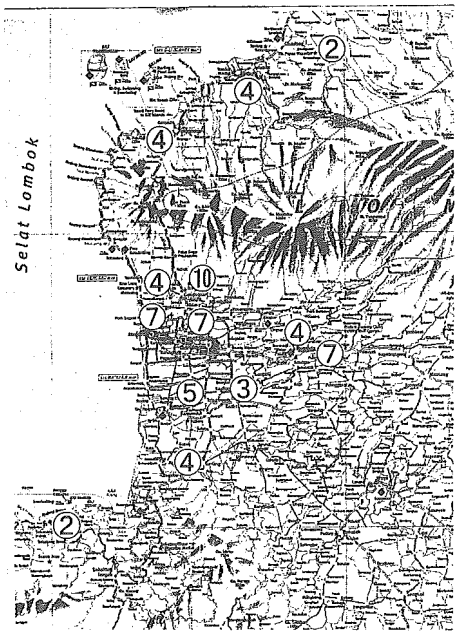

Fig. 3 Number of cerebral malaria cases at Mataram hospital shown by the place of residence

September mentioned in the local report [2]. This type of transmission is thought to be caused by *An. sundaicus* rather than *An. subpictus* according to a previous study by one of the present authors [3]. We obtained the data on malaria patients hospitalized in the Mataram hospital in 2001 and the first half of 2002. In total, 809 malaria patients were hospitalized. These were composed of 580 falciparum malaria patients including 71 with cerebral malaria, 54 vivax malaria patients and 175 clinical malaria patients. A total of 39 died of falciparum malaria, and 29 of these were cerebral malaria patients. We marked the number of cerebral malaria patients on a map according to the place of residence recorded in their patient-reports (Fig. 3). The cases were distributed equally in coastal and inland areas. This indicates that more careful attention should be given to the inland areas to identify malaria endemic foci.

STUDIES IN WEST LOMBOK

1) Survey areas in West Lombok

Malaria epidemiological study target areas were selected on the basis of discussions between TDC and the Nusa Tenggara Barat (NTB) provincial health office. The subdistricts (kecamatan) Batulayar and Sekotong were selected for the preliminary survey. Subjects for blood and spleen examination were randomly selected from different subvillages (dusun) in the two subdistricts. A total of 36 subvillages, or 10 from Batulayar and 26 from Sekotong, were subjected to the survey. Although the number of persons examined in each subvillage was too small for evaluation, we selected three subvillages in Sekotong for the longitudinal survey, namely, dusun Labuhan Poh from desa (village) Sekotong Barat, and dusun Longlongan and Sayong from desa Sekotong Tengah. (Fig. 1, Fig. 4 and Table 1).

2) Geographical differences among subvillages (Table 1)

At subvillage Labuhan Poh, the land gradually rises away from the coast toward the inland. The largest river in this village passes through the subvillage and creates a wide lagoon during the dry season (Photo. 1). Many branches of

Fig. 4 Geographical distribution of the three subvillages selected for malaria survey at Sekotong

Table 1 Geographical features and population of three subvillages of Sekotong Barat and Tengah in 1992

Subvillage	hill	wet rice field	beach	dry field	population
Labuhan Poh	16.5%	0	21.3%	62.2%	855
Sayong	30.6%	67.7%	1.7%	0	1,247
Longlongan	71.5%	4.3%	16.1%	8.1%	805

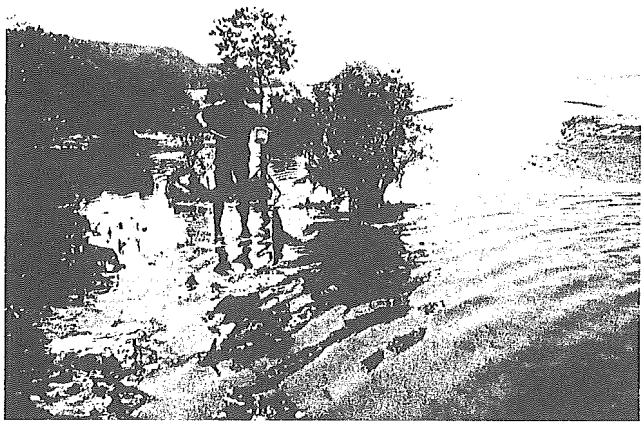

Photo 1. A huge lagoon formed after closing the river's exit to the sea at Labuhan Poh, Sekotong, West Lombok

the river extend into mangrove areas where large mangrove trees have been cut for fuel, leaving many water pools exposed to sunshine and resulting in breeding places for brackish species of *Anopheles* mosquitoes such as *An. sundaicus* and *An. subpictus*. Subvillage Sayong lies on flat ground. Most of the area is occupied by wet rice fields, and in the coastal part of the flat land once covered by mangrove forest, fish ponds were made after the removal of mangrove trees. Subvillage Longlongan has a complex topography; the narrow flat land along the coast, originally mangrove forest, was developed for fish ponds, and the following sharp sloping land leads to a rather flat hilly area where rice fields were developed between stands of grass or bush in the rainy season.

3) Survey methods and subjects

The longitudinal survey was started in August 1992 and carried out five times until June 1993 [4]. At the initial step, in order to determine the seasonal changes in malaria transmission, we intended to collect blood samples from the same subjects randomly selected from all age groups through all the surveys in a year. After the third survey, however, we had to replace the subjects with a new group composed of almost the same proportion of age groups, because of difficulties encountered in obtaining informed consent and cooperation from the former subjects. In the survey, the subjects were usually gathered in one place such as a school or a village health office (pustu) on an appointed date, and a 1–2 ml venous blood sample was obtained from each person along with in a syringe, a drop of blood for thin smear and another drop for thick smear on separate slide-glasses. The blood in a syringe was transferred into a small tube for serum collection. All the samples were carried to TDC for parasitological and serological examination. Medical examination was administered to each person after blood collection, and if necessary, medicines were given. On the same day the entomological survey was conducted, consisting of the examination of breeding sites and larva collection in the daytime, and adult mosquito collection at night.

4) Malaria prevalence in the survey areas

Table 2 and Fig. 5 present the results of the blood examinations. We cannot accurately compare the results of the first three surveys with those of the last two surveys because of the replacement of subjects. In total, the malaria positive rate gradually declined after the first survey in August 1992. However, the malaria transmission trend in each subvillage differed from that in others (Fig. 5). A relatively stable slide positive rate was found in dusun Labuhan Poh in August, October and December 1992, while in other subvillages it varied by month, especially in dusun Longlongan.

Table 2 Results of blood examinations in a longitudinal malaria survey conducted in three subvillages of Sekotong, Lombok from August 1992 to June 1993

	August 1992					October 1992					December 1992				
Subvillage	PR	Number of positive cases				PR	Number of positive cases				PR	Number of positive cases			
	%	Pf	Pv	Pm	Mix	%	Pf	Pv	Pm	Mix	%	Pf	Pv	Pm	Mix
Labuhan Poh	11.5 (14/122)	10	4	0	0	9.8 (12/122)	9	2	0	1	12.3 (15/122)	11	3	0	1
Sayong	11.9 (18/151)	6	11	0	1	6.0 (9/150)	4	5	0	0	6.0 (9/150)	4	5	0	0
Longlongan	21.1 (20/95)	12	8	0	0	6.4 (6/95)	5	1	0	0	2.1 (2/95)	1	1	0	0
Total	14.1 (52/368)	28	23	0	1	7.4 (27/367)	18	8	0	1	7.1 (26/367)	16	9	0	1

	April 1993					June 1993				
Subvillage	PR	Number of positive cases				PR	Number of positice cases			
	%	Pf	Pv	Pm	Mix	%	Pf	Pv	Pm	Mix
Labuhan Poh	3.7 (4/107)	3	1	0	0	1.1 (1/89)	0	0	1	0
Sayong	0.72 (1/139)	1	0	0	0	0 (0/129)	0	0	0	0
Longlongan	1.0 (1/98)	1	0	0	0	9.0 (8/89)	5	2	0	1
Total	1.7 (6/344)	5	1	0	0	2.9 (9/307)	5	2	1	1

PR, positive rate; Pf. *Plasmodium falciparum*; Pv, *Plasmodium vivax*; Pm, *Plasmodium malariae*; Mix, mix infection; ( ), actual number

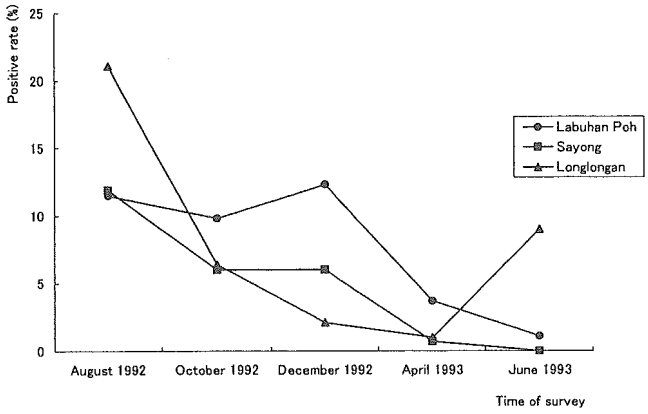

Fig. 5 Slide positive rates in the three subvillages of Sekotong, Lombok from August 1992 to June 1993

This difference may be attributable to the different environmental and geographical conditions of each subvillage (Table1). Especially in dusun Longlongan, the malaria in the hilly area may have a different transmission mode.

