bites, decreasing the average parasite inoculum and therefore the risk of kala-azar, but permitting asymptomatic infection. Having cattle around the household may function in a similar manner to limit but not eliminate exposure, leading to the positive association between cattle density and a positive LST result, in contrast to the inverse association between cattle density and risk of kala-azar.[24]

Our analyses were limited in two major ways. The progressive loss of leishmanin skin test antigen quality in 2003 and 2004 prevented the inclusion of LST conversion without de-

TABLE 4

Prevalence of low and deficient zinc and retinol levels among subject without leishmanial infection, asymptomatic infection, current kala-azar, and kala-azar with onset one or more months after specimen collection

Serum micronutrient level	Group 1, no infection (n = 68) n (%)	Group 2, asymptomatic infection (n = 120) n (%)	Group 3, active kala-azar (n = 16) n (%)	Group 4, subsequent kala-azar (n = 13) n (%)
Zinc < 60 μg/dL	35 (52)	58 (48)	12 (75)	9 (69)
Retinol < 30 mg/dL	34 (50)	50 (42)	12 (80)*	6 (46)
Retinol < 20 mg/dL	12 (18)	16 (13)	9 (60)*	4 (31)
C-reactive protein > 5 mg/L	9 (13)	4 (3)	9 (56)*	4 (31)

* Retinol and C-reactive protein data missing for one subject.

tected seroconversion as a criterion for asymptomatic infection in the longitudinal analysis.[7] Our findings also suggest that except for those with kala-azar and/or very high ELISA results, reversion to negative serologic results usually occurs within months after infection. Because our serosurveys were performed at yearly intervals, we may have missed some instances of seroconversion; our estimate of asymptomatic infection incidence is thus a lower bound, rather than a comprehensive assessment. In addition, the relatively small number of kala-azar cases that occurred among those for whom we had zinc and retinol data decreased the statistical power of that analysis.

Nevertheless, our findings add to the published evidence that diet and micronutrient status play a critical role in the susceptibility of *Leishmania*-infected individuals to progress to kala-azar.[9,37,38] The characteristics that help determine whether an infection leads to overt disease appear to include age and dietary factors such as intake of iron- and zinc-rich red meat. Our data are consistent with earlier epidemiologic studies that demonstrated a higher risk of kala-azar in younger individuals[39] and in those with protein-energy malnutrition.[9,40] Recent experimental data in protein energy-, zinc-, and iron-deficient mice suggest that this effect may be mediated primarily through the functional failure of the lymph node barrier and increased early visceralization of the parasite.[37] Immunogenetic factors have been clearly implicated in the risk of symptomatic disease in *L. infantum/chagasi* infection.[10] These factors are also likely to be important in *L. donovani* infection. Nonetheless, modifiable factors such as dietary micronutrient intake may be more immediately relevant from the standpoint of public health.

We found that patients with active kala-azar had significantly lower retinol and zinc levels, and much higher levels of the acute-phase reactant C-reactive protein. The well-known association of many infections with lower retinol levels is usually attributed to reduced retinol transport and synthesis as a direct result of the inflammatory process, but increased excretion may also be a contributor.[41–43] The trend for lower retinol and zinc levels in those who later developed kala-azar likely reflects in part an early disease state in a proportion of these individuals, as shown by elevated C-reactive protein levels. However, it is also possible that lower meat intake and poor zinc and retinol status may characterize a group with an increased risk of disease progression. Moreover, uninfected community members had poor micronutrient status: more than 50% had zinc levels below the commonly accepted threshold for deficiency. Differences in micronutrient levels between clinical groups may thus be obscured by the poor status of the population as a whole, a concept expressed by Rose 20 years ago as the sick population.[44,45] Improved micronutrient status in the population through supplementation and improved dietary intake may have the potential to decrease the proportion of infected individuals who progress to kala-azar and to decrease the burden of visceral leishmaniasis in highly affected areas.

