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LATE PHASE SPONTANEOUS PROLIFERATION OF CULTURED PERIPHERAL BLOOD LYMPHOCYTES ASSOCIATED WITH HTLV-I INFECTION: INVOLVEMENT OF CELLULAR RESPONSES AGAINST THE INFECTED CELLS

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Abstract: Common features of patients with human T lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) compared to asymptomatic HTLV-I carriers include a genetically determined high cellular immune responsiveness to HTLV-I and a distributional bias of viral activation between the blood flow and central nervous system (CNS). It had been proposed that increased and altered *in vitro* late phase spontaneous proliferation (SP) of peripheral blood lymphocytes, which is concomitant with *in vitro* viral activation, is associated with the pathogenesis of HAM/TSP. To assess whether SP might epitomize the peculiar cellular inflammation in the CNS of patients with HAM/TSP, fractionated peripheral blood lymphocytes from HAM/TSP patients were employed to reconstitute this phenomenon *in vitro*. Although CD8+ cells had no inherent responsive potential in the absence of exogenous interleukin-2 (IL-2), the SP observed in CD4+ cell cultures was facilitated by the addition of autologous CD8+ cells to the cultures. It could be shown that proliferative responses of the CD8+ cells appeared against cultured and irradiated autologous CD4+ cells but not against purified HTLV-I virions. These findings clearly demonstrate that the cellular response against the infected cells is involved and is one of the major components of the late phase SP, and support the view that this phenomenon may represent an *in vitro* counterpart of the susceptibility to HAM/TSP.

Key words: Human T lymphotropic virus type I (HTLV-I); HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP); peripheral blood lymphocyte; spontaneous proliferation

INTRODUCTION

Spontaneous proliferation (SP) of peripheral blood mononuclear cells (PBMCs) is characterized by an increase in thymidine incorporation into the cells under culture conditions which are devoid of mitogenic stimuli. SP observed in the PBMCs cultured less than 3 days reflects the state of *in vivo* lymphocyte activation (Lane and Fauci, 1985; Griffin *et al.*, 1989). This early phase SP has been reported in patients with a number of acute viral infections (measles, varicella, Epstein-Barr virus, and cytomegalovirus) (Griffin *et al.*, 1989; Arneborn and Biberfeld, 1983; Gavosto *et al.*, 1959; Epstein and Brecher, 1965; Rinaldo Jr *et al.*, 1977), in volunteers immunized with various bacterial or viral antigens (Crowther *et al.*, 1969), in human immunodeficiency virus type 1 (HIV-I)-infected individuals (Lane and Fauci, 1985; deShazo *et al.*, 1989), and in patients with multiple sclerosis (Brinkman *et al.*, 1984). In contrast, late phase SP, which is apparent on day 3 and peaks on days 5-7, is one of the hallmarks of human T lymphotropic virus (HTLV) infection. This late phase SP had originally been described and attributed to an increase in large blastoid lymphocytes in cultures of PBMCs derived

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from HTLV-I-infected healthy donors (Yasuda *et al.*, 1986). In patients with HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a chronic neurological disorder characterized by signs of bilateral pyramidal tract lesions, the late phase SP was first referred to as "autologous proliferative response" or "spontaneous lymphoproliferative response" (Usuku *et al.*,1988; Jacobson *et al.*, 1988). It could be demonstrated that the late phase SP is more intense in HAM/TSP patients than in asymptomatic HTLV-I carriers (Itoyama *et al.*, 1988; Eiraku *et al.*, 1992), and alterations in lymphocyte subset populations in the late phase SP were also noted in patients with HAM/TSP compared to asymptomatic carriers (Eiraku *et al.*, 1992). Lymphocytes from HTLV-II carriers also exhibit late phase SP (Prince *et al.*, 1990). However, the HTLV-II SP is significantly lower than that observed in HTLV-I infection (Wiktor *et al.*, 1991), and this may reflect molecular and biological differences (Prince *et al.*, 1994) including their *in vivo* cellular tropism (Ijichi *et al.*, 1992) between these closely related viruses which might be ultimately linked to the clinical outcomes in infected individuals (Hall *et al.*, 1994).

In viro interactions between the virus and the cellular immune-responses in the late phase SP in HTLV-I infections have been suggested to be important (Usuku *et al.*, 1988), and the HTLV-I activation could be demonstrated in cultured PBMCs derived from infected individuals (Hinuma *et al.*, 1982; Minato *et al.*, 1988). However, the role of cellular responses in the HTLV-I late phase SP remains poorly understood. To investigate this we have analyzed the *in vitro* response of CD8+ lymphocytes to cultured and irradiated autologous CD4+ cells from individuals with HAM/TSP.

Materials and Methods

Patients.

A total of 6 patients who met WHO diagnostic criteria for HAM/TSP (WHO, 1989) and two HTLV-I seronegative normal donors were studied. PBMCs were isolated from heparinized peripheral blood samples on density gradients using Ficoll-Hypaque, and the cells were viably cryopreserved in liquid nitrogen until tested using methods previously described (Usuku *et al.*, 1988; Eiraku *et al.*, 1992; Ijichi *et al.*, 1995). Trypan blue dye exclusion tests demonstrated that the thawed PBMCs had a viability of more than 95%.

Cell separations.

T cell subsets were prepared using a double-negative selection procedure employing washed polystyrene magnetic beads coated with mouse monoclonal antibodies (mAbs) (Dynabeads; Dynal, Oslo, Norway). The thawed PBMCs were incubated with either anti-CD8 or -CD4 mAb coated beads (20-25 beads/target cell) in phosphate buffered saline (PBS) containing 2% (v/v) fetal calf serum (FCS) for 30 min at 4°C with gentle mixing. Rosetted cells were removed using a magnetic particle concentrator according to the manufacturer's instructions. The nonrosetted cells were collected and further purified by the second negative selection step using the same conditions as in the first step to obtain the CD4+ T lymphocyte and CD8+ T lymphocyte enriched cell populations respectively (CD4+ cells and CD8+ cells). The purity of the fractionated cell populations was confirmed by flow cytometric analysis where the maximum contamination of cells was 1.0 %.

Cell cultures.

The components of the late phase SP was reconstituted using fractionated viable cells. A series of graded mixtures of CD4+ and CD8+ cells from a patient with HAM/TSP was incubated for 7 days in RPMI-1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO BRL), and 10% (v/v) FCS in 96-well U-bottom plates without any mitogenic stimuli (RPMI medium). Triplicate wells were used for each culture condition.

Responsive transformation of uninfected lymphocytes was evaluated as the proliferation of CD8+ cells responding to autologous irradiated CD4+ cells. The *in vitro* emergence of HTLV-I activation in the precultured CD4+ cells was previously demonstrated by detection of HTLV-I p19 and p24, increase in proviral and viral RNA amount, and using assays of reverse transcriptase activities in culture supernatants (Ijichi *et al.*, 1995). As HTLV-I antigen presenting cells, CD4+ cells were obtained from a patient with HAM/TSP and cultured for 10 days in RPMI medium containing 2 μg/ml phytohemagglutinin (PHA-P, Sigma, St. Louis, MO), and 10 I.U./ml interleukin-2 (IL-2) (Genzyme, Cambridge, MA). The concentration of IL-2 was maintained by half medium changes every 3 days. Viable cells were isolated by density gradient centrifugation, washed with PBS, resuspended in RPMI medium and irradiated (5000 rad). The proliferative response of autologous CD8+ cells was evaluated by incubating triplicate wells containing 1.0×10^5 CD8+ cells and the CD4+ cells (1.0×10^5) in 96 well plates in the presence 10 I.U./ml IL-2 for 3 to 7 days.