5) Entomological observation in the survey area

The results of the entomological examination also showed a wide variety (Table 3 and 4). As expected, a relatively stable number of adult *Anopheles subpictus* mosquitoes were captured at all three subvillages, especially at dusun Labuhan Poh (Table 3), but *An. sundaicus*, *An. barbirostris* and *An. aconitus*, which have been recognized as malaria vectors in Indonesia, were captured sporadically once or twice in five surveys conducted one year except *An. sundaicus* at dusun Labuhan Poh [4, 5]. The fluctuation in the number of captured mosquitoes suspected to be malaria vectors did not correspond to the parasitological data (Table 2, 3 and Fig. 5). The most stable larva collection of the brackish *Anopheles* species was obtained at lagoon and mangrove areas in dusun Labuhan Poh but at fish-ponds in dusun Sayong and Longlongan (Table 4). These results, taken with the parasitological data, indicate that *An. subpictus* (and additionally *An. sundaicus*) play a major role in malaria transmission in these subvillages. The previous intensive study on mosquito fauna in Lombok island by Lee et al.

Table 3 Adult collection of *Anopheles* species known as malaria vector in three subvillages of Sekotong (1992-1993)

SUBVILLAGE	COLLECTION METHOD	Aug 92	Oct 92	Dec 92	Apr 93	Jun 93
		(species / no. mosq. collected per night*)				
LABUHAN POH	Outdoor Human Bait (OHB)	sub/14 sun/4	sub/9 sun/8	sub/6 sun/2	bar/14 sub/4	sub/6 sun/3
	Indoor Human Bait (IHB)	sub/2	sub/11 sun/22	sub/6 sun/5	0 0	sub/4
	Indoor Resting (IR)	0	sun/5	0	0	0
	Bednet Trap (BT)	0	sub/7 sun/16	0	0	0
	Cattle Bait (CB)	sub/11 sun/1 bar/1	sub/12	sub/1	sub/2 acon/1	sub/5 sun/1
SAYONG	OHB	sub/7	sub/14 sun/3	sub/10 acon/5	sub/8 acon/1	sub/1
	IHB	0	sub/4	sub/9 acon/2	acon/2	0
	IR	0	0	0	0	0
	BT	bar/1	0	0	0	0
	CB	sub/5	sub/16	sub/6	acon/4 sub/9	sub/9
LONGLONGAN	OHB	sun/3	sub/15 sun/3	sub/4	sub/19	0
	IHB	0	sub/3 sun/1	sub/17 sun/1	sub/3	sub/1
	IR	0	0	0	0	sub/1
	BT	sun/1	0	0	0	—
	CB	sub/7 bar/1	sub/17	sub/8	sub/10 acon/7	sub/9 acon/1

*40 min per hour from 6 pm–12 pm.
sub, *Anopheles subpictus*; sun, *An. sundaicus*; acon, *An. aconitus*; bar, *An. barbirostris*

Table 4 Type of breeding place and density of *Anopheles* larvae (per dip) in three subvillages of Sekotong

Type Br. Pl.	*Anoph.* Species	Aug. 92	Oct. 92	Dec. 92	Apr. 93	Jun. 93
Labuhan Poh						
I. Lagoon	1. *An. subpictus*	n. d.	1.87	1.30	1.40	0.95
	2. *An. sundaicus*	n. d.	0.70	0.40	0	0.30
II. River	1. *An. flavirostris*	1.00	0	0	0	0
	2. *An. minimus*	0.50	0	0	0.70	0
	3. *An. vagus*	0	0	0	0.11	0.10
	4. *An. subpictus*	1.56	0	1.50	0	0
	5. *An. sundaicus*	0.12	0	0	0	0
III. Mangrove	1. *An. subpictus*	1.00	0	1.60	0.67	(−)
	2. *An. sundaicus*	0	0	0.40	0	(−)
IV. Rice field	1. *An. vagus*	(−)	(−)	1.40	(−)	0.10
V. Fishpond	1. *An. subpictus*	(−)	(−)	(−)	0.10	0
Sayong						
I. Fishpond	1. *An. subpictus*	0.80	0.80	1.00	0.05	0.90
	2. *An. sundaicus*	0	0	0	0.05	0
	3. *An. annularis*	0	0	0	0.05	0
II. Rice field	1. *An. aconitus*	0	0	0.40	0.60	0.25
	2. *An. barbirostris*	0.70	0	0	0	0
	3. *An. vagus*	0	1.50	2.20	0	0.28
III. Fresh water	1. *An. barbirostris*	0.60	0	0	0	0
	2. *An. annularis*	0	0	3.40	0.60	0
Longlongan						
I. Well	1. *An. barbirostris*	0.40	0	0	0	0.35
	2. *An. annularis*	0	0	0	0.10	0
	3. *An. vagus*	0	0	1.20	3.00	0.70
II. River	1. *An. aconitus*	0.02	0	0	0	0
	2. *An. barbirostris*	0.77	0	0	0.40	0
	3. *An. vagus*	0.85	0.67	1.80	0	0.23
III. Ricefield	1. *An. barbirostris*	0	0.05	0	0.25	0.01
	2. *An. annularis*	0	0	0	0.01	0.01
	3. *An. vagus*	1.00	0.80	4.20	2.07	0.55
IV. Fishpond	1. *An. subpictus*	0.08	1.38	1.20	1.80	1.03

(−), no water at the time examined; Br. Pl., Breeding Place; n. d., not done

identified three *Anopheles* species, *An. annularis*, *An. barbirostris* and *An. subpictus*, as potential vectors [6]. Recently, Miyagi et al also found *An. subpictus* and *An. sundaicus* in coastal areas and *An. barbirostris*, *An. leucosphyrus* group and *An. minimus* in fresh water and cited them as potential vectors [7]. In 2001, Sukowati, S. et al., Health Ecology Research Center, NIHR&D found *Plasmodium falciparum* (*P. f.*) sporozoite-positive *An. subpictus* in this area (report to the Indonesian health ministry). In the subvillage Longlongan, in addition to the coastal area, malaria was found in the hilly area where more than half of the population of this subvillage live, but we could not determine the vector mosquitoes there. Because of windy conditions and the collection confined to one night during the survey, the entomological staff were able to capture only a few adult mosquitoes. From the larvae examination we inferred two probable transmission vectors. One is *An. barbirostris*, the larvae of which were found in rice-fields, stagnant water along small rivers and wells, and the other is *An. subpictus*, which was consistently found in fish ponds along the coast and is thought to be able to move back and forth between the coast and the hills with the wind.

6) Endemic situation of malaria

We selected subjects equally from all the age groups to determine the degree of endemicity. Our results showed no difference in malaria prevalence among age groups [4] (data, not shown), indicating a hypo-or meso-endemic pattern in the area. The additional serological examination of antibodies to *P. f.* crude antigens using ELISA also demonstrated a meso-endemic pattern at dusun Labuhan Poh (Fig. 6), that is, the positive rate was low (about 20%) at the age of 0 but rose to nearly 100% at the age of 6 or over. In this area, three *Plasmodium* species were detected, that is, about

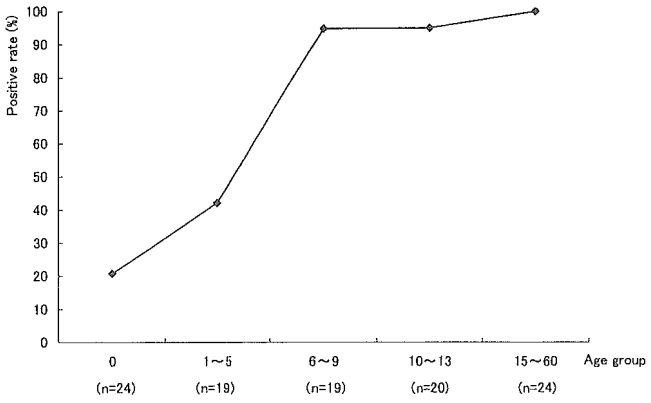

Fig. 6 Sero-positive rate to P. f. crude antigens among persons examined in subvillage Labuhan Poh in October 1992

60% *P. falciparum*, 40% *P. vivax* and only one *P. malariae* (Table 2). The *P. malaria* case was confirmed by PCR using the ribosomal DNA sequence [8]. Our results did not confirm the peak of transmission between July and September as described in the local report [2].

STUDIES IN SUMBAWA

1) Survey areas in Sumbawa

In Sumbawa, four subvillages in different subdistricts were examined for prevalence of malaria from 1996 to 1999 (Fig. 1). One subvillage, dusun Medang, is a small island accessible in one hour from Sumbawa Besar by small motorboat. In this subvillage, the preliminary spleen examination was

Table 5 Parasite positive rate and spleen rate in the longitudinal survey conducted in three subvillages of Sumbawa

	December 1996					July 1997				
Subvillage	Positive Rate (%)	Number of positive cases Pf	Pv	Mix	Spleen Rate (%)	Positive Rate (%)	Number of positive cases Pf	Pv	Mix	Spleen Rate (%)
Penyaring	7.1 (8/112)	7	1	0	0	—	—	—	—	—
Labangka IV	14.3 (16/112)	10	6	0	8.0	7.1 (8/112)	4	4	0	14.3
Stowe Brang	33.9 (38/112)	8	25	5	25.9	15.3 (17/111)	13	3	1	34.2
Total	18.5 (62/336)	25	32	5	11.3	11.2 (25/223)	17	7	1	16.0*
	March 1998					October 1998				
Subvillage	Positive Rate (%)	Number of positive cases Pf	Pv	Mix	Spleen Rate (%)	Positive Rate (%)	Number of positive cases Pf	Pv	Mix	Spleen Rate (%)
Penyaring	1.8 (2/112)	2	0	0	0	0.9 (1/112)	1	0	0	0
Labangka IV	0.9 (1/112)	1	0	0	0	13.0 (9/69(74))**	4	3	2	23.0
Stowe Brang	8.0 (9/112)	7	2	0	12.5	1.8 (2/112)	1	1	0	4.5
Total	3.6 (12/336)	10	2	0	4.2	14.1 (12/293)	6	4	2	7.4
	December 1998					February 1999				
Subvillage	Positive Rate (%)	Number of positive cases Pf	Pv	Mix	Spleen Rate (%)	Positive Rate (%)	Number of positive cases Pf	Pv	Mix	Spleen Rate (%)
Penyaring	0.9 (1/112)	1	0	0	0	3.6 (4/111)	3	1	0	0
Labangka IV	5.3 (5/95)	1	4	0	1.1	5.4 (6/111)	2	3	1	0
Stowe Brang	0 (0/112)	0	0	0	0	ND (−/108)	ND	ND	ND	0.9
Total	1.9 (6/319)	2	4	0	0.3	4.5 (10/222)	5	4	1	0.3

P. f., *Plasmodium falciparum*; P. v., *Pl. vivax*; Mix, mix infection

−,data lost

* The number of subjects in Penyaring was assumed to be 112.

