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Retrospective Malaria Diagnosis by Indirect Fluorescent Antibody Titration on Japanese Patients

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Abstract

Sera were obtained from 84 microscopically diagnosed Japanese cases of imported malaria, and subjected to the indirect fluorescent antibody test (IFAT). Antigens of *Plasmodium falciparum* and *P.vivax* were used for the serological diagnosis of each serum, and it was possible to differentiate the species of the infected parasite by the higher titer shown by the corresponding antigen. Three patients did not develop antibody at the early acute stage of infection, however specific titers were eventually boosted. None of 207 Japanese healthy donors, who had never been abroad, showed the positive IFAT titers to both antigens. Eight patients out of the 84 cases were followed up and the persistence of the titers was studied. The profiles of the antibody development were useful for retrospective diagnosis of the infected species of malaria. Three infected Japanese emigrants settled at an Amazonian colony were also tested for 4 years. Persistence of the titer was longer in the emigrants than that manifested by imported malaria patients in Japan. The measurement of the level and persistence of antibodies in the non-immune Japanese cases provide an elementary information for the seroepidemiology of malaria in the tropics.

Key words: IFAT, *Plasmodium falciparum, Plasmodium vivax,* retrospective diagnosis

Introduction

The IFAT has been used for the diagnosis of malaria and also for epidemiological studies in the tropics (Voller & Draper, 1982). However interpretation of the obtained titers is still a topic of discussion among authorities (Bruce-Chwatt *et al.*, 1972). This may be due to the considerable variation in the serological response of infected people according to their immunological competence (Kuvin *et al.*, 1962; Lupascu *et al.*, 1966) and their cumulative experience of malaria infection (Bruce-Chwatt *et al.*, 1972; Draper & Sirr, 1980). The serological dynamics of the indirect fluorescent antibodies in Japanese patients who are not immune to malaria are worth studying to clarify the specificity of the tests and the persistence of antibodies produced in response to the infection. In the present study, the amount and persistence of antibody in non-immune Japanese imported cases are reported. Agreement of the IFAT diagnosis with the microscopic parasite identification allowed differentiation of the infected species and retrospective diagnosis of past episodes by means of the IFAT. The present study shows a reference model of IFAT diagnosis of uncomplicated malaria and provides elementary information for seroepidemiological assessment in the field.

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Patients, Materials and Methods

From February 1974 to March 1990, with the collaboration of many clinical doctors, we examined the malarial antibodies and blood-smear slides of 579 suspected fever cases. Eighty-four Japanese patients, of ages 18 to 65, with confirmed *P.f.* or *P.v.* infection by microscopic studies comprised the test group of the present

paper. All samples were taken at the time of admission. Eight patients were consecutively studied during and after curative treatment. Two hundred and seven Japanese volunteers, of ages 18 to 40, who had never been abroad, were sampled and served as a control group. Sera from three Japanese settlers in an Amazonian colony who contracted malaria in 1984, 1984, and 1985 were taken successively in 1986, 1987, 1988, and 1989 and subjected to the IFAT. All blood donors agreed to be subjected to malaria IFAT and were informed of the results.

The IFAT was carried out according to a modified method of Voller & O'Neill (1971). Briefly, a 4-fold dilution system was applied to each sample. Serum samples at each dilution were overlaid on both *P.f.* and *P.v.* antigen spots prepared on glass slides. The *P.f.* antigen had been prepared from infected splenectomized *Aotus* monkeys during 1974 to 1979. *P.f.* parasites obtained from a continuous culture (Trager & Jensen, 1976) have been used since 1980. The *P.v.* antigen had been obtained also from the *Aotus* monkeys. After encountering difficulties in obtaining susceptible *Aotus,* we used infected blood of a *P.v.* patient with approximately 1% parasitemia. The erythrocytes were washed well with PBS (pH. 7.2) (Schulzer *et al.*, 1969). After incubation and washing, thirty-times-diluted fluorescein conjugated rabbit anti-human IgG (Behring, West Germany) was placed on each spot. Following the final washing, titers were determined by reading with an incident light illuminating type fluorescent microscope (Olympus, model BH-RFC, Japan). Parasite estimations were based on the microscopic examination of Giemsa-stained thick or thin smears. The IFAT and microscopic observations were respectively carried out by different workers.