To compare the potential of purified virus virions

with that of infected cells as the stimulant for CD8+ cells, 5.0×10^4 irradiated CD4+ cells prepared as described above (precultured for 13 days) or 8 μg/ml HTLV-I virions (EISAI, Tokyo, Japan) were added to autologous CD8+ cells (5.0×10^4) in 5 patients with HAM/TSP. The cells were incubated for 5 days in RPMI medium containing 10 I.U./ml IL-2 in triplicate wells. To assay the mitogenic effect of HTLV-I virions on normal cells, PBMCs and CD4+ cells isolated from two seronegative healthy donors were incubated for 5 days with 8 μg/ml virions in the absence of IL-2.

^{3}H-thymidine incorporation assay.

Cultures were maintained at 37 °C in a 5% CO_2 atmosphere. The cells were pulsed with ^{3}H-thymidine ([^{3}H] TdR; 37 kBq/well) at 16 hr before terminating the culture and then harvested on glass micro filter papers. Incorporated radioactivity was measured in a liquid scintillation counter (TRI-CARB-4430; Packard Instrument CO. Inc., IL), and data are shown as the mean ± SD. To evaluate the mitogenic activities of the purified virions and irradiated CD4+ cells, stimulation indices were calculated using the formula: mean cpm of cultures with stimulation/mean cpm of cultures without stimulation.

RESULTS

Reconstituted late phase SP (Fig. 1).

Cultured viable CD4+ cells (3.0 and 1.0×10^5 cells/well) obtained from a representative patient with HAM/TSP showed remarkable increase in [^{3}H] TdR incorporation without any mitogenic stimuli. In the absence of exogenous IL-2, the CD8+ cells (3.0×10^5 cells/well) from the same patient had no proliferative response without viable CD4+ cells. However, the increase in [^{3}H] TdR incorporation observed in CD4+ cell cultures was facilitated by the addition of autologous CD8+ cells (2.5×10^4 to 2.0×10^5 cells/well) in a dose dependent manner, showing that the extraordinary excess of CD8+ cell population (at 0.5 of the CD4+/CD8+ cell ratio) induces the greatest facilitation in cell proliferation in these conditions.

Proliferative response of CD8+ cells against irradiated autologous CD4+ cells.

In contrast to the transformation of CD4+ cells, the proliferative response of CD8+ cells to autologous HTLV-I-infected cells was not concomitant with the induction of HTLV-I antigens in the proliferating cells (Ijichi *et al.*, 1995). Preliminary studies have demonstrated that the *in vitro* proliferation of CD8+ cells depends on the presence of viable CD4+ cells co-cultured, which include infected cells and may be supplying growth factors, and the CD8+ cell response against irradiated CD4+ cells requires preincubation of the CD4+ cells and supplementation of exogenous IL-2 in the medium (Ijichi *et al.*, 1995; Nagai *et al.*, 1995). Because IL-2-induced proliferation of the CD8+ cells (alone) varies according to the in vivo expression of IL-2 receptors on the cells (Ijichi *et al.*, 1995; Nagai *et al.*, 1995), the responsive proliferation was assessed as stimulation indices. To analyze the proliferative response of uninfected CD8+ cells against autologous infected cells, the proliferative potential of CD8+ cells cocultured with precultured and irradiated CD4+ cells was inves-

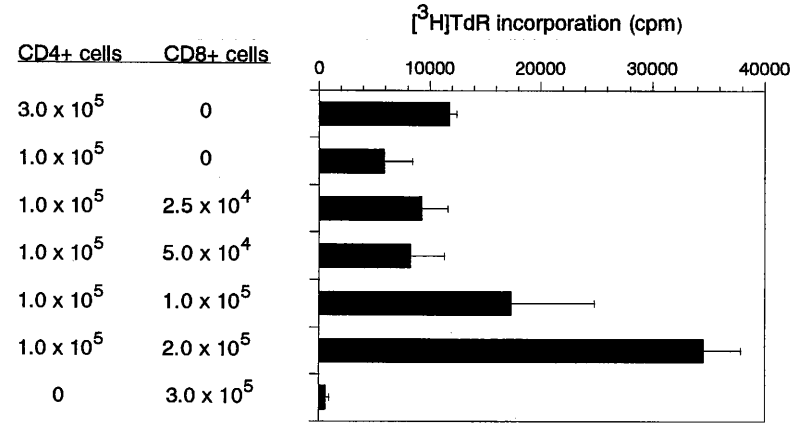

Figure 1. Reconstituted cultures to show the each role of CD4+ cells and CD8+ cells for the HTLV-I late phase SP. Fractionated viable cells were cultured in the absence of IL-2 for 7 days. The results are expressed as the means ± SD in triplicate cultures.

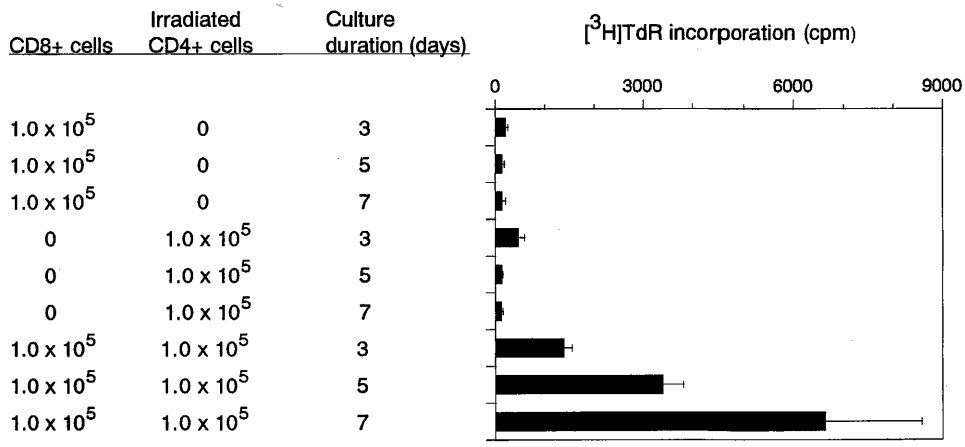

Figure 2. CD8+ cell response against the autologous irradiated CD4+ cells (precultured) in the presence of exogenous IL-2. The results are expressed as the means ± SD in triplicate cultures.

tigated (Fig. 2). The representative case in Fig. 2 is HAM3 in Table 1, whose CD8+ cells alone did not exhibit high incorporation of thymidine in the presence of exogenous IL-2. Control cultures showed low [^{3}H] TdR incorporation into the viable CD8+ cells and the irradiated CD4+ cells cultured alone for 3 to 7 days in

Table 1 Cell responses against purified HTLV-I virions or irradiated autologous CD4+ cells possessing HTLV-I antigens