** 74 persons were subjected to spleen examination, but only69of these underwent blood examinations.

ND, not done

conducted on 161 1st and 2nd grade school-children and showed a 42.9% spleen rate (meso-endemic), but afterwards neither blood nor mosquito examinations was conducted because of the risk of the available boat capsizing. Therefore, three subvillages were selected for the longitudinal survey. The methods were the same as those used in Lombok.

2) Malaria prevalence in the Sumbawa survey areas

The slide positive rates and spleen rates at three subvillages are shown in Table 5. All three subvillages are located along the coast. Subvillage Penyaring and Stowe Berang face the ocean to the north and subvillage Labangka IV to the south. The former two subvillages are geographically similar. They have mangrove beaches and flat lands. The mangrove beaches were developed for fish ponds in both subvillages. Despite the environmental similarities, subvillage Penyaring showed a very low slide-positive rate and 0% spleen rate, while dusun Stowe Berang showed rather high positive rates for both examinations. Subvillage Labangka IV showed a medium endemic pattern with seasonal epidemics.

3) Entomological observation and epidemiological analysis

The entomological examination clearly demonstrated a high density of adults and larvae of *An. subpictus* at subvillage Stowe Berang but a very low density at subvillage Penyaring (data, not shown). This was due to the difference in breeding sites between the two subvillages, namely, many abandoned fish-ponds with algae and weeds were found at the former (Photo. 2) while most of the fish ponds were well maintained at the latter. The sharp decline in positive rates for spleen and blood examinations at dusun Stowe Berang from October 1998 was due to two malaria control projects conducted from January 1998 for a year, that is, the distribution of insecticide impregnated mosquito-nets and the cleaning of abandoned fish-ponds (Fig. 7). Our reports in 1996 and 1997 note that these control projects were conducted by the Sumbawa district health office, and suggest that the control methods worked effectively. In subvillage Labangka IV, an outbreak of malaria was observed just before our survey in October 1998. This subvillage has a very narrow sandy beach with a steep cliff rising behind. A rather flat hilly area spreads away from the cliff. The entomological survey found that the captured *Anopheles* mosquitoes were exclusively *An. subpictus* and that there were several lagoons on a small beach where *An. subpictus* larvae bred. According to staff in the Sumbawa district health office, the outbreak may be related to the custom of villagers to gather around the cliff (cape) at night to catch a species of bird during this season.

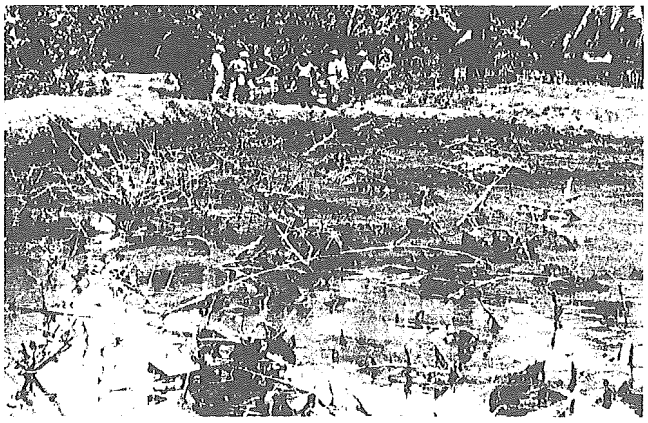

Photo 2. An abandoned fish pond at Stowe Brang, Utan, Sumbawa

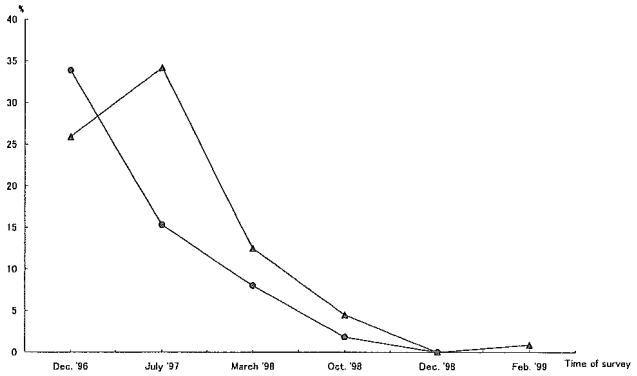

Fig. 7 Changes in slide-positive rate (●) and spleen rate (▲) in Stowe Brang, Sumbawa during the three-year period after malaria control activities in January 1998

SUMMARY

Although our data are still insufficient to determine the full range of epidemiological features, we can draw the following conclusions about malaria in Lombok and Sumbawa.

1) Malaria endemic areas are located mainly along the seacoast and less frequently inland.

2) The degree of endemicity is hypo-endemic to meso-endemic.

3) The main transmission vectors are *Anopheles subpictus* and *An. sundaicus*, which breed in brackish water.

4) Although similar species of vector play a role in transmission in coastal endemic foci, the mode and the season of transmission vary with the ecological characteristics of the vector and social and environmental conditions.

5) Small endemic foci are found in hilly areas inland, but the responsible vector species have not been determined.

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ORIGINAL ARTICLE

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Glucose-6-phosphate dehydrogenase (G6PD) mutations in Cambodia: G6PD Viangchan (871G > A) is the most common variant in the Cambodian population

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Abstract We conducted a survey of malaria diagnoses and glucose-6-phosphate dehydrogenase (G6PD) testing in remote areas of Cambodia. Blood specimens from 670 people were collected by the finger-prick method. Of these people, 24.9% were found to have malaria, and 7.0% of people were G6PD deficient. In the Khmer, the largest ethnical population in Cambodia, the G6PD deficiency rate of males was 12.6% (25/199) whereas the rates in the minorities of the Tum Pun and the Cha Ray were 1.1% (1/93) and 3.2% (2/63), respectively. Of the G6PD-deficient subjects, 97.9% (46/47) were G6PD Viangchan (871G > A), and only one case (2.1%) was G6PD Union (1360C > T). Since G6PD Mahidol (487G > A) is common in Myanmar according to our previous study, the current finding suggests that the Cambodian population is derived from homogeneous ancestries and is different from the Myanmar population. All G6PD Viangchan cases were linked to two other mutations of 1311C > T and IVS-11 nt93T > C in the *G6PD* gene.

Keywords Cambodia · Glucose-6-phosphate dehydrogenase deficiency · Khmer · Malaria primaquine · Viangchan

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Introduction

We have introduced rapid diagnosis methods for malaria (Kawamoto and Billingsley 1992) and glucose-6-phosphate dehydrogenase (G6PD) deficiency (Hirono et al. 1998; Tantular and Kawamoto 2003) to malaria endemic areas in Southeast Asian countries. Using these methods, patients are notified of the results of blood examination within 30 min and are able to receive antimalarial medicine, including primaquine (Tantular et al. 1999; Jalloh et al. 2004). Primaquine can kill gametocytes, the sexual stage of malaria parasites, which are the cause of malaria transmission to mosquitoes. Once primaquine is introduced in a community, new malaria cases decrease because the number of malaria-infective mosquitoes decreases (Matsuoka et al. 1987). However, when G6PD-deficient persons take primaquine, a hemolytic attack can occur. Without G6PD, erythrocytes cannot prepare a sufficient amount of reduced pyridine nucleotide, and reduced glutathione and cannot prevent oxidant attack by primaquine. Thus, primaquine should not be administered to malaria patients before confirming their G6PD activity status.

G6PD deficiency is one of the most frequent hereditary abnormalities. The *G6PD* gene exists on the X-chromosome distributed in 13 exons (Chen et al. 1991). Almost all G6PD deficiencies are caused by one amino-acid change caused by a point mutation of the genomic DNA, and 140 molecular variants of the *G6PD*

genotype have been identified (Beutler and Vulliamy 2002). The incidence of G6PD deficiency is said to be high in malaria-endemic areas. More than 10% of the male population is G6PD deficient in African and Southeast Asian countries where malignant malaria *Plasmodium falciparum* is endemic. In contrast, in Japan, northern China and north European countries, where falciparum malaria is historically not endemic, the incidence of G6PD deficiency is less than 0.1%.