Results

Specificity and differential serodiagnosis by the IFAT

Each member of the test group of 84 Japanese patients was diagnosed as having acute malaria by microscopic observation on admission at his or her respective hospital. All smeared blood specimens were sent to the authors' laboratory for re-checking, together with sera from the respective patients. The IFAT titers of each patient and 207 Japanese healthy individuals, who never had malaria, are illustrated in Fig. 1. Forty individuals were microscopically diagnosed as having falciparum malaria, and 44 as having vivax malaria. The figure shows that the infected parasite of the respective patient is in principle identified by the higher result between the titer against *P.f.* antigen (*P.f.* titer) and the titer to *P.v.* antigen (*P.v.* titer). Differential serodiagnosis between the two species could not be made in six patients who manifested the same titers against both antigens. However all results showed no contradictory diagnoses between the serological method and microscopic observations.

In addition to the above results, 6 patients with ovale malaria were tested. These patients always manifested *P.v.* titers equal to or higher than *P.f.* titers. Two malariae cases were also tested. One showed negative titers to both *P.f.* and *P.v.* antigens, and the other manifested 1:4096 to both antigens. On the other hand, the control group of 207 healthy individuals showed no IFAT titers at all. The results confirmed the

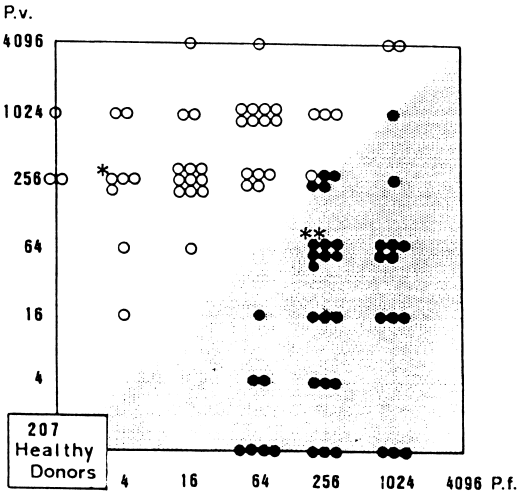

Fig. 1. *P.f.* titers and *P.v.* titers of sera taken from *P.f.* infected (●) and *P.v.* infected (○) patients. The three cases represented by (*) showed negative titers at the first test but eventually reached the respective points.

specificity of malaria IFAT against the homologous antigen, thus justifying serological species diagnosis so far as falciparum malaria and vivax malaria were concerned.

The time lag of the serological response

A falciparum patient was tested on the second day after the first fever attack. The serum titer at this point was <1:4 to each antigen. However, the antibody titer elevated to 1:16 to *P.f.* on the 6th day. On the 24th day, antibodies had risen to 1:256 to *P.f.* and 1:64 to *P.v.* Another falciparum patient, Case F4 in Fig. 2, showed a considerable time lag before antibody was detected. Although parasitemia was observed, the IFAT was negative on the 7th day after the first fever attack. In a test done on the 19th day, the *P.f.* titer and *P.v.* titer were 1:256 and 1:64 respectively. A vivax malaria patient showed a titer at 1:4 to vivax antigen and a negative titer to falciparum antigen on the 5th day after the first fever attack. One day later (the 6th day), the *P.v.* titer had risen to 1:256, but the *P.f.* titer was <1:4.

In the above 3 cases the antibody was not detectable at the early stage of infection, but eventually the specific titer was higher than the cross reacting titer in each case. Considerable variation in the time lag until serological response occurs was suggested from these observations.