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SHORT REPORT: THERAPEUTIC EFFICACY OF ARTEMETHER-LUMEFANTRINE FOR THE TREATMENT OF UNCOMPLICATED *PLASMODIUM FALCIPARUM* MALARIA IN BANGLADESH

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Abstract. Bangladesh faces increasing levels of chloroquine resistance, and drug sensitivity to sulfadoxine-pyremethamine is already compromised. Therefore, the Ministry of Health recently changed the national treatment guidelines to artemisinin-based combination therapies. The purpose of this study was to determine the baseline therapeutic efficacy of artemether-lumefantrine used as a six-dose regimen for the treatment of uncomplicated *Plasmodium falciparum* malaria. Sixty-seven patients were enrolled in the study; the cure rate in a 42-day follow-up after an adjustment by polymerase chain reaction was 94.3%. The treatment led to rapid fever (mean ± SD = 25.82 ± 12.14 hours) and parasite (30.36 ± 19.43 hours) clearance. These data suggest that artemether-lumefantrine is a highly efficacious and well-tolerated treatment for uncomplicated *P. falciparum* malaria in Bangladesh.

Multidrug resistance of *Plasmodium falciparum* is spreading throughout Asia and is impeding efforts to control malaria. The World Health Organization (WHO) reports a worsening malaria situation in Bangladesh, particularly in the hilly and forested areas in the Hill Tract Districts with reported chloroquine and sulfadoxine-pyrimethamine resistance.[1] Resistance to chloroquine in the southeastern regions of Bangladesh has been known for a relatively long time, but until very recently chloroquine was still the first-line treatment for uncomplicated *P. falciparum* malaria.[2–4] Recently, the Bangladesh Ministry of Health and Family Welfare decided to introduce an artemether-lumefantrine combination for the treatment of uncomplicated *P. falciparum* malaria.

Combinations of chemotherapeutic agents are generally used to accelerate therapeutic response, improve cure rates, and protect the component drugs against resistance. Artemether-lumefantrine has been given priority as a first-line artemisinin-based combination therapy (ACT) recommended by the WHO for the treatment of uncomplicated *P. falciparum* malaria. However, financial constraints and shortcomings in production of adequate drug quantities limit the availability of ACT in extremely poor regions such as Bangladesh. Since data on the efficacy of ACT in Bangladesh is limited, we conducted a baseline study to determine the therapeutic efficacy of artemether-lumefantrine for treatment of uncomplicated *P. falciparum* malaria in the southeastern parts of the country.

The study was performed at the field site of the International Center for Diarrheal Disease Research, Bangladesh in Chakaria, Cox's Bazar district and Lama, Bandarban district, in the Chittagong Hill Tracts, a malaria-endemic area in southeastern Bangladesh, between July and September 2005. The study was carried out by the International Center for Diarrheal Disease Research, Bangladesh in collaboration with the Medical University of Vienna, Austria, and the Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand. Written informed consent was obtained from all study participants and the study protocols were reviewed and approved by the Ethical Review Committee of the International Center for Diarrheal Disease Research, Bangladesh and the Human Use Review Committee of the United States Army.

All patients ≥ 18 years of age with laboratory-confirmed monoinfections with *P. falciparum* were invited to participate in the study. Pregnant women and patients with prior antimalarial treatment within the preceding eight weeks were excluded. All patients agreed to undergo a 42-day follow-up. A total of 83 subjects were screened and 67 (80.7%) were enrolled in the study. Twenty-two (32.8%) patients were men and 45 (67.2%) were women. No patient was lost to follow-up during the 42-day follow-up. The median age was 22 years (range = 18–50 years).

All patients received treatment with artemisinin-lumefantrine. Artemisinin-lumefantrine tablets (Coartem®; Novartis, Basel, Switzerland), each containing 20 mg of artemether and 120 mg of lumefantrine as a coformulated combination, were administered in six consecutive doses: four tablets each at 0 hours and 8 hours on the first day, then twice a day on two consecutive days (total = 480 mg of artemether and 2,880 mg of lumefantrine).

The study design essentially followed the WHO guidelines for the assessment and monitoring of antimalarial drug efficacy with an extension of follow-up until day 42.[5] Patients were asked to return to the study center on days 1, 2, 3, and 7 and then weekly until day 42. If the patients did not return for the scheduled visits or missed an appointment they were seen at their homes by the study staff. Blood smears were prepared and body temperature was recorded at each visit. In case of any symptoms consistent with malaria, patients were advised to return to the center immediately.