During the surveys of malaria and G6PD deficiency, we collected G6PD-deficient blood samples after receiving informed consent and read the genomic DNA of *G6PD*. We have described *G6PD* variants in various countries or ethnic groups in Asian countries (Iwai et al. 2001; Matsuoka et al. 2003a, b, 2004). In Cambodia, the rapid tests for malaria and G6PD activity have not been introduced yet. We intended to introduce these methods in remote areas in Cambodia. We also tried to obtain genomic information on the G6PD molecule. This report is the first description of *G6PD* variants in Cambodian people.

Materials and methods

Between 2003 and 2004, surveys were conducted by the National Malaria Center, Cambodia, at 12 villages in three provinces in Cambodia. These were Battambang Province, Kampot Province, and Rattanakiri Province. A small test unit was opened at each site, and local staff announced to the residents that we would perform rapid, on-site examinations for malaria and G6PD activity. This study was approved by the Department of Health, Cambodia.

People were first registered by names, ages, genders, and ethnicity. Next, three drops of blood were collected from the fingertip: one for malaria diagnosis, one for hemoglobin concentration, and one for G6PD test. Malaria was diagnosed by the acridine orange staining method (Kawamoto and Billingsley 1992), and hemoglobin concentration was measured using a battery-powered HemoCue machine (Angelhorm, Sweden). For G6PD activity test, we used the G6PD Assay Kit (Dojindo Laboratories, Tokyo, Japan). Five microliters of blood was added to a G6PD test tube containing 800 µl of sample reagent. The contents were vigorously mixed to destroy red blood cells in the tube and incubated for 20 min at ambient temperature. When G6PD activity was normal, orange color appeared and became dark. When G6PD activity was deficient, the faint red color of hemoglobin did not change during the incubation time. The reaction was stopped by adding 10 µl of 1 N HCl. Judgment was made by comparison of the color with positive and negative control tubes (Tantular and Kawamoto 2003).

Anemia is a potential cause of misdiagnosis of G6PD deficiency. In this field study, we measured hemoglobin concentration as well as G6PD activity. When we met a case showing low G6PD activity and low hemoglobin level (less than 8.0 g/dl), we tested for G6PD activity again using an increased amount of blood in the G6PD test tube. For instance, 10 µl of blood was added when the Hb level was 6.0 g/dl; 7.5 µl of blood was added when the Hb level was 8.0 g/dl. With this alteration, we could divide individuals into (1) full activity of G6PD, (2) partial deficiency group, and (3) complete deficiency group.

All results were reported to each individual within 30 min after taking blood. When malaria patients were found, we gave them artesunate and mefloquine. We did not give primaquine to malaria patients in these surveys because the national health policy of Cambodia does not currently allow administration of primaquine to malaria patients.

When we found a person whose G6PD activity was low, we asked the person if we could take 0.2 ml of venous blood to read the *G6PD* gene. We explained the purpose of the investigation and received informed consent. Blood samples collected were stored at 4°C and brought back to Japan, and G6PD activity was confirmed by another G6PD test developed by Fujii et al. (1984). The DNA sequence of *G6PD* was then identified. Since genomic *G6PD* consists of 13 exons, we prepared primers for each exon (Hirono et al. 1994), amplified the exon by PCR, and read the DNA sequence (ABI PRISM 310; PE Biosystems, CT, USA). Both strands of each exon were sequenced.

Results and discussion

We conducted malaria diagnosis and treatment with G6PD activity testing in remote areas of Cambodia. Blood samples from 670 people were collected by the finger-prick method at 13 sites in four provinces (Fig. 1). Among those tested, we found 167 malaria cases and 47 cases of G6PD deficiency (Table 1). We detected 29 complete G6PD-deficient cases among 360 males (8.1%) and two completely and 16 partially G6PD-deficient cases among 310 females (5.8%). In the Khmer, representing more than 90% of the population of Cambodia, the G6PD deficiency rate among males was 12.6% (25/199) and that among females 13.8% (17/123).

Partial G6PD deficiency in females was difficult to find because anemia is common in malaria-endemic areas. Anemic cases show partial G6PD deficiency because the number of red blood cells in the sample volume (5 µl) in an anemic case is lower than that in a nonanemic case. Thus, we tried to adjust the sample volume by measuring the hemoglobin level. We measured hemoglobin levels in each person. When we met a subject who showed low G6PD activity and low hemoglobin level (less than 8.0 g/dl), we tested her G6PD activity one more time, increasing the amount of blood added to the G6PD test tube. In this way, we could separate the group that showed G6PD deficiency due to anemia and succeeded in diagnosing partial deficiency in the female group.

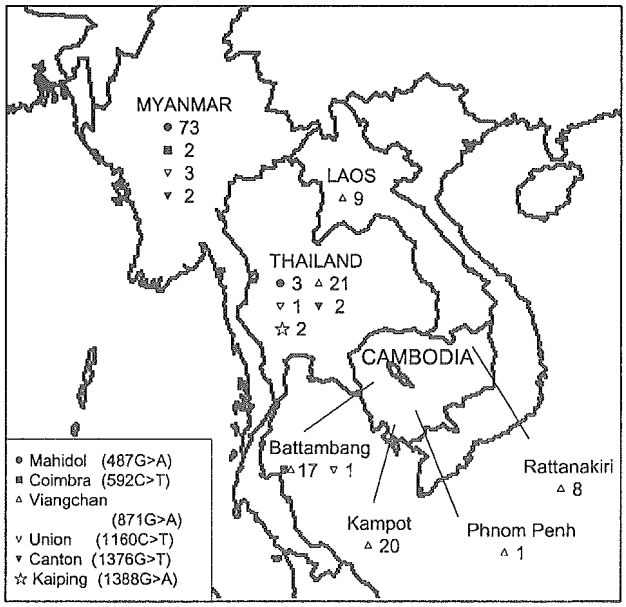

Fig. 1 Distribution and frequencies of glucose-6-phosphate dehydrogenase (*G6PD*) variants in Southeast Asian countries. *Each number* indicates the number of G6PD-deficient cases confirmed by sequence analysis. Data of Laos, Myanmar, are from our previous reports (Iwai et al. 2001; Matsuoka et al. 2004). Data of Thailand are from the report by Nuchprayoon et al. (2002)

The G6PD deficiency rates in males in malaria-endemic areas were 10.5% (45/430) in Myanmar and 3.9% (25/648) in Indonesia (Jalloh et al. 2004). Compared with these rates, the rate of 8.1% (29/331) in Cambodian males was reasonable. Comparing ethnic groups, 12.6% (25/199) in Khmer males was statistically higher whereas the rates in minorities of the Tum Pun and the Cha Ray were 1.1% (1/93) and 3.2% (2/63), respectively ($P < 0.01$). Everett et al. (1977) reported that the rate of G6PD deficiency in Khmer males was 14.2% (15/106). Compared with their report, our result of 12.6% was reasonable. We cannot explain at present why the rates of G6PD deficiency in the Tum Pun and the Cha Ray were low. There is a hypothesis that the rate of G6PD deficiency becomes high in places where falciparum malaria is endemic (Ruwende et al. 1995). If this hypothesis is correct, the Tum Pun and the Cha Ray perhaps moved from malaria-free areas in recent years. Or perhaps falciparum malaria was introduced in their habitant areas in the recent years.

During these activities, we received informed consent from G6PD-deficient persons to analyze their *G6PD* gene. We analyzed 47 cases and found that 46 (97.9%) were G6PD Viangchan (871G > A) (Table 1). These results indicate that the Cambodian population was derived from a homogeneous ancestry. Among 47 cases of G6PD Viangchan, 42 were Khmer, two were Tum Pun, two were Cha Ray, and one was Indian. This is the first case of G6PD Viangchan found from an Indian. He did not know when his ancestor moved from India to Cambodia or whether one of his ancestors married with a Khmer person. We found one case of G6PD Union heterozygote (1360C > C/T). Her mother was Khmer and her father was from Laos. We could not obtain further information on her parents.

In Myanmar, we carried out malaria diagnosis and G6PD activity test in remote areas. We analyzed 80 cases of G6PD variant from Myanmar and obtained a result that 91.3% (73/80) of G6PD variants were G6PD Mahidol (487G > A) (Matsuoka et al. 2004). This suggests that the Myanmar population is derived from homogeneous ancestries. Interestingly, we found no cases of G6PD Viangchan in Myanmar. Similarly, we found no cases of G6PD Mahidol in Cambodia in the present study, indicating that Myanmar and Cambodian people are different from each other in terms of *G6PD* variant.

In Thailand, G6PD Viangchan (871G > A) was the most common variant in the Thai population (Nuchprayoon et al. 2002). In that study, 21 cases of G6PD Viangchan (67.7%) were found among 31 cases of G6PD deficiency while G6PD Mahidol comprised 9.7% (3/31). When we compare that report and our results, we

Table 1 Incidence of malaria and G6PD deficiency in Cambodia

Province	Villages	Ethnicity	Male				Female				
			No. of tested	Malaria positive (%)	G6PD activity		No. of tested	Malaria positive (%)	G6PD activity		
					N[a]	CD[a] (%)			N[a]	PD[a] (%)	CD[a] (%)
Battambang	1 and 2	Khmer	84	17 (20.2)	73	11 (13.1)	53	2 (0.4)	46	7 (13.2)	0 (0)
Kampot	3 and 4	Khmer	98	29 (29.6)	86	12 (12.2)	59	16 (27.1)	51	6 (10.2)	2 (3.4)
Rattanakiri	5–7	Tum Pun	93	31 (33.3)	92	1 (1.1)	115	31 (27.0)	114	1 (0.9)	0 (0)
	8–10	Cha Ray	63	18 (28.6)	62	2 (3.2)	72	18 (25.0)	72	0 (0)	0 (0)
	11 and 12	Khmer	17	3 (17.6)	15	2 (13.3)	11	2 (18.2)	9	2 (22.2)	0 (0)
Phnom Penh	NMC[b]		5	0 (0%)	4	1 (20.0)					
Total			360	98 (27.2)	331	29 (8.1)	310	69 (22.3)	292	16 (5.2)	2 (0.6)

[a] *N* Normal activity, *PD* partially deficient, *CD* completely deficient. Sequencing analysis of *G6PD* confirmed that PD was heterozygote and CD was hemizygote in males or homozygote in females

[b] Samples were from staff of the National Malaria Center. The ethnicity was three Khmers, one Chinese, and one Indian

conclude that the Thai population might consist of people from Myanmar and people from the Khmer.