Persistence of antibodies in imported malaria cases

Follow-up IFAT studies were carried out on eight patients. All patients had fever attacks on admission. All sera except the serum of Case F4 showed positive titers ranging from 1:256 to 1:4096 at the time of admission. Patient antibody-level courses are illustrated in Figs. 2 and 3. Clinical treatment was begun on all of these patients from day 0 (D-0). Fig. 2 demonstrates the courses of antibody levels in falciparum cases. The solid bar represents *P.f.* titer and the open bar stands for *P.v.* titer. The profiles of the antibody development of two cerebral malaria are shown in the Cases F1 (28 years of age) and F4 (31). Indirect fluorescent antibody titers of RI and RII chloroquine

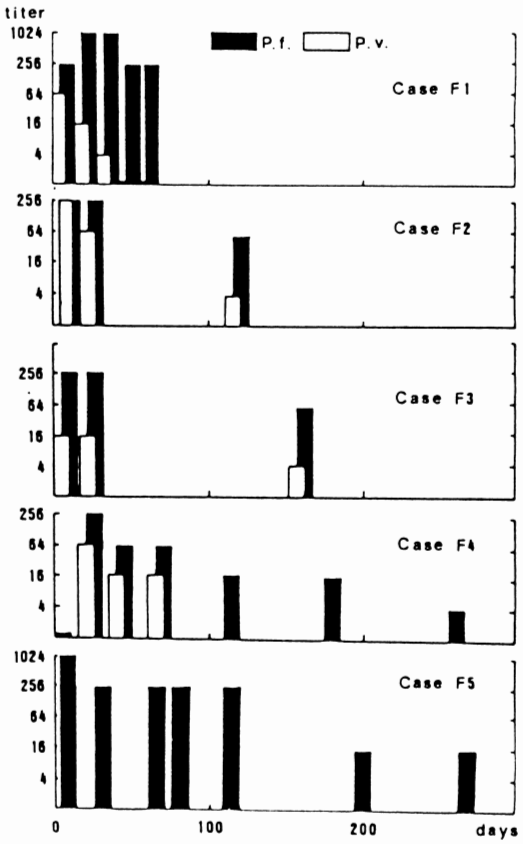

Fig. 2. The persistence of malarial antibodies in patients with falciparum malaria. D-0 is the day of admission.

resistances were shown in Case F2 (39) and Case F3 (28) respectively. All patients from Case F1 to F4 had never had malaria before the respective episodes. Case F1 showed the *P.f.* titer at equal to or higher than 1:256, whereas the *P.v.* titer was at 1:64 and turned negative on D-30. Case F2 showed a titer of 1:256 against both antigens on D-6, but *P.v. titers came down faster than those of P.f.* A follow-up study on Case F4 was continued until D-259 at random intervals. On D-110 the *P.v.* titer became negative, while the *P.f.* titer persisted until termination of the study.

Case F5 was a 29-year-old woman engaged in Chimpanzee research. She stayed at Kasoge near Lake Tanganyka in Tanzania from 1982–1984. There she experienced fever episodes, in April 1983, and then again in July and September 1984. She recovered from the respective fever attacks

by presumptive self-treatment with chloroquine or sulfamonomethoxine + pyrimethamine. After she left Tanzania for Japan in September, she had fever every other month. On 26 November 1984 microscopic examination revealed *P.f.* infection, and she was hospitalized. No clinical symptoms, such as chill, anemia, jaundice, or hepatosplenomegaly were observed, except slight headache and fever spikes of 37.4–38.3°C. Such slight manifestations associated with *P.f.* infection are very exceptional in Japanese patients. The antibody-level course after her admission is illustrated in Fig. 2. The *P.f.* titer remained at 1:256 on D-112, and persisted at 1:16 on D-265. No *P.v.* titer was demonstrated throughout the entire course of the study.

Fig. 3 demonstrates the persistence of antibodies in vivax malaria cases. Cases V1, V2, and V3 had fever attacks after returning to Japan from malaria-endemic areas. Case V2 (33) showed a 1:1024 titer against *P.v.* antigen at the onset of the disease, a 1:64 titer even on D-260, and a <1:4 titer on D-428. Case V3 (41) manifested the highest *P.v.* titer (1:4096) at admission, and maintained a positive titer until D-439.