Cells	[^{3}H] TdR incorporation (cpm) into cells stimulated by	
	Purified HTLV-I virions	Irradiated CD4+ cells
(Cultures in the absence of IL-2)		
ND1 PBMCs 5.0×10^4	2539±556(7.7)	n.d.
ND2 PBMCs 5.0×10^4	8077±1221(16.3)	n.d.
ND1 CD4+ cells 1.0×10^5	24207±13183(69.8)	n.d.
ND2 CD4+ cells 1.0×10^5	16412±4529(61.2)	n.d.
(Cultures in the presence of IL-2)		
ND1 CD8+ cells 1.0×10^5	24311±1141(0.8)	n.d.
ND2 CD8+ cells 1.0×10^5	52756±7780(0.9)	n.d.
HAM1 CD8+ cells 5.0×10^4	4553±439(1.0)	31232±5148(7.2)
HAM2 CD8+ cells 5.0×10^4	22516±3794(0.8)	60394±5007(2.0)
HAM3 CD8+ cells 5.0×10^4	1956±510(1.1)	17723±2539(10.1)
HAM4 CD8+ cells 5.0×10^4	5714±1884(1.0)	11799±470(2.0)
HAM5 CD8+ cells 5.0×10^4	22178±3177(0.8)	45531±3227(1.7)

The results are expressed as the means ± SD (stimulation indices) in triplicate cultures. Significant stimulation indices are underlined. Abbreviations: ND1 and ND2, normal donors; HAM1-HAM5, patients with HAM/TSP; n. d., not done.

this patient. The CD8+ cells cocultured with the irradiated autologous CD4+ cells exhibited an increase in thymidine incorporation, and the proliferation of CD8+ cells was found to be most remarkable in late phase of culture (day 7).

The late phase responsive proliferation of the CD8+ cells cocultured with irradiated autologous CD4+ cells was confirmed in a total of 5 patients with HAM/TSP (Table 1 and Figure 2). Cell free purified HTLV-I virions were found to have mitogenic activity on normal donor PBMCs and CD4+ cells. However, the stimulation index data demonstrated that the virions had essentially no stimulatory effect on the CD8+ cells in these patients (Table 1). The quantity of purified virus virions employed (8 μg/ml) was found to be optimal to activate normal donor PBMCs (data not shown).

DISCUSSION

Although HTLV-I antigens have a variety of viro-immunological properties as a mitogen, a *trans*-activator, and antigens, the infection is characterized by the viral latency in circulating infected cells in the host (Schüpbach, 1989). Peripheral blood lymphocytes obtained from HTLV-I-infected individuals exhibit late phase SP accompanied by *in vitro* HTLV-I activation, and patients with HAM/TSP are characterized by high and altered late phase SP as mentioned above. Because purified T lymphocytes can exhibit the late phase SP, this phenomenon is different from the autolegous mixed lymphocyte reaction (AMLR) (Minato *et al.*, 1989; Lal *et al.*, 1992), and recently, the HTLV-I SP has been reported as a T cell colony-forming cell abnormality (Lunardi-Iskandar *et al.*, 1993). *In vitro* spontaneous expression of HTLV-I RNA and viral antigens in cultured PBMCs has been demonstrated in healthy HTLV-I carriers and patients with HAM/TSP (Hinuma *et al.*, 1982; Minato *et al.*, 1988; Mann *et al.*, 1994), and HTLV-I viral particles have been demonstrated in extracellular spaces of cul tured PBMCs (Gessain and Gout, 1992). As such, the IL-2-independent growth of infected clones (Höllsberg *et al.*, 1992), mitogenic activity of HTLV-I virions (Gazzolo and Dodon, 1987; Dodon and Gazzolo, 1987; Dodon *et al.*, 1989; Maguer *et al.*, 1993; Cassé *et al.*, 1994), and T cell activation induced by cell-to-cell contact with the infected cells (Wucherpfennig et al., 1992; Kimata *et al.*, 1993) may be involved in the HTLV-I SP. The mitogenic activity of HTLV-I virions and the mitogenic surface structure(s) on HTLV-I-infected cells requires the CD2/lymphocyte function-associated antigen 3 (LFA-3) pathway for the activation of responder T cells (Dodon *et al.*, 1989; Wucherpfennig *et al.*, 1992; Kimata *et al.*, 1993), and the subsequent autocrine or paracrine secretion of IL-2 from responding cells is essential for cell proliferation (Dodon and Gazzolo, 1987; Wucherpfennig *et al.*, 1992). In addition, it has been suggested that other soluble factors may contribute to the SP (Lal and Rudolph, 1991; Lal *et al.*, 1991). Other studies have suggested that responsive T cell proliferation in HTLV-I SP may be a human lymphocyte antigen (HLA) haplotype-linked response against HTLV-I virions (Usuku *et al.*, 1988; Sonoda, 1990), and it had been suggested that an inhibitory effect of CD8+ cells on the *in vitro* HTLV-I expression in infected autologous CD4+ cells is a HLA haplotype-linked response (Sonoda, 1990). Moreover, cell responses in HTLV-I SP have recently been shown to involve a different signal transduction pathway from the growth of HTLV-I-infected cells (Mann *et al.*, 1994). Therefore, the proliferation of CD8+ cells in the HTLV-I SP has received much attention as an *in vitro* epitome of the cellular inflammation in the central nervous system (CNS) of HAM/TSP patients (Eiraku *et al.*, 1992), and the view supports the proposed implication of the anti-HTLV-I cytotoxic T lymphocytes in the pathogenesis of HAM/TSP (Moore *et al.*, 1989; Jacobson *et al.*, 1990).

In the present study we could demonstrate the following. (i) Not only the *in vitro* activation of infected CD4+ cells but also the proliferative response of CD8+ cells against infected cells is involved and is one of the major components of the HTLV-I late phase SP. (ii) The presence of viable CD4+ cells is critical for the HTLV-I virion-related T cell activation. (iii) The CD8+ cell response to infected cells requires either the presence of viable CD4+ cells or exogenous IL-2.

The HTLV-I late phase SP might bear an important relationship to the observed cellular inflammation *in situ* around HTLV-I-infected cells in HAM/TSP, and may be involved in the critical pathological process of this disorder (Ijichi *et al.*, 1993). Although it has been extremely difficult to detect HTLV-I antigens in the affected tissues of patients (Gessain and Gout, 1992), the presence of integration and activation of HTLV-I in the CNS of HAM/TSP patients has been demonstrated by *in situ* hybridization techniques (Hara *et al.*, 1994; Kashio *et al.*, 1994; Kuroda *et al.*, 1994; Lehky *et al.*, 1995). The cell responses involved in the HTLV-I late phase SP presented here may be an *in vitro* equivalent of the cellular inflammation in the affected tissues in

patients with HTLV- I -associated diseases.

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HEMOLYSIN PRODUCTION BY *VIBRIO CHOLERAE* AS EXAMINED BY HEMOLYTIC AND ANTIGENIC ACTIVITIES

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Abstract: The hemolytic and antigenic activities of 36 strains of *Vibrio cholerae* O1 biotype El Tor, and 63 strains of *Vibrio cholerae* non-O1 isolated in Argentina were studied. Only 8% in stationary culture and 11% in shaking culture were hemolytic when the heart infusion broth (HIB) culture supernatants of *V. cholerae* O1 were examined. However, these percentages increased to 94% and 22% respectively when the strains were grown in HIB supplemented with 1% glycerol. On the other hand, most strains of *V. cholerae* non-O1 (97%) were hemolytic in both stationary and shaking HIB culture supernatants. The antigenic activity detected by reversed passive latex agglutination method (RPLA) varied markedly from strains and culture conditions; ranging from below the detection limit to 16,000 ng/ml. The optimal condition of hemolysin production varied with the strain, but a stationary culture was preferable to obtain high hemolytic activity and shaking culture to obtain a large amount of hemolysin antigen. The comparison of the hemolytic and antigenic activities showed that the hemolysin is readily inactivated in the culture supernatant, especially in the case of *V. cholerae* O1. These findings suggested the presence of some potent inactivation factor (s) in El Tor vibrios.