In Malaysia, Ainoon et al. (2003) demonstrated nine *G6PD* variants among 84 cases of G6PD deficiency in Malaysian Malays. The main variants were G6PD Viangchan (38.1%), G6PD Mediterranean (27.4%), and G6PD Mahidol (15.5%). They concluded that Malaysian Malays had various ancestral contributions.

In Indonesia, we reported that people in Flores Island showed a heterogeneous ancestry (Matsuoka et al. 2003b) because we found five *G6PD* variants among 15 G6PD-deficient persons. Flores Island belongs to the Sunda Archipelago where people might have come from Eurasian countries, African countries, Philippine Islands, and Pacific Islands. Historically, Flores Island might have accepted many tribes from different origins. Thus, people in Thailand, Malaysia, and Indonesia are of heterozygous ancestry. However Myanmar and Cambodians show homozygous ancestries in terms of *G6PD* variant.

G6PD Viangchan (871G > A) is said to link to two other mutations in *G6PD*. One is nucleotide 1311C > T on exon 11 and another is a T > C substitution in intron 11, 93 bp downstream of exon 11 (IVS11 nt93) (Beutler et al. 1992). We read these two points in all cases and confirmed that all cases of G6PD Viangchan (871G > A) had these two mutations. This result is in accordance with the data from the other countries, e.g., Laos (Iwai et al. 2001), Thailand (Nuchprayoon et al. 2002), and Malaysia (Ainoon et al. 2003), describing that all cases of G6PD Viangchan (871G > A) linked to 1311C > T on exon 11 and T > C in nt93 on IVS11. In the case of G6PD Union (1360C > T), *G6PD* contained the wild type (1311C and IVS-11 nt93T). Moreover, in the 16 cases of G6PD Viangchan heterozygote (871G > G/A), all cases showed a heterozygote pattern (1311C > C/T and IVS-11 nt93T > T/C). These results suggest that G6PD Viangchan (871G > A) is firmly linked to 1311C > T and IVS-11 nt93T > C and that the wild type of Cambodian people has a wild type of 1311C and IVS-11 nt93T.

In this study, we did not give primaquine to malaria patients found in the surveys because the national health policy of Cambodia does not allow administration of primaquine to malaria patients at present. Primaquine is a unique medicine to kill the malaria gametocyte, which is the cause of transmission from malarial patients to mosquitoes. When a malaria patient is confirmed to have normal G6PD activity, he/she should take primaquine together with other malaria medicine. It inhibits the malaria parasites' transmission to mosquitoes, and it prevents the community from malaria infection. We recognize that there are a great many G6PD-deficient people in Cambodia, so the G6PD activity test is essential before using primaquine on malaria patients.

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Nasal Immunization with a Malaria Transmission-Blocking Vaccine Candidate, Pfs25, Induces Complete Protective Immunity in Mice against Field Isolates of *Plasmodium falciparum*

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Malaria transmission-blocking vaccines based on antigens expressed in sexual stages of the parasites are considered one promising strategy for malaria control. To investigate the feasibility of developing noninvasive mucosal transmission-blocking vaccines against *Plasmodium falciparum*, intranasal immunization experiments with *Pichia pastoris*-expressed recombinant Pfs25 proteins were conducted. Mice intranasally immunized with the Pfs25 proteins in the presence of a potent mucosal adjuvant cholera toxin induced robust systemic as well as mucosal antibodies. All mouse immunoglobulin G (IgG) subclasses except IgG3 were found in serum at comparable levels, suggesting that the immunization induced mixed Th1 and Th2 responses. Consistent with the expression patterns of the Pfs25 proteins in the parasites, the induced immune sera specifically recognized ookinetes but not gametocytes. In addition, the immune sera recognized Pfs25 proteins with the native conformation but not the denatured forms, indicating that mucosal immunization induced biologically active antibodies capable of recognizing conformational epitopes of native Pfs25 proteins. Feeding *Anopheles dirus* mosquitoes with a mixture of the mouse immune sera and gametocytemic blood derived from patients infected with *P. falciparum* resulted in complete interference with oocyst development in mosquito midguts. The observed transmission-blocking activities were strongly correlated with specific serum antibody titers. Our results demonstrated for the first time that a *P. falciparum* transmission-blocking vaccine candidate is effective against field-isolated parasites and may justify the investigation of noninvasive mucosal vaccination regimens for control of malaria, a prototypical mucosa-unrelated mosquito-borne parasitic disease.

Malaria causes high mortality and morbidity in tropical and subtropical countries, killing more than 3 million people annually (4, 29). The emergence of drug-resistant parasites and insecticide-resistant mosquitoes has raised continued public health problems worldwide. Given their complex life cycle and the discrete nature of immune responses to each developmental stage, the malaria parasites provide many potential targets for the development of prophylactic vaccines. Transmission-blocking vaccines the target sexual stages of the parasites (i.e., gametocyte, gamete, zygote, and ookinete) (6). Transmission-blocking antibodies ingested together with the gametocytes block parasite development in the mosquito midgut, preventing parasite transmission to other susceptible individuals. Thus, transmission-blocking vaccines are expected to prevent the spread of escape mutants that could be emerging during the course of antimalaria drug treatment or other prophylactic vaccines targeting asexual stages of the parasites. A leading transmission-blocking vaccine candidate antigen against *Plasmodium falciparum* is the ookinete surface protein Pfs25 (17, 18), and a clinical-grade recombinant Pfs25 expressed in *Pichia pastoris* is now available (33).

Mucosal vaccination with nonreplicating particles or recombinant proteins in combination with effective mucosal adjuvants has demonstrated their ability to induce local protective immunity against mucosal pathogens (32). Nasal vaccines in particular are by far the most effective mucosal vaccines, capable of priming a full range of local as well as systemic immune responses against protective antigenic epitopes (13, 14). In addition, this type of topically administrable, needle-free, noninvasive vaccine may be safer than injection-based parenteral vaccines by reducing the risk of infection from blood-borne pathogens, and may also be cost-effective because ad-

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ministration does not require highly trained medical or veterinary personnel.

Although mucosal vaccines have several attractive features over parenteral vaccines, their targets had been almost exclusively limited to mucosal infections, and their potential applicability to nonmucosal pathogens such as arthropod vector-borne parasites and viruses seemed to be unappreciated. However, previous studies with malaria parasites (1, 5, 15, 23, 24, 27, 30) and Japanese encephalitis virus (unpublished data), which are prototypical mosquito-borne infectious protozoa and virus, respectively, indicated that mucosal vaccines could be effective alternative immunization methods.

In this study we evaluated the ability of transmission-blocking mucosal vaccines against field isolates of *P. falciparum*. Our results indicate that recombinant Pfs25 is sufficiently immunogenic when coadministered intranasally with a mucosal adjuvant to achieve robust immune protection against parasite transmission, suggesting that noninvasive mucosal vaccines are a promising alternative approach for malaria prevention.

MATERIALS AND METHODS

Mice and immunization schedule. Six-week-old female BALB/c (*H-2*^{d}) and A/J (*H-2*^{a}) mice (Japan SLC, Hamamatsu, Japan) were used for intranasal immunization experiments. Groups of six to seven mice were intranasally immunized three times at weeks 0, 3, and 5 with 20 μg of *Pichia pastoris*-expressed recombinant Pfs25 (33) mixed with 1 μg of cholera toxin (CT; Sigma-Aldrich) as a mucosal adjuvant. The volume of the mixture was adjusted to 15 μl with phosphate-buffered saline (PBS) and administered to external nares with micropipette without anesthesia. As negative controls mice were intranasally administered with 1 μg of CT alone or PBS.

Serum and mucosal sample collection. Blood was collected from immunized mice one week after the third immunization by cardiac puncture under complete anesthesia confirmed by eyelid reflex responses. Immune sera prepared from the collected blood were used for antibody titer analysis and transmission-blocking assays. Nasal secretions were collected from exsanguinated animals immediately after sacrifice by washing the nasal cavities several times with 200 μl of PBS. The samples were centrifuged to remove insoluble debris and supernatant was immediately analyzed for specific antibodies. For collection of intestinal secretory antibodies, a fraction of the small intestine (approximately 3 cm long) was excised and cut perpendicularly to open the intestinal tubes. The excised samples were immersed in 0.5 ml of PBS and vigorously vortexed, followed by centrifugation to remove insoluble debris. Supernatant was used for antibody analysis.