In both falciparum and vivax cases, specific antibody titers to homologous antigen were always higher than cross-reacting antibody titers to the heterologous antigen. In *P.v.* cases, the cross-reacting titer to *P.f.* antigen was always lower than the *P.v.* titer. However, tailing of cross-reacting antibody was observed until the late stage of convalescence, which was not found in *P.f.* infections. Both specific antibody types persisted at a high level for 1 to 2 months and then gradually lowered. Although many more follow-up studies are required, it seems from the present results that *P.v.* titers tended to persist for a longer time than did *P.f.* titers.

Long-lasting positive titers in Japanese patients at an Amazonian settlement

From 1986 to 1989, malaria serological surveys were conducted in a hypoendemic Amazonian colony where Japanese emigrants had settled in the early 1960's (Daini Tomé-Açu).

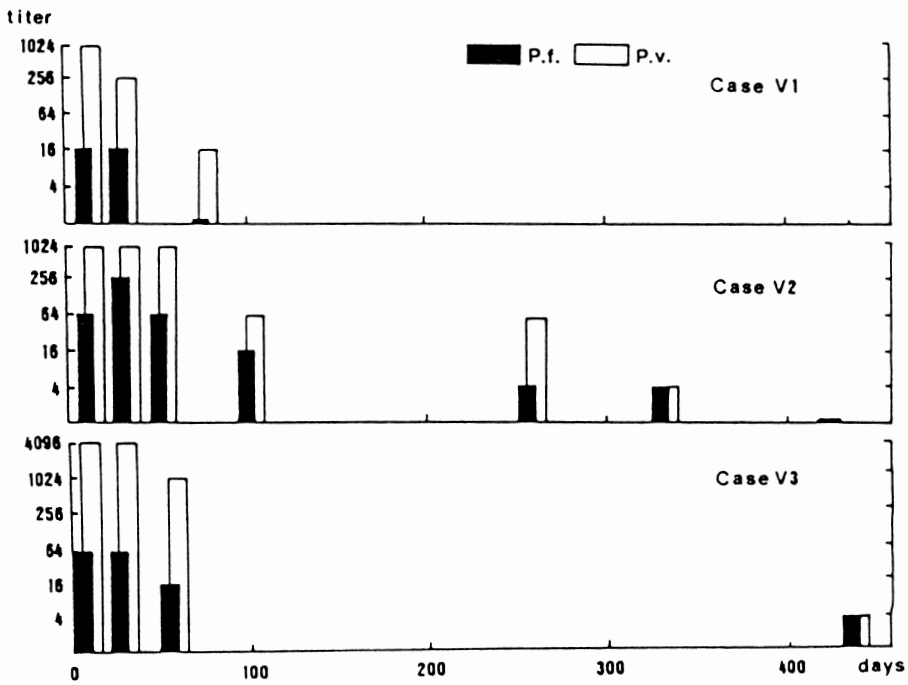

Fig. 3. The persistence of malarial antibodies in patients with vivax malaria. D-0 is the day of admission.

Table 1. Four-year follow-up study of malaria antibodies in 3 patients in an Amazonian settlement.

Case	malaria attack	Year	IFAT titers P.f.	P.v.
1	1985	1986*	40	neg.
		1987*	40	40
		1988	64	64
		1989	64	64
2	1984	1986*	640	neg.
		1987*	40	10
		1988	64	64
3	1984	1988	16	neg.

*Blood samples were taken by filter paper method.

Three farmers who contracted falciparum malaria in 1984 and 1985 were respectively followed for 4 years. According to their memories the 3 examinees had no malaria attacks during the study period. The results are shown in Table 1. Long-lasting positive titers are shown in each case. Particularly in Case 1 (49 years of age) and Case 2 (48), the *P.f.* titer and *P.v.* titer, at 1:64, persisted even 4 years after the attacks. Although the titer was low, Case 3 (47) also showed a 4-year-persisting *P.f.* titer. This follow-up study shows that specific antibodies in patients with a single incidence of malaria in an endemic area persisted much longer time than those in a non-endemic area.

DISCUSSION

Malaria has been eradicated in Japan since 1961. All Japanese are now non-immune to malaria. A Japanese patient with a single incidence of malaria provides an excellent reference model to study the elementary profile of specific antibody development.