INTRODUCTION

Since Gotschlich discovered the hemolytic properties of *Vibrio cholerae* O1 in 1905 (Pollitzer, 1959), *V. cholerae* O1 (cholera vibrio) was classified into two biotypes, non-hemolytic classical biotype and hemolytic El Tor biotype. On the other hand, most *V. cholerae* non-O1 are hemolytic (Sakazaki, *et al.*, 1967). The hemolysin of *V. cholerae* non-O1 and O1 El Tor were purified by Yamamoto et al. (1986), and they proved that both hemolysins were identical. Although the role of hemolysin in the illness is not elucidated, it is cytotoxic and enterotoxic (Ichinose *et al* ., 1987; McCardell *et al.*, 1985). Recently identified new cholera vibrios (*V. cholerae* O139 synonym Bengal) are also hemolytic and the hemolytic activity is neutralized by anti-El Tor hemolysin antisera (Higa *et al.*, 1993). The hemolytic activities vary with the strain and culture condition. Hemolytic activities of *V. cholerae* non-O1 are more potent than those of *V. cholerae* O1 El Tor. El Tor cholera invaded South American Continent in 1991, and rapidly spread through the continent and Central America by Weekly Epidemiological Record(1992). According to the weekly epidemiological record from WHO, in Argentina there were 553 cases in 1992, 2080 in 1993, and 889 in 1994. In the epidemiological studies for cholera in Argentina, a number of *V. cholerae* O1 El Tor were isolated as well as *V. cholerae* non-O1 from the cases with watery diarrhea. Among the *V. cholerae* non-O1 isolates, one strain of non-toxigenic *V. cholerae* O139 was found (Rivas *et al.*, 1993). In this communication, we describe the production of hemolysin by *V. cholerae* strains isolated in Argentina as quantitatively examined by hemolytic as well as antigenic activities.

MATERIALS AND METHODS

1. *Bacterial Strains*: *Vibrio cholerae* non-O1 strain N037 isolated from a diarrheal patient in Thailand was used for purification of hemolysin. Thirty-six strains of *V. cholerae* O1 El Tor and 63 strains of *V. cholerae* non-O1 isolated in Argentina were used to study hemolysin

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production.

2. *Culture conditions*: Heart infusion broth (HIB) was used in stationary and shaking conditions to determine hemolysin production. The organisms were cultured at 30°C for 18 hr stationary in a large test tube or with shaking in a 100 ml Erlenmyer's flask containing each 10 ml of HIB. HIB supplemented with 1% glycerol (HIBG) was used for El Tor strains, producing a very small amount of hemolysin, to confirm the productivity.

3. *Purification of hemolysin*: The strain N037 was cultured in a 3L Erlenmyer's flask containing 300 ml of heart infusion broth (HIB) at 37°C for 18 hr with shaking. The culture supernatant was salted out with 60% saturated ammonium sulfate. The precipitate was dissolved in TEA buffer [50mM Tris-HCl, 1mM EDTA, 3mM NaN_3, pH 8.0] and dialyzed against the same buffer. The sample was fractionated by Sephadex G-100 column chromatography using TEA buffer for elution. The fractions with hemolytic activity were pooled and concentrated by ultrafiltration using an Amicon PM10 filter membrane. The concentrated material was fractionated by Sephacryl S-200 column chromatography using TEA buffer for elution. When the purity of hemolysin was not satisfactory, Sephacryl S-200 column chromatography was repeated once again.

4. *Preparation of anti-serum*: A rabbit was immunized with 100 μg of purified hemolysin every 2 weeks. One milliliter of antigen emulsified with an equal volume of Freund's complete adjuvant was injected multisite-subcutaneously. For the boosting injection, 100 μg of hemolysin and Freund's incomplete adjuvant was used.

5. *Preparation of hemolysin coupled affinity gel column*: The hemolysin solution was dialyzed against coupling buffer [0.2M $NaHCO_3$, 0.5M NaCl, pH 8.3] and 1 ml containing 9 mg of hemolysin was applied to 1 ml volume of Hi Trap Affinity Column NHS-activated (Pharmacia Biotech.). Then, the column was washed with buffer A [0.5M ethanolamine, 0.5M NaCl, pH 8.3] and successively with buffer B [0.1M sodium acetate, 0.5M NaCl, pH 4.0]. The column was equilibrated with storage buffer [0.05M Na_2HPO_4, 0.1% NaN_3, pH 7.0], and kept in a cold room until use.

6. *Preparation of anti-hemolysin IgG*: Anti-hemolysin anti-serum was heated at 56°C for 30 min and was salted out with 20% saturated ammonium sulfate. The supernatant was salted out again with 33% saturation. The precipitate was suspended in 10 mM phosphate buffered saline pH 7.4 (PBS) and dialyzed against PBS. This crude IgG fraction was adjusted to OD=0.7 at a wavelength of 280 nm, and 1 ml of the IgG solution was applied to the hemolysin conjugated Hi Trap Affinity Column equilibrated with PBS. The flow rate of IgG solution was 2 drops/sec. The column was washed with PBS until OD_{280} returned to the base line. Anti-hemolysin specific IgG was eluted with elution buffer [0.2M glycine, 0.5M NaCl, pH 2.7]. The eluted sample was neutralized with 0.1M NaOH.

7. *Preparation of sensitized latex*: SLD 59 latex (Takeda) prepared in a 0.1% suspension was mixed with an equal volume of 5% glutal aldehyde and incubated overnight at 4°C with shaking. The latex was washed and suspended in PBS to a final concentration of 0.5%. The affinity purified anti-hemolysin IgG was dialyzed against 3% sucrose in PBS. The latex and IgG were mixed at the concentrations of 0.1% and 5 to 10μg/ml, respectively. The mixture was incubated at 4 °C for 1 hr with shaking. Then the sensitized latex was washed with PBS containing 0.8% bovine serum albumin (BSA) and 0.1% sodium azide. Finally, the latex was suspended in adjusting buffer [PBS containing 0.8% BSA, 0.1% sodium azide, and 0.0005% polyvynylalcohol] at the concentration of 0.5%.

8. *Determination of hemolytic activities*: Fifteen ml of blood agar (1.3% agar, 7% sheep blood, 0.03% sodium azide in 10mM PBS) was prepared in a plastic petri dish 9 cm in diameter, and about 20 wells each 3 mm in diameter were made within one plate. The HIB culture supernatants of *V. cholerae* were applied to the wells (about 15 μl to each well) and incubated at 37 °C for 18 hr. The diameter of the hemolytic zone was measured.

9. *Titration of hemolysin antigen*: The amount of hemolysin antigen was quantitatively determined by reversed passive latex agglutination (RPLA). The culture supernatants were serially diluted 2-fold with the diluent [10mM PBS containing 0.8% BSA, 0.01% polyvinylpyrrolidone, and 0.1% sodium azide] in the U-bottom microdilution plates. An equal volume (25μl) of 0.025% sensitized latex was added to each well, mixed on the plate vibrator and incubated overnight at room temperature. The titers were defined as the reciprocal of the highest dilution in which agglutination was observed or the number of wells with agglutination. Purified hemolysin protein (1 or 0.1μg/ml) was used as the standard in each plate to determine the amount of

hemolysin.