Antibody titer determination by enzyme-linked immunosorbent assay (ELISA) for serum, nasal and intestinal secretions. Serum and mucosal samples were analyzed for the presence of specific antibodies by ELISA as described previously (1). Briefly, 96-well ELISA plates (Sumilon, Sumitomo Bakelite, Japan) were coated with recombinant Pfs25 proteins (33). The plates were washed with PBS containing 0.05% Tween-20 (PBST) three times and blocked with 1% BSA in PBS. Serial dilutions of serum samples were applied to wells in duplicates. Secondary antibodies specific for each mouse antibody isotype (immunoglobulin G [IgG], IgM and IgA) and IgG subclass (IgG1, IgG2a, IgG2b, and IgG3) was used for detection. Optical density (OD) was measured by microplate reader (Bio-Rad Laboratories) at 415 nm. The OD_{415} value of 0.1 was used as the baseline to determine the endpoint titers for specific serum IgG. In some experiments, serum antibody levels were expressed as OD_{415} measurement after making appropriate dilutions as indicated. Antibodies present in nasal secretions were analyzed by diluting the nasal washings by 15-fold with PBS before applying the samples to microtiter plates. To analyze specific antibodies in intestinal secretions, intestinal washings collected as described above were diluted with PBS by 2-fold prior to ELISA. Student's *t* test was performed to compare antibody levels of serum and mucosal samples between different test groups.

Recognition of native parasite by immunofluorescence assay. All human materials used in this study were reviewed and approved by the Institutional Ethics Committee of the Thai Ministry of Public Health and the Human Subjects Research Review Board of the United States Army. For purification of gametocytes, peripheral blood was collected by heparinized syringes under written informed consent from patients who came to the malaria clinics in the Mae Sod district in the Tak province of northwestern Thailand. Infection with *P. falciparum* was confirmed by Giemsa stain of thick and thin blood smears. Cultured *P. falciparum* parasite preparations rich in zygotes and small numbers of ookinetes were spotted on slides and fixed with acetone as previously described (25). The slides were blocked with PBS containing 5% nonfat milk and incubated with Pfs25/CT immune sera. The slides were washed with ice-cold PBS for 5 min and incubated with fluorescein isothiocyanate-conjugated anti-mouse antibody, followed by washing with ice-cold PBS. Slides were examined by confocal scanning laser microscope (Nikon C-1).

Transmission-blocking assays. Peripheral blood was collected from four volunteer patients as described above. Their parasitemia were ranging from 0.04 to 0.18%, and gametocytemia from 0.002% to 0.011%. Collected blood was aliquoted into tubes (300 μl/tube) and plasma was removed. Mouse immune sera were diluted (2-, 8- and 32-fold) with heat-inactivated normal human AB serum prepared from malaria naïve donors. Each diluted test serum was mixed with *P. falciparum*-infected blood cells as described above (1:1 vol/vol ratio) and incubated for 15 min at room temperature. The mixture was placed in a membrane feeding apparatus kept at 37°C to allow starved *Anopheles dirus* A mosquitoes (Bangkok colony, Armed Forces Research Institute of Medical Sciences) to feed on the blood meals for 30 min. Unfed mosquitoes were removed and only fully engorged mosquitoes were maintained for a week by giving 10% sucrose water in the insectary.

For each mouse test immune serum, 20 mosquitoes (i.e., a total of 80 mosquitoes for four patients' blood samples) were dissected and analyzed by staining with 0.5% mercurochrome to count the number of oocysts developed within the mosquito midgut under the microscope. Mann-Whitney *U* test was used to examine the difference in oocyst counts per mosquito between control and immunized groups. Fisher's exact probability test was used to examine the difference of infection rates between control and immunized groups. *P* values less than 0.05 were considered statistically significant.

RESULTS

Systemic and mucosal antibody responses induced in mice by intranasal immunizations. Immunization with Pfs25/CT resulted in a significant increment of specific anti-Pfs25 serum IgG responses ($P < 0.01$) (Fig. 1A). Higher IgG responses were induced in A/J than BALB/c mice, but the difference did not reach statistically significant level ($P = 0.23$). We also evaluated specific reactivity of immune sera against denatured forms of the recombinant Pfs25 proteins. We found that mucosally induced antibodies exhibited strict conformation-dependent reactivity, in which both A/J and BALB/c mouse immune sera recognized only native proteins, while no specific reaction was observed against denatured forms of the proteins ($P < 0.001$) (Fig. 1B). Mucosal immunization induced comparable levels of IgG1, IgG2a and IgG2b, but not IgG3 (Fig. 1C). Immunoglobulin isotypes other than IgG (i.e., IgM, IgE, and IgA) were also detected, although at relatively low levels in Pfs25/CT immune sera, but not in immune sera derived from intranasal administration of CT alone or PBS (Fig. 1D).

Next we analyzed mucosal antibody responses (Fig. 2). Intranasal immunization with Pfs25/CT induced significant levels of anti-Pfs25 IgA and IgG ($P < 0.01$) in nasal washings, whereas control mice given CT alone or PBS did not develop Pfs25-specific mucosal antibodies (Fig. 2A). IgG subclass analysis for nasal washings revealed a similar pattern seen for serum IgG subclasses (Fig. 2B). Mucosal adjuvant CT-specific IgA and IgG were also detected in nasal washings of mice immunized with Pfs25/CT or CT alone, but not in mice given PBS (Fig. 2C). Intranasal immunization also resulted in low but detectable levels of Pfs25-specific IgA and IgG in intestinal secretions of Pfs25/CT immunized group, but not in CT or PBS group, and the induced IgG subclass patterns were similar to that for nasal IgGs (data not shown).

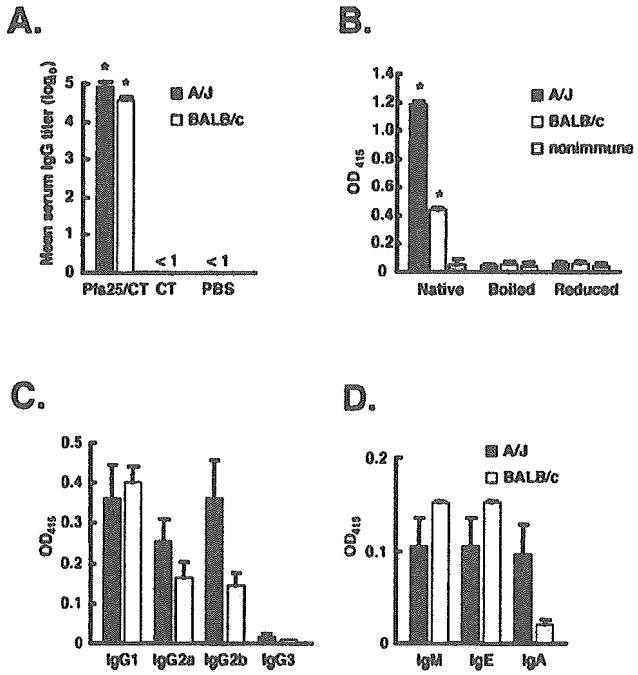

FIG. 1. Serum antibody responses induced by intranasal immunization of BALB/c and A/J mice with Pfs25/CT, CT alone, or PBS were analyzed by ELISA. Groups of six to seven mice were immunized three times at weeks 0, 3, and 5, and immune sera were collected at week 6 for analysis. A. Pfs25-specific serum IgG responses. Serum IgG titers were defined as the highest serum dilution giving 0.1 OD_{415}, and the data were expressed as the mean titers ± standard error. *, $P < 0.01$ versus CT alone or PBS. B. Conformation-dependent serum IgG responses. Data are expressed as the average OD_{415} ± standard error for immune sera diluted to 1:20,000. *, $P < 0.001$ versus boiled or reduced form of Pfs25 proteins. C. Serum IgG subclass analysis for Pfs25/CT immune sera. Data are expressed as the average OD_{415} ± standard error for immune sera diluted to 1:9,000. D. Analysis of serum Ig isotypes other than IgG (i.e., IgM, IgE, and IgA). Data were expressed as the average OD_{415} ± standard error for immune sera diluted to 1:3,000.

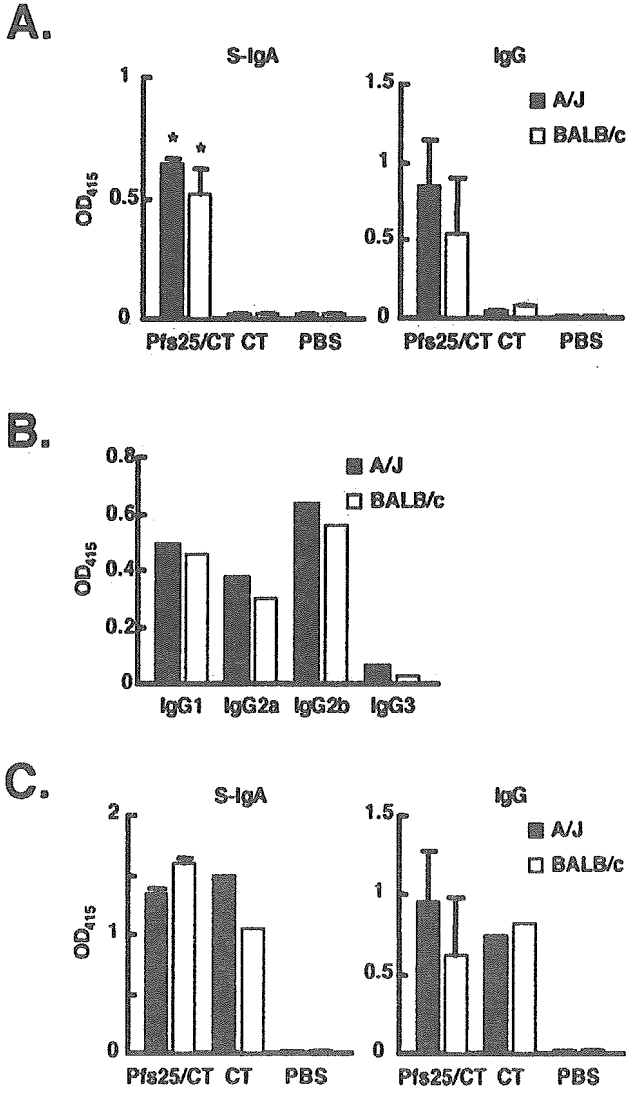

FIG. 2. Mucosal antibody responses induced by intranasal immunization of mice with Pfs25/CT, CT alone, or PBS were analyzed by ELISA. Nasal wash samples were collected immediately after exsanguination by washing the nasal cavities several times with 200 μl of PBS. The collected samples were diluted 15-fold with PBS prior to analysis. A. Pfs25-specific secretory IgA (S-IgA) and IgG in nasal secretions. Data are expressed as the average OD_{415} ± standard error. *, $P < 0.01$ versus CT alone or PBS. B. IgG subclass analysis of nasal IgG collected from mice immunized with Pfs25/CT. Results are expressed as the average OD_{415} of the pooled nasal washings. C. CT-specific secretory IgA and IgG in nasal secretions. Results are expressed as the average OD_{415} ± standard error.