Absence of parasitemia until day 42 irrespective of axillary temperature was categorized as an adequate clinical and parasitologic response (ACPR). Patients who had parasitemia and an axillary temperature ≥ 37.5°C on any day from day 4 to

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day 42 were classified as late clinical treatment failures (LCFs), and parasitemia on any day from day 7 to day 42 and an axillary temperature < 37.5°C were classified as late parasitologic failures (LPF). Patients who were parasitemic on day 3 with an axillary temperature ≥ 37.5°C or ≥ 25% of the parasite count on day 0 were categorized as early treatment failures (ETF).[5]

Fever clearance time was defined as the time from drug administration until the body temperature decreased to < 37.5°C and remained so for 48 hours. Parasite clearance time (PCT) was defined as the time from drug administration until the first in a series of negative blood smears. To determine whether the genotype before and after reappearance of parasites in the peripheral blood was identical, indicating a recrudescence, gene loci of pre-treatment and post-treatment sample pairs were compared by a polymerase chain reaction (PCR).

Kaplan-Meier analysis was performed to calculate the proportion of aparasitemic patients for each point in time (later referred to as cure rates). Patients with reinfection diagnosed by PCR and patients who developed *P. vivax* parasitemia during the follow-up were censored.

From a total of 83 screened subjects, 67 were enrolled in the study. Fifty-nine subjects were evaluable for primary endpoint on day 42. Seven patients (10.4%, 95% confidence interval [CI] = 4.3–20.3%) were censored after being treated with chloroquine and primaquine for *P. vivax* parasitemia, which appeared in the course of the follow-up. One patient (1.5%, 95% CI = 0.0–8.0%) was censored after a reappearing *P. falciparum* parasitemia could not be confirmed by PCR.

The overall cure rate after PCR adjustment calculated by Kaplan-Meier analysis after 42 days was 94.3%. The cure rates for day 7, 14, 21, 28 and 35 were 100.0, 100.0, 100.0, 98.3, and 94.7%, respectively. Nine (15.2%, 95% CI = 7.2–27.0%) of the 59 enrolled patients showed reappearance of parasites. All showed relatively late reappearances of parasites within a median time of 34 days (range = 25–39 days). After PCR adjustment, six (10.2%, 95% CI = 3.8–20.8%) had acquired a novel infection, whereas three (5.1%, 95% CI = 1.1–14.1%) were classified as LTFs. Two (3.4%, 95% CI = 0.4–11.7%) of these patients were classified as LCFs, and one (1.7%, 95% CI = 0.0–1.1%) was classified as an LPF. None of the patients had an ETF. Geometric mean parasite densities were not significantly higher ($P > 0.05$) in patients who later showed reappearance of parasites (parasitemia = 0.28%) than in patients who were classified as ACPRs (parasitemia = 0.27%). All patients with a recurrent *P. falciparum* parasitemia were treated according to national guidelines. The mean ± SD time until fever clearance was 25.82 ± 12.14 hours. The mean ± SD PCT was 30.36 ± 19.43 hours and was not significantly longer in patients who had a reappearance of parasites ($P > 0.05$) or in patients who experienced treatment failure ($P > 0.05$) than in those classified as cured.

Treatment was directly observed by the study team and by village volunteers. The overall compliance was good, no patient was lost to follow-up, and no severe adverse events were observed.

Our data suggest that artemisinin-lumefantrine may be superior to previously tested drug combinations (i.e., quinine plus sulfdoxine-pyremethamine) regarding cure rate and parasite and fever clearance.[6] We have previously reported PCR-adjusted cure rates with quinine plus sulfdoxine-pyremethamine in a 42-day follow-up of 87.3% in a comparable group of patients.[7] Because of their short half-lives and to protect the efficacy of artemisinin derivatives, they must be combined with longer-acting antimalarial drugs. Lumefantrine is a highly lipophilic substance and the oral bioavailability varies considerably between individuals and increases greatly if the drug is administered after a meal rich in fat.[8] This remains a limitation to the effective usage of the drug because uptake may be reduced in fasting patients.