10. *Protein assay*: The protein concentration was determined by Lowry's method (Lowry *et al.*, 1951).

RESULTS

1. *Sensitization of latex*: Various concentrations of anti-hemolysin IgG were examined at a constant concentration of latex to find the optimal concentration to sensitize the latex. At an IgG concentration lower than 1 μg/ml, the detection limit of hemolysin was higher than 10 ng/ml. However, IgG at a concentration higher than 10 μg/ml tended to cause autoagglutination. Therefore, to sensitize latex we used IgG at a concentration of 5μg/ml. The detection limit of hemolysin was 1 to 2 ng/ml.

2. *Hemolytic activities of V. cholerae*: Among 36 *V. cholerae* O1 (El Tor strains), hemolytic activity was found in the culture supernatants of only 3 strains (8%) in stationary culture and 4 strains (11%) in shaking culture. However, 61 out of 63 *V. cholerae* non-O1 strains (97%) in both culture conditions were hemolytic. Hemolytic activities of non-O1 *V. cholerae* in stationary culture were generally greater, than those in shaking culture (Table 1).

Comparing each strain, stronger activity was seen in stationary culture for 43 strains, but in shaking culture for 10 strains. The other 10 strains showed equal activity in both culture conditions.

3. *Antigenic activities of hemolysin*: Although most El Tor strains were not hemolytic in the culture conditions used (HIB), large amounts of hemolysin antigen were detected from the non-hemolytic culture supernatants. The El Tor strains produced more hemolysin antigen than the non-O1 strains (Table 2, Figure 1). Generally, the shaking culture produced more hemolysin antigen than the stationary culture, except for a few strains.

Table 1 Hemolytic activities of the culture supernatant

		Diameters of hemolytic zone (mm)					
		non	~4	~6	~8	~10	10~
O1, El Tor (n=36)	ST	33	1	2	0	0	0
	SH	32	0	2	2	0	0
non-O1 (n=63) HIB	ST	2	0	6	12	28	15
	SH	2	0	23	14	14	10
O1, El Tor HIBG	ST	2	2	30	2	0	0
	SH	28	2	6	0	0	0

Numerals indicate number of strains (36 O1 and 63 non-O1 strains in total), ST: stationary test tube culture, SH: shaking flask culture, HIB: heart infusion broth, HIBG: HIB supplemented with 1% glycerol, (El Tor strains were also cultured in HIBG). Diameter of hemolytic zone includes the size of well (3mm)

Table 2 Hemolysin production in HIB

Organisms (No. examined)	Culture conditions	Hemolysin antigen ng/ml (RPLA)	
O1 El Tor (n=36)	ST	min.	6.25
		max.	6400
		mean	764
	SH	min.	40
		max.	6400
		mean	1744
non-O1 (n=63)	ST	min.	0
		max.	6000
		mean	224
	SH	min.	6.25
		max.	6400
		mean	903

abbreviations: same as in Table 1

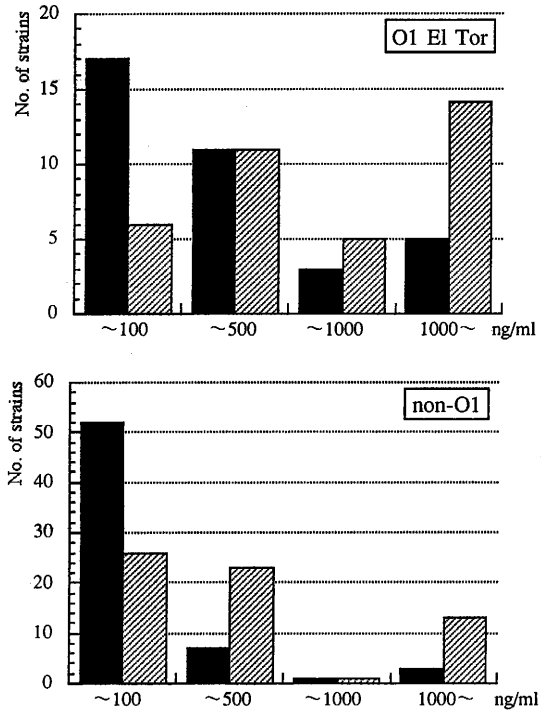

Figure 1 The amount of hemolysin produced in heart infusion broth as determined by reversed passive latex agglutination (RPLA) method. Solid column indicates stationary test tube culture, shaded column indicates shaking flask culture. Non-O1 strains do not include O139.

Table 3 Hemolysin production
(36 El Tor strains in HIBG)

Culture conditions	Hemolysin antigen ng/ml (RPLA)	
ST	min.	31.3
	max.	16000
	mean	1282
SH	min.	7.8
	max.	16000
	mean	1849

abbreviations: same as in Table 1

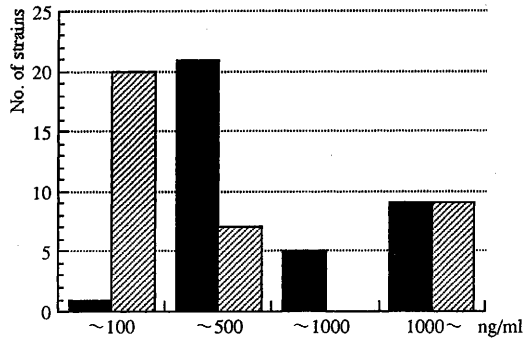

Figure 2 The amount of hemolysin produced by El Tor vibrios in heart infusion broth supplemented with 1% glycerol as determined by reversed passive latex agglutination (RPLA) method. Solid column indicates stationary test tube culture, shaded column indicates shaking flask culture.

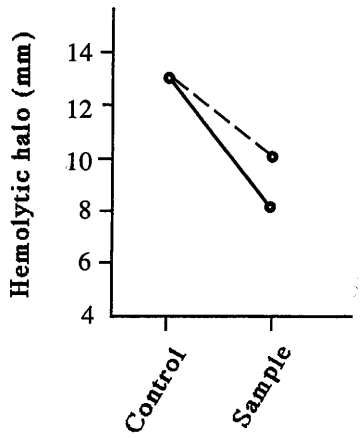

Figure 3 Inactivation test of hemolytic activity. The culture fluid of non-O1 vibrio strain (Arg 45) was incubated with fresh HIB at 37°C for 1 hr (volume ratio of 2 : 1). The diameter of hemolytic halo was reduced by adding the culture fluid of El Tor vibrio strain, Arg 57 (solid line), or Arg 62 (broken line).

4. *Hemolysin production in HIBG*: Since most El Tor strains were not hemolytic in HIB culture, HIB was supplemented with 1% glycerol (HIBG). In HIBG, 34 strains of 36 (94%) were hemolytic by stationary test tube culture, but 8 strains (22%) were hemolytic by shaking flask culture. A larger amount of hemolysin antigen was produced than in HIB. The average amount of hemolysin produced in the stationary test tube cultures was 1282 ng/ml in HIBG and 764 ng/ml in HIB, and that in the shaking flask cultures was 1849 ng/ml in HIBG and 1744 ng/ml in HIB (Table 2, Table 3, Figure 2). However, the amount of production varied markedly from strain; ranging from 7.8 ng/ml to 16,000 ng/ml.

5. *Inactivation test of hemolytic activity*: Hemolytic activity of non-O1 culture supernatant was markedly inhibited by mixing El Tor culture supernatant (Figure 3).