Recognition of native Pfs25 proteins expressed at ookinete stage of *P. falciparum*. To evaluate antibody specificity of mucosally induced immune sera to native Pfs25 proteins, Pfs25/CT immune sera were reacted with cultured ookinete preparations. As indicated in Fig. 3, the immune sera specifically recognized ookinetes but not gametocytes. The fluorescent signal was localized on the surface of ookinetes, confirming that induced antibodies were capable of recognizing native Pfs25 proteins on the surface of ookinetes. The result was consistent with the expression patterns of the Pfs25 proteins in the parasites.

Evaluation of transmission-blocking activity. Transmission-blocking assays were performed using pooled immune sera of mice intranasally immunized with Pfs25/CT, CT alone or PBS. Pfs25/CT immune sera, but not control CT or PBS serum, had significant transmission-blocking activities, indicated by profound reduction of the numbers of oocysts developed in mosquito midgut (Table 1). Dilution of the immune sera resulted in reduction of transmission-blocking activities. In addition, Pfs25/CT immune sera significantly reduced mosquito infection rate defined as percent of infected mosquitoes in a total number of mosquitoes examined (Table 1). Serum dilution resulted in an increase in the infection rate.

We also analyzed the complete transmission-blocking rate, defined as the percentage of volunteers whose infected blood did not establish any parasite infection in mosquitoes (Table 1). All four human blood samples failed to transmit any parasite to mosquitoes when mixed with Pfs25/CT immune sera, but dilution of the immune sera gradually reduced the complete transmission-blocking rate.

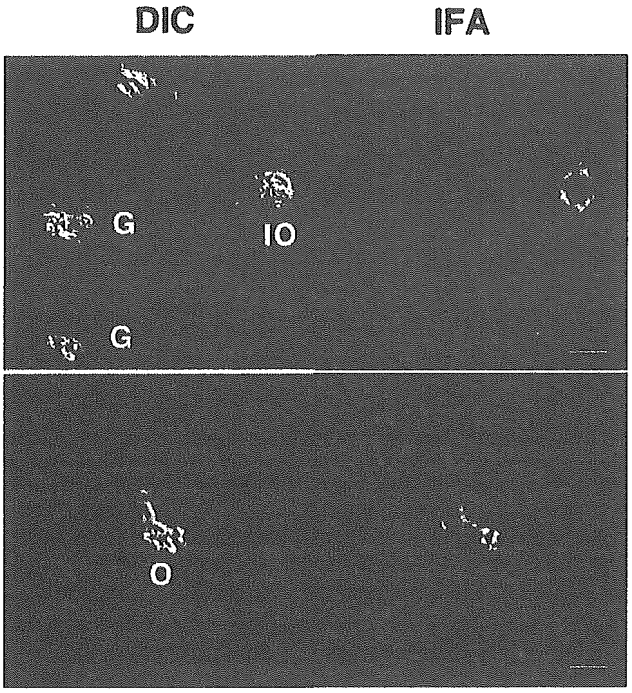

FIG. 3. Ookinete-specific reactivity of Pfs25/CT immune sera was confirmed by immunofluorescence analysis. The immune sera specifically recognized native Pfs25 proteins expressed on the surface of *P. falciparum* ookinetes. The immune sera did not react with gametocytes. DIC, differential interference contrast microscopy. IFA, fluorescence confocal scanning laser microscopy. G, gametocyte. IO, immature ookinete. O, mature ookinete. Bar = 5 μm.

We found strong correlations of Pfs25-specific serum antibody levels (Fig. 1) with the oocyst counts (correlation coefficient $r = -0.717$), with the mosquito infection rate ($r = -0.832$), and with the complete transmission-blocking rate ($r = 0.878$). In contrast, no correlation was observed between CT-specific antibody levels and the transmission-blocking activities.

DISCUSSION

With the prospect of developing noninvasive malaria transmission-blocking vaccines, we have previously demonstrated that intranasal immunization of recombinant Pvs25, an orthologous gene product of Pfs25 and a vaccine candidate against *P. vivax* malaria, induced a robust systemic immune response, conferring a significant protection against parasite transmission to mosquitoes (1). In this study we demonstrated a potent mucosal immunogenicity and protective efficacy of recombinant Pfs25 proteins (33). In addition, this is the first report indicating that a *P. falciparum* transmission-blocking vaccine candidate is effective against field-isolated parasites in a malaria-endemic area.

Our recent studies with rodent malaria *P. yoelii* also demonstrated that intranasal immunization of mice with recombinant Pys25 proteins (25) provided complete transmission-blocking immunity in both active and passive immunization regimens (unpublished data). Unlike the results of our previous studies with *P. vivax* (1) and *P. yoelii* (unpublished data), we found that the serum IgG subclasses induced by Pfs25/CT immunization were not strongly biased towards IgG1. It rather induced comparable levels of IgG1, IgG2a, and IgG2b (Fig. 1C), implying that mixed Th1 and Th2 responses were induced. Malkin et al. observed that the presence of heat-labile components in the membrane feeder enhanced the transmission-blocking activity of Pvs25 antisera (21). Because all heat-labile components were inactivated by heat treatment during the transmission-blocking assays performed in our present and previous studies, we might have underestimated the levels of actual transmission-blocking activity of mucosally induced immune sera. However, regardless of the types of immunity induced, specific antibody titers were the best correlate for protection, and no such correlation was found between CT-specific antibody titers and protective efficacy: these observations were consistent with other transmission-blocking vaccine studies (2, 7, 10, 11, 16, 18–20). Taken together, transmission-blocking activity was clearly correlated with levels of vaccine antigen-specific serum IgG, regardless of the reper-

TABLE 1. Transmission-blocking efficacy against Thai *Plasmodium falciparum* isolates

Mouse strain	Immunization	Serum dilution[a]	Median no. of oocysts with quartiles and ranges					P^b		Infection rate (%)[c]	P^d		Complete blocking rate (%)[e]
			Min	Lower	Median	Upper	Max	Vs. PBS	Vs. CT		Vs. PBS	Vs. CT	
A/J	PBS	1:2	0	0	0	9.0	108		0.0615	35/80 (43.8)		0.0505	0/4 (0)
	CT	1:2	0	0	0	3.8	68	0.0615		24/80 (30.0)	0.0505		0/4 (0)
	Pfs25/CT	1:2	0	0	0	0	0	<0.0001	<0.0001	0/80 (0)	<0.0001	<0.0001	4/4 (100)
		1:8	0	0	0	0	0	<0.0001	<0.0001	0/80 (0)	<0.0001	<0.0001	4/4 (100)
		1:32	0	0	0	0	10	0.0001	0.0529	17/80 (21.3)	0.0019	0.1386	0/4 (0)
BALB/c	PBS	1:2	0	0	4.5	28.5	130		0.3348	46/80 (57.5)		0.3752	1/4 (25)
	CT	1:2	0	0	2.0	19.5	109	0.3348		43/80 (53.8)	0.3752		0/4 (0)
	Pfs25/CT	1:2	0	0	0	0	0	<0.0001	<0.0001	0/80 (0)	<0.0001	<0.0001	4/4 (100)
		1:8	0	0	0	0	12	<0.0001	<0.0001	6/80 (7.5)	<0.0001	<0.0001	2/4 (50)
		1:32	0	0	0	0	12	<0.0001	<0.0001	19/80 (23.8)	0.0001	0.0001	1/4 (25)

[a] Dilution of test immune sera used for transmission-blocking assays.
[b] Determined by the Mann-Whitney *U* test for comparison of oocyst numbers between the test immune sera and PBS and CT sera.
[c] Number of infected mosquitoes/total number of mosquitoes examined.
[d] Determined by Fisher's exact probability test for comparison of mosquito infection rate between the test immune sera and PBS or CT sera.
[e] Number of patients whose blood samples did not result in any oocyst development in any mosquito examined/total number of volunteer patients ($n = 4$).

toire of IgG subclasses induced, strain of mouse used, difference in *Plasmodium* species targeted, or type of immunization method employed.

In contrast to the general perception that mucosal vaccines are much less effective for the induction of systemic antibody responses than parenteral vaccines, we found that intranasal vaccines, when coadministered with a strong mucosal adjuvant like CT, are not necessarily considered inferior to parenteral vaccines at least in a murine model (1, 2, 16, 18; unpublished data). Systemic antibodies raised against *Pichia pastoris*-expressed recombinant Pfs25 proteins (33) by intranasal immunization specifically recognized native proteins expressed on ookinete surface (Fig. 3), but barely recognized heat-denatured or reduced form of proteins (Fig. 1B), indicating that at least some conformational epitopes were retained in the course of intranasal immunization and correctly presented to the immune system in an intact form for the induction of biologically active antibodies that functioned as transmission-blocking agents within the mosquito midgut.