The four-dose regimen of artemisinin-lumefantrine showed cure rates greater than 95% in a 28-day follow up in Chinese, African, and Indian patients with either known semi-immunity to malaria or non-drug resistant infections, but showed lower cure rates of 83.3% and 81% in Thailand.[9,10] Studies from Thailand clearly showed that a six-dose regimen is essential for areas with a high percentage of multidrug-resistant *P. falciparum* parasites to reach high levels of efficacy.[10,11] The cure rate of 94.3% in a six-dose regimen after a 42-day follow-up in our study is consistent with previous studies in Laos,[12] along the Thailand-Myanmar border,[13] and in the Chittagong Hill Tracks,[14] with showed cure rates of 93.6%, 98.8%, and 97%, respectively.

The PCT is known to be short in the course of a treatment with artemisinin derivates.[15] We observed rapid parasite clearance as well as fever clearance, which is consistent with previously reported data. Most patients cleared fever (79.1%, 95% CI = 67.4–88.1%) and parasites (83.58%, 95% CI = 72.5–91.5%) within one day of drug administration, and the time to complete parasite clearance was not significantly longer in patients who developed recrudescence than in those classified as cured. This suggests little influence of parasite density on the cure rate.

In the artemisinin-lumefantrine drug combination, lumefantrine is the slower and longer acting partner with a comparatively longer half-life. Lumefantrine has rarely been used in Bangladesh and closely related drugs such as mefloquine were never used as part of the national treatment guidelines. Reduced bioavailability because of limited drug uptake and absorption may therefore have a stronger influence on the cure rate than reduced drug sensitivity.

In conclusion, our data suggest that artemisinin-lumefantrine is a highly efficacious combination with rapid fever and parasite clearance. However, its use in Bangladesh is still constrained by a relatively high cost and difficulties with supplies. Alternative treatments should therefore be explored.

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Malaria parasite induces tryptophan-related immune suppression in mice

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SUMMARY

Plasmodium spp. cause the worst parasitic diseases in humans and evade host immunity in complicated ways. Activated catabolism of tryptophan in dendritic cells is thought to suppress immunity, which is mediated by an inducible rate-limiting enzyme of tryptophan catabolism, indoleamine 2,3 dioxygenase (IDO), via both tryptophan depletion and production of toxic metabolites. In various infections, including malaria, IDO is known to be activated but its biological significance is unclear; therefore, we investigated whether malaria parasites induce IDO to suppress host immune responses. We found that enzymatic activity of IDO was elevated systematically in our mouse malaria model, and was abolished by *in vivo* IDO inhibition with 1-methyl tryptophan. Experimental infection with *Plasmodium yoelii* showed that IDO inhibition slightly suppressed parasite density in association with enhanced proliferation and IFN-γ production by $CD4^+$ T cells in response to malaria parasites. Our observations suggest that induction of IDO is one of the immune mechanisms of malaria parasites.

Key words: cellular immunity, indoleamine 2,3-dioxygenase, *Plasmodium yoelii*.

INTRODUCTION

Efforts to develop an effective vaccine against malaria, which kills 2–3 million African children each year, would be greatly aided by universal recognition of the host–parasite interactions, especially the parasite immune evasion mechanisms. Their complicated and delicate evasion mechanisms include passive avoidance strategies, such as intracellular parasitism, antigenic variations and diversity, and active immune suppression (Hisaeda *et al.* 2005). The latter includes induction of T cell function failure (Xu *et al.* 2002), immunosuppressive regulatory T cells (Hisaeda *et al.* 2004), or bone marrow dysfunction (Helleberg *et al.* 2005).

Dendritic cells (DCs) are antigen-presenting cells that play a central role in both innate and adaptive immune responses. Several pathogens interfere with the host immune response by targeting different functions of DCs, including their maturation and migration (Rescigno *et al.* 2001). Erythrocytes infected with several species of *Plasmodium* activate DCs in terms of their maturation and T cell stimulation (Ing *et al.* 2006), but *Plasmodium falciparum*- and *Plasmodium yoelii*-infected erythrocytes also suppress DC maturation and T cell responses (Urban *et al.* 1999; Ocana-Mongner *et al.* 2003; Millington *et al.* 2006), suggesting a role for DCs in malaria-induced immune suppression.