DISCUSSION

The present study revealed that the hemolysin produced by *V. cholerae* is readily inactivated in the culture supernatant, especially in the case of *V. cholerae* O1 El Tor. Therefore, the hemolytic activity of the culture supernatant is not correlated with the amount of hemolysin as examined by antigenic determination. There was no hemolytic activity in the 18 hr culture supernatant of most El Tor vibrios, but, a large amount of hemolysin protein was antigenically detected in the non-hemolytic culture supernatant. Besides, when El Tor vibrios were cultured for 4~6 hr, most culture supernatants were hemolytic (Data not shown). This means that some inactivation factors appear in the culture fluid within 18 hr. The factor(s) that inactivates the hemolysin of El Tor vibrios has not yet been identified, and it is unknown why the hemolysin produced by *V. cholerae* non-O1 (identical to El Tor hemolysin) maintained its hemolytic activity for 18 hr. The inactivation is not clearly understood but there may be a certain protease specific for El Tor vibrios, although non-O1 produces a protease similar to El Tor vibrios (Honda *et al.*, 1987). The non-O1 *V. cholerae* has stronger hemolytic activity than El Tor vibrios up to 18 hr of culture. Nevertheless, more hemolysin was produced by El Tor vibrios than by non-O1 *V. cholerae*. These findings also suggest the presence of some potent inactivation factor(s) in El Tor vibrios. The inactivation test of hemolytic activity supported this theory (Figure 3). Most El Tor vibrios cultured in heart infusion broth supplemented with 1% glycerol, became hemolytic even in an 18 hr culture but less hemolytic

than non-O1 *V. cholerae* strains in HIB culture, and in addition, El Tor vibrios cultured in HIBG produced more hemolysin than those cultured in HIB. This inactivation mechanism is characteristic of El Tor vibrios. *V. cholerae* non-O1 in shaking culture gave a larger amount of hemolysin and weaker hemolytic activity than in stationary culture, which indicated the presence of a certain factor which inactivates hemolysin to some extent but not completely.

The antigenically determined, biologically inactive hemolysin of El Tor vibrios consisted of a protein with the molecular weight of 60kDa and some other proteins with smaller molecules in SDS-PAGE (data not shown). The molecular weight of 60kDa is coincident with that of mature El Tor hemolysin. El Tor vibrios have an epidemic potential but non-O1 *V. cholerae* (excluding O139) including cholera toxin producing strains do not. Although the factors involved in the epidemic potential are unknown, it is attractive to speculate the relationship between the hemolysin inactivation mechanism and epidemic potential.

The optimal condition of hemolysin production varied with the strain, but a stationary culture was preferable to obtain high hemolytic activity and shaking culture was preferable to obtain a large amount of hemolysin antigen. If the inactivation mechanism could be blocked by some techniques in shaking culture, a large amount of biologically active hemolysin should be obtained.

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DESCRIPTION OF A NEW SPECIES OF *SIMULIUM* (*SIMULIUM*) FROM SABAH, MALAYSIA (DIPTERA: SIMULIIDAE)

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Abstract: The female and male of *Simulium* (*Simulium*) *beludense* sp. nov. are described based on reared adult specimens collected from Sabah, Malaysia. This new species was previously misidentified as *S.* (*S.*) *nigripilosum* Edwards of the *melanopus*-group because of a great similarity of their external morphology. The female genitalia examined in this study easily separate this new species from *S.* (*S.*) *nigripilosum*. It is also indicated that *S.* (*S.*) *beludense* sp. nov. is not assignable to the *melanopus*-group, but is rather closely related to *S.* (*S.*) *argentipes* Edwards from Peninsular Malaysia, *S.* (*S.*) *bidentatum* (Shiraki) from Japan and China, and *S.* (*S.*) *canlaonense* Delfinado from the Philippines, all of which belong to the different group so far undetermined.

Key words: black fly, Simuliidae, *Simulium*, Sabah, Malaysia

Smart and Clifford (1969) gave the descriptions of the male, pupa and mature larva, as well as the redescription of the female of *Simulium* (*Simulium*) *nigripilosum* originally described by Edwards (1933) from female and male specimens collected from Sabah, Malaysia. Takaoka (1983) illustrated for the first time the genitalia of the holotype female of *S.* (*S.*) *nigripilosum* loaned from British Museum (Natural History), London (abbreviated hereafter BMNH) and assigned it to the *melanopus*-group on the basis of the characteristic genitalia, particularly paraproct. The male genitalia of this species were also illustrated at the same time by Takaoka (1983) based on the reared adult specimen (also loaned from BMNH). This was one of the male adult specimens reared from the pupae collected by Smart in 1964 and determined by Smart and Clifford (1969). While revising the *melanopus*-group of the subgenus *Simulium* s.str. in Southeast Asia, I found the pupa of *S.* (*S.*) *nigripilosum* described by Smart and Clifford (1969) was atypical among the *melanopus*-group by having the eight-filamented gill and corbicular cocoon in place of the six-filamented gill and simple shoe-shaped cocoon, respectively. The subsequent examination of the reared female specimens loaned again from BMNH revealed that their genitalia were completely different from those of the holotype *S.* (*S.*) *nigripilosum* illustrated by Takaoka (1983).

In this paper, all the pupae, larvae and reared adults thought as "*S.* (*S.*) *nigripilosum*" by Smart and Clifford (1969) are regarded as a new species, and the female and male are described below. It should be also noted that the illustration of the male genitalia given by Takaoka (1983) was not of *S.* (*S.*) *nigripilosum* but of the new species.

Simulium (*Simulium*) *beludense* sp. nov.

Figs. 1-13

DESCRIPTION. **Female**. Body length ca. 2.5 mm. ***Head***. Narrower than width of thorax. Frons brownish black, shiny, with a few dark stout hairs along each lateral margin and above antennal base; frontal ratio 1.15:1.00:1.10; frons-head ratio 1.0:4.2. Fronto-ocular area (Fig. 1) narrowly developed, with round lateral tip. Clypeus brownish black, shiny, thickly white pruinose, largely bare medially, covered moderately with dark stout hairs only near and along lateral margins. Antenna (lost in holotype) composed of 2+9 segments, brown except scape and pedicel orange in one paratype ♀ from Pegalan River, but yellow on basal 1/2 and brown on apical 1/2 in the other paratype ♀ from Tuaran River. Maxillary palp brownish black, composed of 5 segments with proportional length of 3rd, 4th and 5th segments 1.0:1.0:2.5; 3rd segment (Fig. 2) of moderate size; sensory vesicle medium in size, elliptical, with rugged surface, 0.24 × length of 3rd segment, with