An excellent agreement between serological diagnosis and microscopic identification of infected parasites was confirmed in this study's Japanese patients. By using *P.f.* and *P.v.* antigens, differential diagnosis of each malaria type was feasible at the early stage as well as the later stage of infection. At the early acute stage of infection it has been pointed out by previous reports that antibody is not detectable in some cases (Wilson *et al.*, 1970). This fact was also affirmed in the present study. However specific antibodies always became elevated in later examinations. Practically, the diagnosis is definitely made at the early stage of infection by blood smear examination. The time lag in the serological response creates no problem in making a retrospective diagnosis, which is always required at the late or convalescent stage of infection when parasites are no longer in the peripheral blood and the specific antibody has been boosted. The usefulness of serological diagnosis should be stressed in retrospective diagnosis after parasites have been eliminated. The follow-up studies on 8 patients exemplified the feasibility of retrospective differential diagnosis. If a patient's serum shows equal titers to each antigen in a first examination, a second IFAT study done several weeks later will present a higher titer to the homologous antigen and a lower cross-reacting titer. The present result is useful for clinicians in deciding on the administration of radical primaquine treatment (Voller & Draper, 1982; Draper & Sirr, 1980; WHO, 1972; Suzuki, 1985) for a suspected relapsing-type of recent past malaria, including *P. ovale* infection. The findings were also true in the sera taken from several non-Japanese imported malaria patients from endemic areas (data are not shown).

The persistence of antibodies in non-immune Japanese patients presented in Figs. 2 and 3 can be a reference to estimate the time of past infection of individual patients. Titers of 1:256 and over seemed to reflect an attack within the past 2 months, and titers of 1:64, 1:16, and 1:4 covered attacks around 2–6 months, 9 months, and 1 year prior, respectively. It seemed that the *P.v.* titer tended to persist longer than the *P.f.* titer. Similar results were reported on malaria cases in U.S. servicemen by Wilson *et al.* (1970) and in U.K. residents and immigrants by Draper & Sirr (1980). In some studies (Lunn *et al.*, 1966; Collins *et al.*, 1964) the IFAT titer remained for longer periods in donors induced by injection with sporozoites or blood-stage parasites; other workers (Bruce-Chwatt *et al.*, 1980; WHO, 1972; Kuvin & Voller, 1963; Luby *et al.*, 1967) reported 7–30 years' persistence of antibodies in individuals who were thought to have experienced cumulative infection. In the present report, Case F5 in Fig. 2 repeated at least 4 malarial attacks in 2 years in an African area where *P.f.* was highly prevalent. It is notable that, in this patient, the high titer (1:256) persisted much longer time than that shown by other patients with a single incidence of malaria. Interestingly, the patient manifested very slight malarial symptoms as compared to patients in endemic areas. This case suggested that the baseline antibody titer would be elevated by repeated infections in a short period.

The persistence of antibody was longer in Japanese patients living in an Amazonian settlement. Although they claimed an absence of repeated malarial attacks, considerably high titers were maintained for 4 years. These cases suggest that even a single incidence of malaria can cause the persistence of a high level of a specific antibody titer for a long time. The explanation for this finding is difficult, and the following speculation may be sound. The examined cases became infected in a remote colony and they were eventually transported to a hospital by air. The retardation of treatment and degree of parasitemia may be other factors that induce the prolonged persistence of antibody.

The present study thus confirmed the excellent compatibility of the IFAT results with each malaria infection. The IFAT should be the standard reference technique to estimate the reliability of any new serological method. The present report can also be used as a reference model to estimate the time of past malaria transmission that occurred in a hypoendemic population of persons who are close to non-immune Japanese in terms of antibody response. The antibody measurement of a community to survey period prevalence is advisable for estimating the time and degree of endemicity (Voller, 1971). Particularly for a study in a remote inaccessible place, one-point serological assessment at an appropriate time is rather practicable than repeated blood observation. Simple serological methods such as ABC-ELISA (Kano *et al.*, 1989; Sato *et al.*, 1990), which can be used even in a peripheral health post in the tropics, are desired for the malaria control programs, as long as the compatibility with the IFAT is guaranteed.

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