A series of reports has shown that activated catabolism of tryptophan in DCs induces peripheral immune tolerance, such as feto-maternal tolerance (Munn *et al.* 1998), tumour growing microenvironment (Munn *et al.* 2004), and prevention of autoimmune diseases (Sakurai *et al.* 2002; Gurtner *et al.* 2003). Activated catabolism of tryptophan, an essential amino acid for mammals, is mediated by the inducible rate-limiting enzyme indoleamine 2,3-dioxygenase (IDO), which is also reported to play various roles in host–pathogen relationships. Activated IDO reduces tryptophan and increases its metabolites, including kynurenine, which might have an important role in antimicrobial activity, especially against microbes unable to synthesize tryptophan. On the other hand, tryptophan starvation and accumulation of toxic metabolites leads to apoptosis or anergy of host immune cells, resulting in immunosuppression (Munn *et al.* 2004). It has been reported that infection with microbes, even those that synthesize tryptophan, such as malaria parasites, activates host IDO (Sanni *et al.* 1998). However, the biological significance of IDO activation in these

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infections remains unclear. We demonstrate here that malaria infection in mice activated IDO, and inhibition of IDO during infection partially protected mice against lethal infection with malaria parasites.

MATERIALS AND METHODS

Mice and parasites

Female 8-week-old C57BL/6 mice were purchased from Kyudo, Tosu, Japan. A rodent malaria parasite, *P. yoelii* was used in this study. Commonly, 25 000 *P. yoelii* L or NL strain (PyL or PyNL)-infected red blood cells (RBCs) per mouse were intraperitoneally injected, and the percentage parasitaemia (ratio of parasitized RBCs to total RBC population) was monitored by microscopical evaluation of thin blood films stained with Giemsa solution. All experiments using mice were conducted according to the guidelines for animal experimentation of Kyushu University.

When required, parasitized RBCs were isolated using the Percoll enrichment technique, as previously described (Tosta *et al.* 1980). Briefly, blood of malaria-infected mice was collected with heparin, and passed through a cellulose column to remove white blood cells (WBCs). The RBC solution was added to 70% (v/v) Percoll/PBS and centrifuged, and parasitized RBCs were collected from the interface.

Tryptophan and kynurenine measurement

Tryptophan and kynurenine concentration was measured by high performance liquid chromatography (HPLC). Mouse sera were kept at −30 °C until measurement. All samples were pre-treated with 10% (v/v) 2·4 M perchloric acid, mixed and kept at 4 °C for 15 min. The cells were centrifuged at 10000 ***g*** for 2 min, and the supernatants were used for analysis after passing through 0·45 μm filters. HPLC was performed as previously described, with slight modification (Pawlak *et al.* 2002).

In vivo *IDO inhibition*

1-methyl tryptophan (1mT; Aldrich), the strongest competitive inhibitor of IDO (Cady *et al.* 1991) was first dissolved in 1 M hydrochloric acid, at a ratio of 1 g 1mT in 10 ml of 1 M HCl, and then dissolved in drinking water at 5 mg/ml 1mT in water. The pH was adjusted with 5 M sodium hydroxide solution. This method gave a 2×10^{-3} μM 1mT concentration in serum, as in the study of Uyttenhove *et al.* (2003). This 1mT treatment was started 5 days before malaria infection, and continued until the end of each experiment.

In vitro P. yoelii *culture with 1mT or chloroquine diphosphate*

In vitro culture of *P. yoelii* was started at 10% haematocrit, 5% parasitized RBCs/total RBCs, in RPMI1640 supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 mM *N*-2-hydroxylethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 50 mM $NaHCO_3$, 2 mM L-glutamine, and 10% inactivated non-infected mouse serum. Chloroquine diphosphate (Sigma), an anti-malaria agent, was used as an assay control. Incubation was carried out under mixed gas (N_2 90%, O_2 5%, CO_2 5%) at 37 °C for 20 h. 1mT was added at 2×10^{-2}–10^{-7} M, chloroquine diphosphate was 5×10^{-5}–10^{-10} M. The IC_{50} of chloroquine diphosphate to *P. yoelii* is 5×10^{-8} M (Fujioka *et al.* 1990).