The highly efficacious transmission-blocking activity observed with mucosally induced immune sera supports the potential of applying mucosal vaccines to malaria prophylactics. Further, our recent mucosal immunization studies with various antigens such as formalin-inactivated Japanese encephalitis virus vaccine and the paramyosin antigen of *Schistosoma japonicum* demonstrated that mucosal vaccines induced strong and long-lasting humoral as well as cellular immunity comparable to that with parenteral vaccines when strong adjuvants like CT were coadministered (unpublished data). These results indicate that CT is a strong immune potentiator that may be able to induce immunological memory against heterologous antigens in a rodent model. However, CT needs to be precluded from clinical use due to its enterotoxicity and potential hazardous effects on olfactory nerves (12). Therefore, the particular vaccination regimen presented in this study using CT as an adjuvant needs to be considered as a model system to prove the effectiveness of mucosal vaccines against malaria transmission.

Since the mucosal immunogenicity of Pfs25 may not depend on a particular mucosal adjuvant or delivery system, specific targeting or immunomodulation of professional antigen-presenting cells such as dendritic cells and B cells with other, potentially safer agents (3, 8, 9, 22, 26, 28, 31) than CT may offer new approaches for the development of malaria vaccines and warrant further evaluation of mucosal and other less invasive vaccination regimens as alternative strategies for malaria control in the future.

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Erythrocyte surface glycosylphosphatidyl inositol anchored receptor for the malaria parasite

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Abstract

Parasitophorous vacuole formation is a critical step for the successful invasion of host erythrocytes by the malaria parasite. Rhoptry proteins are believed to have essential roles in vacuole formation, although their biological roles are poorly understood. To understand the molecular interactions between parasite rhoptry proteins and the erythrocyte during invasion, we have characterized the binding specificity of the high molecular mass rhoptry protein (RhopH) complex to erythrocytes using the rodent malaria parasite, *Plasmodium yoelii*. RhopH complex binding to erythrocytes was species-specific, observed with mouse but not rabbit or human erythrocytes. Binding is abolished following treatment of erythrocytes with trypsin or chymotrypsin. Because host cell cholesterol-rich membrane domains are recruited into the nascent parasitophorous vacuole, we evaluated a possible role of RhopH complex binding to the cholesterol-rich membrane domain-associated glycosylphosphatidyl inositol (GPI)-anchored protein. Using chimeric mice harboring GPI-deficient erythrocytes, RhopH complex binding to GPI-deficient mouse erythrocytes was undetectable, indicating involvement of GPI-anchored protein in *Py*RhopH complex binding. Furthermore, a significant reduction of *P. yoelii* parasite infection of GPI-deficient erythrocytes was observed in vivo, probably due to inefficient invasion. We conclude that the major erythrocyte receptor for *Py*RhopH complex is a protein attached to the erythrocyte surface via GPI-anchor and that GPI-deficient erythrocytes are resistant to *P. yoelii* invasion.

Keywords: Erythrocyte; GPI anchor; Invasion; Malaria; *Plasmodium yoelii*

1. Introduction

Malaria is one of the most prevalent and deadly global infectious diseases and is caused by the obligate intraerythrocytic stages of the protozoan parasite, *Plasmodium*. To facilitate erythrocyte invasion, *Plasmodium* merozoites discharge the contents of apical organelles called rhoptries that are involved in the formation of an intraerythrocytic parasitophorous vacuole in which the parasites reside and develop. The parasitophorous vacuole membrane (PVM) contains erythrocyte proteins found in detergent-resistant membrane [e.g., Duffy antigen receptor for chemokines (DARC) and glycosylphosphatidylinositol (GPI)-anchored proteins], but excludes most transmembrane proteins (e.g., glycophorin A) [1].

Abbreviations: DARC, Duffy antigen receptor for chemokines; EDTA, ethylenediaminetetraacetic acid; GPA, glycophorin A; mAb, monoclonal antibody; PBS, phosphate buffered saline

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A number of rhoptry proteins have been identified, including a complex of high molecular mass proteins containing three distinct polypeptides, RhopH1, RhopH2 and RhopH3 (the RhopH complex) [2–5]. The RhopH genes have been cloned from the human malaria parasite, *Plasmodium falciparum*, as well as the rodent malaria parasite, *Plasmodium yoelii* [6–10]. The *P. falciparum* RhopH (*Pf*RhopH) complex binds erythrocytes and distributes into the erythrocyte and parasitophorous vacuolar membranes [11,12]. *Pf*RhopH appears to be essential for parasite development: antibodies against the *Pf*RhopH complex partially inhibit growth of *P. falciparum* in vitro and in vivo [13–15], and attempts to disrupt the *Pf*RhopH3 gene locus were unsuccessful, suggesting a resulting lethal phenotype [16]. Taken together, these results strongly indicate that the RhopH complex has a critical role in erythrocyte invasion; however, the molecular interactions of this protein complex are poorly understood. For example, erythrocyte surface binding of the *P. falciparum Pf*RhopH complex was observed only with erythrocytes of the non-susceptible mouse, but not those of the susceptible human host, for which the RhopH complex receptor has not been identified.

To explore a biological role of the rhoptry proteins, we are focusing to characterize the erythrocyte receptors for the RhopH complex using a rodent malaria parasite. In this report we describe a flow cytometric-based erythrocyte binding assay and show that the *P. yoelii* RhopH complex specifically binds to erythrocytes of a susceptible mouse host. Using chimeric mice harboring GPI-deficient erythrocytes, we further show a possible role of the GPI-anchored protein for RhopH complex binding and determine that GPI-deficient erythrocytes are resistant to *P. yoelii* infection.

2. Materials and methods

2.1. Parasites and parasite extracts

Parasite infected blood was collected from *P. yoelii* 17X (lethal)-infected BALB/c mice and leukocytes were removed by passing through a CF11 column. Schizont-stage parasites were enriched by differential centrifugation over 50% Percoll (Amersham Pharmacia Biotech Inc., UK), washed twice in phosphate buffered saline (PBS), pH 7.4, and stored at −80 °C. Parasite proteins were extracted by three times repeated freeze-thaw at −80 °C from schizont-rich pellets in PBS, pH 7.4, containing protease inhibitors [PI; 1 μg ml^{-1} of leupeptin, 1 μg ml^{-1} of pepstatin A, 100 μM 4-(2-aminocthyl)benzenesulfonyl fluoride hydrochloride] and 1 mM EDTA (ethylenediaminetetraacetic acid), and a soluble fraction was obtained by centrifugation at 21,600 × *g* for 10 min to make a final concentration used for erythrocyte binding assays corresponding to 1×10^7 parasites μl^{-1}.

2.2. Monoclonal antibodies

The monoclonal antibodies mAb#25 (IgG1), #32 (IgG2b) and #16 (IgG1) recognizing *P. yoelii* RhopH2, RhopH3 and yPys25, respectively, were described [8,17].

2.3. Erythrocytes

Female BALB/c, DBA/2 and C57BL/6 (B6) mice were obtained from Charles River Japan Inc. GPA wild type (GPA$^{+/+}$) and knockout (GPA$^{-/-}$) mice and DARC wild type (DARC$^{+/+}$) and knockout (DARC$^{-/-}$) mice were generated and maintained in Tokyo University as described [18,19]. Mice harboring GPI-deficient erythrocytes were generated and maintained in Osaka University. Briefly, B6 mice were lethally irradiated and fetal liver cells were transplanted from mice with a disrupted *Pig-a* gene locus, which was essential for the GPI biosynthesis. Control mice were generated in a similar manner except that bone marrow was transplanted from a normal mouse [20]. Mouse blood was collected from tail snip bleeds into an excess amount of PBS containing 50 mM EDTA, washed extensively with PBS to remove serum and buffy coat, stored in PBS containing 1% bovine serum albumin (BSA), and used for binding assays within 1 day of preparation. Erythrocytes were also collected from two rabbits (JW/CSK; Japan SLC Inc., Japan) and two human donors (O-type/Rh-positive) by venous puncture and processed in a similar manner.

2.4. Erythrocyte enzyme treatments

Erythrocytes were incubated in RPMI1640 medium without sodium bicarbonate (designated as RPMI1640) with 1 mg ml^{-1} trypsin (Sigma, St. Louis, MO) or 1 mg ml^{-1} chymotrypsin (Sigma) for 2 h at 37 °C with gentle rocking. The erythrocytes were then washed once with RPMI1640, incubated with 1 mg ml^{-1} soybean trypsin inhibitor (STI; Sigma) or 10 mM *N*-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma), respectively, for 10 min at room temperature (RT) with gentle rocking, and washed once with RPMI1640. Control erythrocytes were treated with STI or TPCK only.

Erythrocytes were also incubated with 100 or 200 mU ml^{-1} neuraminidase (1 U ml^{-1}; *Vibrio cholera*, CalBiochem, San Diego, CA) for 2 h at 37 °C and washed three times with RPMI1640. Two control treatments were devised as follows. In one tube, erythrocytes were incubated with a corresponding volume of the buffer. In a second tube, EDTA with a final concentration of 10 mM was added in addition to the neuraminidase solution, to inactivate neuraminidase activity by chelating calcium. Fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (WGA) recognizing *N*-acetylneuraminic acid (NANA) residues was used to monitor erythrocyte surface desialylation.