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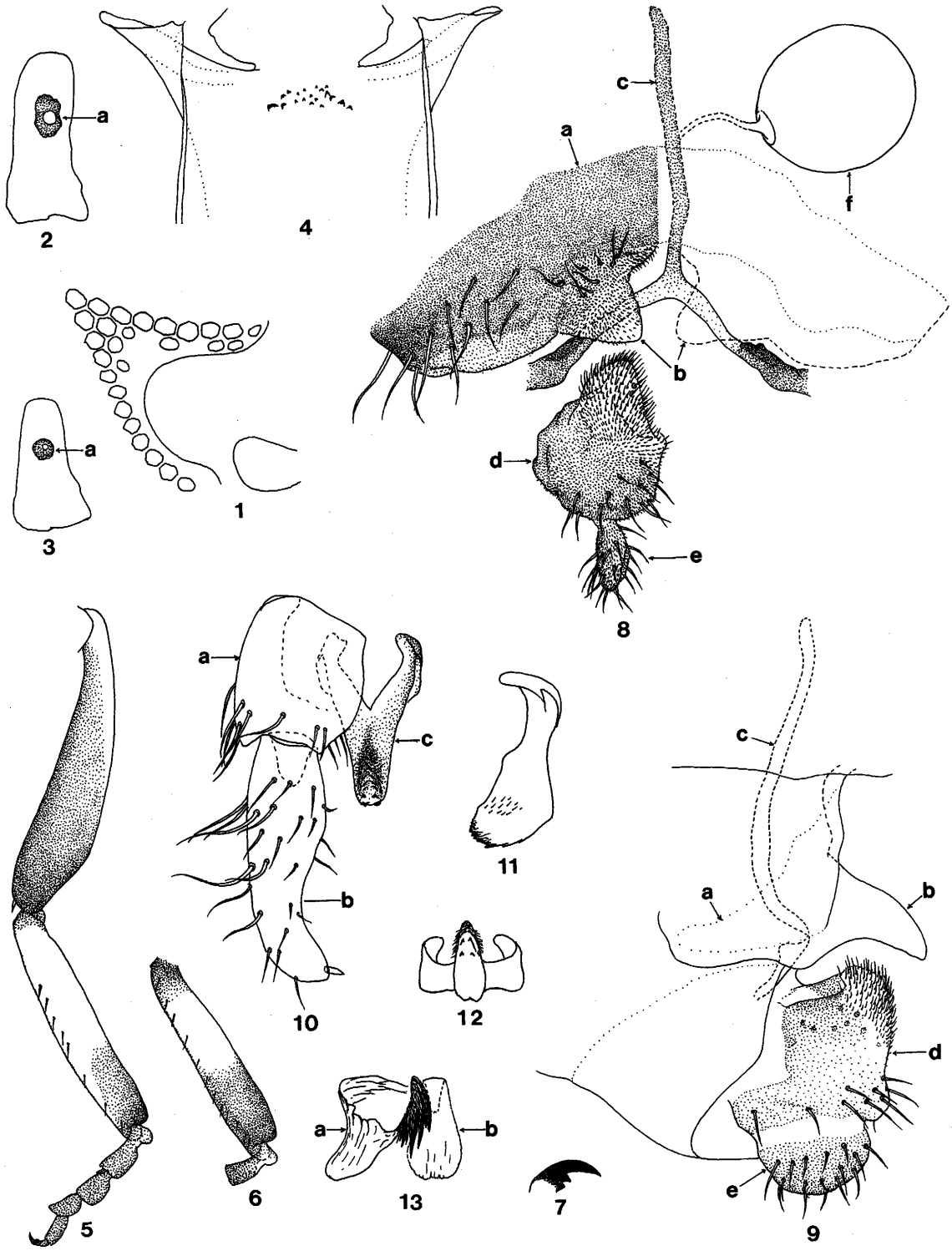
medium round opening medially. Maxillary lacinia with 11 or 12 inner and 12 or 13 outer teeth. Mandible with ca. 22 inner and 10 outer teeth. Cibarium (Fig. 4) with ca. 20 minute tubercles. ***Thorax***. Scutum dark brown, shiny, thinly white pruinose, covered moderately with fine copper-colored pubescence, interspersed with long, upstanding dark hairs on prescutellar area. Scutellum dark brown, with long dark hairs and copper-colored pubescence. Postscutellum dark brown, shiny, white pruinose, without hairs. Pleural membrane bare. Katepisternum longer than deep, bare. ***Legs***. Foreleg: coxa pale yellow, trochanter and femur brown; tibia whitish yellow with base somewhat darkened and distal 1/5 or 1/4 brown, and with median outer portion largely white sheeny in lights; basitarsus entirely brownish black, dilated (W:L = 1.0:4.1); rest tarsal segments brownish black. Midleg: coxa, trochanter and femur brown; tibia brown with posterior surface along basal 4/5 or a little more white and largely white sheeny when illuminated; tarsi brownish with basal 2/3 of basitarsus yellow. Hind leg: coxa, trochanter and femur brown; tibia (Fig. 5) brown with posterior surface along basal 2/3 white, and largely white sheeny when illuminated; tarsi (Fig. 5) brown with basal 2/3 of basitarsus whitish yellow; basitarsus parallel-sided, W:L = 1.0:5.3, calcipala short, L:W ratio 1.0:1.4; pedisulcus distinct. All tarsal claws (Fig. 7) with small subbasal tooth. All femora and tibiae covered with scale-like setae on outer surface. ***Wing***. Length 2.0 mm; costa with spinules and hairs; subcosta haired except distal 1/3 or 1/4 bare; basal section of vein R bare; hair tuft of stem vein dark brown; basal cell absent. ***Abdomen***. Basal scale brownish black with fringe of pale hairs; all segments brownish black; tergite 2 with a pair of large, dorsolateral silvery iridescent spots broadly connected to each other in middle; tergites 3, 4 and 5 small and dull, tergites 6-8 large, shiny, with dark hairs. ***Genitalia*** (Figs. 8 & 9). Ventral surface of abdominal segment 7 without developed sternite. Sternite 8 well sclerotized, bare medially but with 10-13 dark long stout hairs laterally on each side; anterior gonapophysis tongue-like in shape, membraneous, bent ventrally, covered with 9-11 somewhat long hairs as well as numerous microsetae except narrow portion along inner margin bare; inner borders concave, widely separated in middle from each other. Genital fork of inverted-Y form, with well sclerotized stem; arms narrow, each with strongly sclerotized distal bulge and with or without very short projection directed anterodorsally (if present, discernible when viewed from side). Paraproct enlarged, somewhat longer than wide in ventral view; nearly as long as wide, somewhat produced ventrally and ventroanteriorly, covered with ca. 12 short stout hairs in lateral view; surface of anteroventral corner densely covered with pale setae; inside surface largely concave anteriorly and with several minute setae. Cercus semilunar in lateral view, covered with numerous short hairs. Spermatheca nearly globular, well sclerotized with no definite reticulate pattern, with minute internal setae; tube and small area of tubal base unsclerotized.

Male. Body length 2.6 mm. ***Head***. Slightly wider than thorax. Upper eye consisting of large facets in 15 horizontal rows and 15 vertical columns. Clypeus black, silvery pruinose, strongly iridescent when illuminated, sparsely covered with dark hairs. Antenna composed of 2+9 segments, dark brown except scape, pedicel and a few basal flagellar segments yellowish; 1st flagellomere 1.6 × as long as 2nd one. Maxillary palp composed of 5 segments, proportional lengths of 3rd, 4th and 5th segments 1.0:1.2:2.1; 3rd segment (Fig. 3) of normal size, with small sensory vesicle. ***Thorax***. Scutum brownish black, with silvery and bluish iridescent pattern composed of an anterior pair of large spots on shoulders, a large transverse spot on prescutellar area contiguous to anterior spots by broad band along lateral margins; anterior pair of spots widely separated from each other in middle, curved posteromedially with posteromedial end somewhat pointed; scutum uniformly covered with brown pubescence, interspersed with long upright dark hairs on prescutellar area. Scutellum dark brown with long upright dark hairs. Postscutellum dark brown, white pruinose, shiny and bare. Katepisternum and pleural membrane as in ♀. ***Legs***. Foreleg: coxa yellow; trochanter pale brown; femur brown to brownish black; tibia brownish black with outer surface mostly white, iridescent when illuminated; tarsi blackish; basitarsus somewhat dilated, W:L = 1.0:6.1. Midleg: coxa, trochanter and femur brownish black; tibia brownish black with posterior surface along basal 1/3 white, iridescent on basal 1/2 when illuminated; tarsi brownish black with basal 1/2 or a little more of basitarsus and basal 1/2 of 2nd tarsal segment yellow. Hind leg: brownish black except basal 1/2 of basitarsus yellow; tibia white iridescent at base when illuminated; basitarsus enlarged, W:L = 1.0:4.7, and ca. 0.7 × as wide as tibia and femur (latter two segments subequal in width); calcipala small, pedisulcus distinct. ***Wing***. Length 1.9 mm; other features as in ♀ except subcosta bare. ***Abdomen***. Basal scale brownish black with long dark hairs. Tergites brownish black with dark hairs; segments 2, 4-7 each with a pair of silvery and bluish iridescent spots dorsolaterally; those on segment 2 broadly connected in