Cell purification and culture

Cell purification was performed using a magnetic cell sorting system, according to the manufacturer's instructions (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany). Spleens of mice were prepared as single cell suspensions. To purify $CD4^+CD25^-$ cells, the suspensions were incubated with fluorescein isothyocyanate (FITC)-anti-CD4 and phycoerythrin (PE)-anti-CD25 antibodies, then anti-PE microbeads were added, and $CD25^+$ cells were removed. Anti-FITC microbeads were added to the flow-through and $CD4^+CD25^-$ cells were obtained. For DC purification, FITC-anti-CD11c antibody was used. The purity of each cell subset usually exceeded 85%.

Purified T cells were cultured with parasitized RBCs in the presence of DCs in 200 μl of RPMI1640 supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES, 50 mM $NaHCO_3$, 2 mM L-glutamine, and 10% inactivated foetal bovine serum on round-bottomed 96-well plates. ConA was added as an assay control at 2·5 μM final concentration. DCs were irradiated with 30 Gy before co-culture with other cells. The T cell population was commonly 1×10^5 cells per well, and parasitized RBCs and DCs were added at the ratio described for each experiment. Culture was performed for 68–72 h in 37 °C air supplemented with 5% CO_2, including 10–16 h co-culture with 1 μCi[^{3}H]-thymidine/well. Cells were harvested onto glass fibre filter mats, dried, and [^{3}H]-thymidine intake was measured by liquid scintillation counter.

Interferon (IFN)-γ ELISA

IFN-γ production *in vitro* was measured in the supernatant of $CD4^+CD25^-$ T cells, parasitized RBCs and DC co-culture. Supernatants were collected after 60 h co-culture, which was performed as described above, and kept at −80 °C until

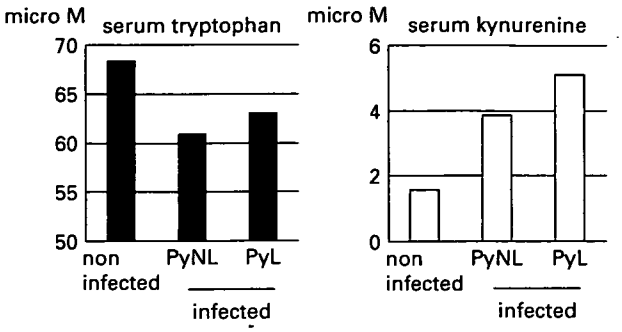

Fig. 1. Tryptophan metabolism is activated in *P. yoelii*-infected mice. Tryptophan (left panel) and kynurenine (right panel) were measured in sera from mice 5 days after infection with PyNL or PyL by HPLC. Data represent micromolar concentrations.

measurement. IFN-γ concentration was measured by ELISA, using the Mouse IFN-γ ELISA Development Kit, DuoSet (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Statistical analysis

We used Student's *t*-test for statistical analysis. $P<0{\cdot}05$ was considered significant.

RESULTS

Infection with P. yoelii *induces hyper-catabolism of tryptophan*

To evaluate the involvement of inducible tryptophan catabolism in the host–parasite relationship during malaria, we first measured its substrate tryptophan, and its metabolite kynurenine. *P. yoelii* has 2 substrains, PyL and PyNL, apparently differing in their virulence for mice. Mice were infected with either of the 2 strains, and serum samples obtained from mice 5 days after infection were analysed with HPLC (Fig. 1). Mice infected with strain Py showed decreased tryptophan and increased kynurenine levels, resulting in a lower ratio of tryptophan to kynurenine. These alterations occurred in mice infected with strains PyL or PyNL, suggesting that infection with malaria parasites induces hyper-catabolism of tryptophan regardless of parasite virulence.

Inhibition of IDO partially protects mice from infection with strain PyL

As IDO is known as an immunosuppressive enzyme, we hypothesized that IDO-mediated hyper-catabolism of tryptophan during malaria leads to less resistance to infection. To test this hypothesis, we used experimentally infected mice treated with IDO inhibitor 1mT. Prior to evaluation of the course of infection, we examined the inhibitory action of 1mT by measuring tryptophan and kynurenin in serum samples from PyL-infected mice treated with 1mT, using HPLC. As shown in Fig. 2A, the treatment suppressed the tryptophan to kynurenine ratio in PyL-infected mice, confirming that infection-induced hyper-catabolism of tryptophan was inhibited by 1mT administration. The mice treated with 1mT exhibited decreased parasite density and a delayed peak of parasitaemia during infection with strain PyL (Fig. 2B, C), although the survival rate was not significantly different among these mice (data not shown). Treatment with 1mT did not alter the course of infection with strain PyNL (Fig. 2D, E). These results demonstrate that inhibition of inducible catabolism of tryptophan by treatment with 1mT enhanced resistance to infection with strain PyL.

1mT does not have a direct parasiticidal effect on Plasmodium *spp.*

As shown in Fig. 2, 1mT treatment of mice was found to significantly enhance protection against strain PyL, probably through cancelling immunological suppression by IDO. To exclude any direct parasiticidal effect of 1mT on malaria parasites, we cultured PyL-parasitized RBCs with various concentrations of 1mT, and analysed parasite growth (Fig. 3). Chloroquine, an anti-malaria drug, reduced parasite growth in a dose-dependent manner. However, 1mT did not affect parasite growth even at concentrations 10 times more than those in the serum of mice treated with 1mT. Taken together with the finding that 1mT did not affect strain PyNL infection, it seems that 1mT does not have any direct effect on parasites.

Inhibited catabolism of tryptophan suppresses malaria-specific immune responses

To analyse the mechanisms involved in the development of effective anti-malaria immunity under inhibition of tryptophan catabolism, we assessed the immune responses in Py-infected mice that had been treated with 1mT. Spleen $CD4^+$ T cells isolated from infected mice were stimulated with parasitized RBCs in the presence of DCs, and were analysed for proliferation and production of IFN-γ. $CD4^+$ T cells from PyL-infected mice treated with 1mT were found to proliferate more than those from infected mice not receiving 1mT, regardless of DC origin (Fig. 4A). In contrast, 1mT treatment of PyNL-infected mice did not promote the immune response, in accordance with our result that 1mT failed to enhance protection against PyNL infection (Fig. 2B, D).

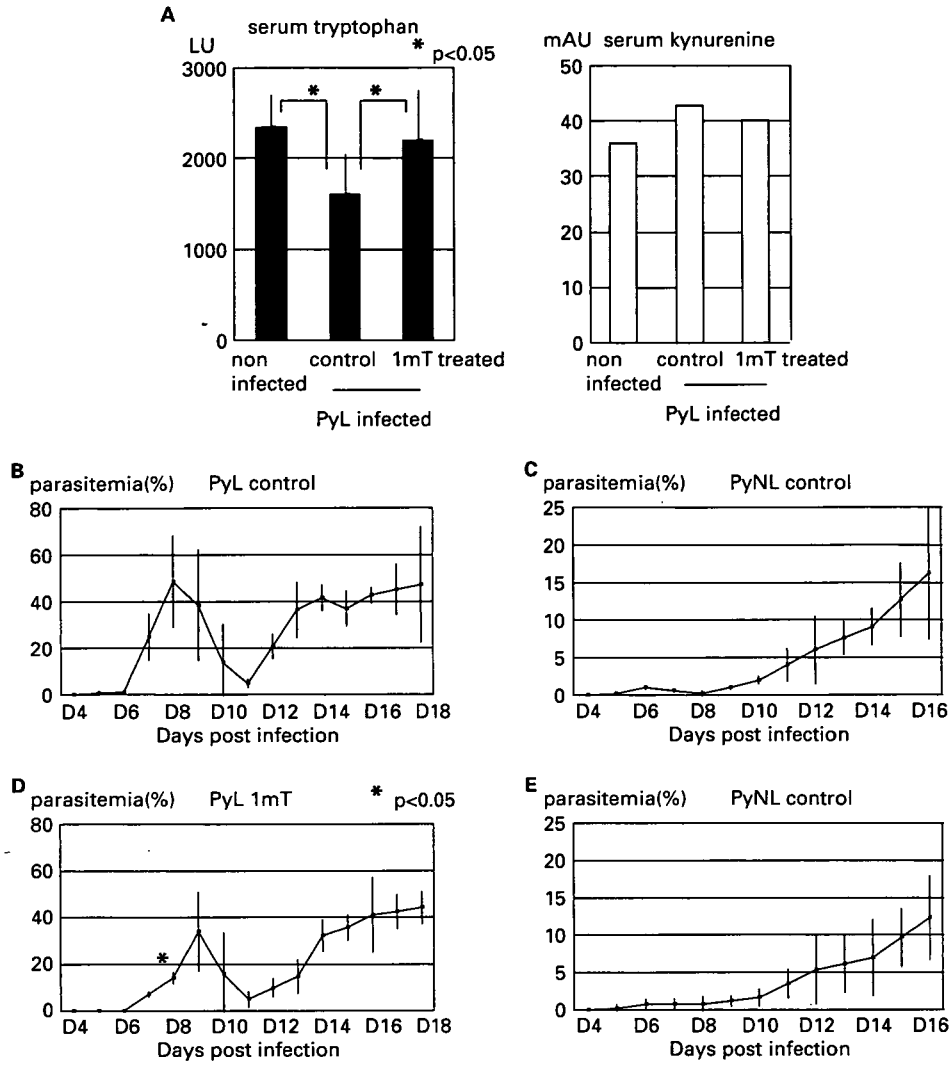

Fig. 2. Effect of IDO inhibition on malaria infection. (A) Serum samples from PyL-infected mice treated with or without (control) 1mT were analysed as in Fig. 1. Data represent luminescence units for tryptophan or milli-absorbance units for kynurenine because the standards for tryptophan and kynurenine were not available in this experiment. Asterisks represent statistical significance ($P<0{\cdot}05$) with Student's t test. (B–E) Frequency of parasitized RBCs (percentage parasitemia) in mice infected with PyL (B, D) or PyNL (C, E), and treated with (D, E) or without 1mT (B, C). Data are presented as the mean ± S.D. from 5 mice per group. Asterisk on (D) represents statistical significance ($P<0{\cdot}05$) with Student's t test between parasitaemia (%) of PyL control and of PyL 1mT at 8 days post-infection. Similar results were obtained in the 2 repeated experiments.

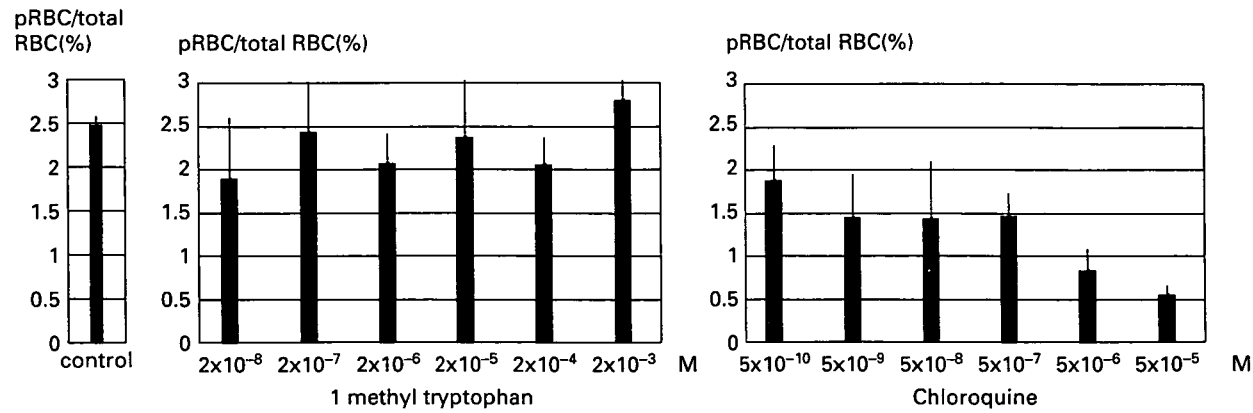

Fig. 3. 1mT does not have direct parasiticidal effect on *Plasmodium* spp. PyL-infected RBCs were cultured with 1mT (centrer panel) or chloroquine (right panel) at the indicated concentrations. Parasitaemia at the culture start point was 5%, and was evaluated after 20 h. Data are presented as the mean ± S.D. from triplicate cultures.