Figs. 1-13. Female and male of *Simulium* (*Simulium*) *beludense* sp. nov. 1, fronto-ocular area of ♀; 2 & 3, 3rd segment of maxillary palp (a, sensory vesicle) (2, ♀; 3, ♂); 4, ♀ cibarium; 5, ♀ hind tibia and tarsi; 6, ♂ hind basitarsus and 2nd tarsal segment; 7, ♀ claw; 8 & 9, ♀ genitalia in situ (a, 8th sternite; b, anterior gonapophyses; c, genital fork; d, paraproct; e, cercus; f, spermatheca) (8, ventral view; 9, lateral view); 10, ♂ genitalia in situ (a, coxite; b, style; c, ventral plate) (ventral view); 11 & 12, ventral plate (11, lateral view; 12, end view); 13, paramere (a) and median sclerite (b) (end view).

middle. ***Genitalia*** (Figs. 10-13). Coxite nearly quadrate, somewhat longer than wide. Style elongate, 1.6 × as long as coxite, narrowed from basal 1/3 to apical tip, and with apical spine; style flattened dorsoventrally and having no basal protuberance. Ventral plate Y-shaped in ventral view, having median process directed ventrally with ca. 10 fine setae on each side; posteroventral surface of median process with 2 marginal rows of several small spines converging apically; basal arms diverged from each other, with apical tip curved inward. Parameres wide, each with several hooks. Median sclerite wide, thin, almost transparent except basal portion brownish.

TYPE SPECIMENS. Holotype ♀ (BMNH 1969-492), depinned, dissected, cleared in KOH and slide-mounted in Berlese; reared from pupa, collected from River Wariu, ca. 15 m in altitude, Kota Belud, Sabah, 10.III. 1964, by J. Smart. Paratypes: 1 ♂, slide-mounted, same data as holotype; 1 ♂, pinned, other data same as holotype; 1 ♀, pinned, reared from pupa, collected from a stream on Tenom-Keningau Road, Pegalan Valley, ca. 180 m in alt., Sabah, 5-7.III.1964, by J. Smart; 1 ♀, pinned, reared from pupa collected from a small tributary of Tuaran River, ca. 60 m in alt., Tuaran, Sabah, 2. III.1964, by J. Smart; 1 ♂, pinned, reared from pupa collected from Tuaran River, above Tamparuli, ca. 14 m in alt., Tuaran, Sabah, 28.II.1964, by J. Smart. All type specimens examined in this study had been previously labelled as "*S.* (*S.*) *nigripilosum*" and "Brit. Mus. 1969 -492" by Smart and Clifford (1969).

ECOLOGICAL NOTE. According to Smart and Clifford (1969), the pupae and larvae of this species were collected on dead leaves caught among the stones in the river which was, at place of collection, unshaded and relatively wide and shallow.

DISTRIBUTION. Sabah.

ETYMOLOGY. This new species *beludense* was named after the locality from where the holotype specimen was collected.

REMARKS. This new species has a great resemblance in external characters to *S.* (*S.*) *nigripilosum*, but distinctly differs from the latter species by the genitalia of the female. The paraproct of this new species is of normal form and lacks a ventrointernal plate (Figs. 8d & 9d) while that of *S.* (*S.*) *nigripilosum* is remarkably produced ventrally and bears a ventrointernal plate (see Fig. 77 in Takaoka, 1983).

S. (*S.*) *beludense* appears to be very close in the adult to *S.* (*S.*) *bidentatum* (Shiraki) from Japan and China (Takaoka, 1976) by sharing many characters, eg., characteristic genitalia of both sexes, the female unpatterned shiny scutum and tarsal claws with a small basal tooth. The pupa and larva of this species, though not available in this study, also appear to resemble those of *S.* (*S.*) *bidentatum*; according to the illustrations of *S.* (*S.*) *beludense* (="*S.* (*S.*) *nigripilosum nec* Edwards") (see Figs. 11A, 10A and 4C of Smart and Clifford, 1969), the pupal gill, cocoon, and larval postgenal cleft are almost the same as those of *S.* (*S.*) *bidentatum* (Takaoka, 1976). However *S.* (*S.*) *bidentatum* differs from *S.* (*S.*) *beludense* in the female by the predominant yellowish leg coloration and in the male by the shape of the ventral plate and the abdominal iridescent spots present only on the segments 2, 5-7. From the morphological similarities of the female genitalia, tarsal claws and scutum, this new species also seems to be closely related to *S.* (*S.*) *argentipes* Edwards from Peninsular Malaysia (Takaoka and Davies, 1995) and *S.* (*S.*) *canlaonense* Delfinado from Negros Island of the Philippines (Takaoka, 1983), both of which have been known only from female adults. However *S.* (*S.*) *beludense* differs from both species by the much darker leg coloration, and also from the former species by the copper-colored pubescence on the scutum and from the latter species by the much produced paraproct.

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A NEW SPECIES AND NEW RECORDS OF BLACK FLIES (DIPTERA: SIMULIIDAE) FROM THAILAND

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Abstract: *Simulium* (*Byssodon*) *siripoomense* sp. nov. is described based on two mature larval specimens collected from Thailand. This species is distinguished from the other known species of this subgenus by the pupal gill histoblast with six slender filaments. Four other simuliid species are newly recorded from this country: i.e., *S.* (*Gomphostilbia*) *decuplum* Takaoka and Davies, *S.* (*G.*) *parahiyangum* Takaoka and Sigit, *S.* (*Simulium*) *brevipar* Takaoka and Davies, and *S.* (*S.*) *quinquestriatum* (Shiraki). Two known species, which had been tentatively identified as *S.* (*S.*) *sakishimaense* Takaoka and *S.* (*S.*) *nitidithorax* Puri, are treated to be conspecific to *S.* (*S.*) *fenestratum* Edwards and *S.* (*S.*) *tani* Takaoka and Davies, respectively. *Simulium* (*G.*) sp. C, one of the five unnamed species previously reported, is found to conform to *S.* (*G.*) *asakoae* Takaoka and Davies.
Key word : black fly, Simuliidae, *Simulium*, Diptera, Thailand

Takaoka and Suzuki (1984) reported 19 species of black flies (Diptera: Simuliidae) from Thailand, including seven new species. No other simuliid species have since been recorded from this country.

The present paper reports the result of black fly collections made in 1989 by the junior author. The material examined consists of larvae, pupae and reared adult specimens preserved in 70 % ethanol. Here identified are a total of 16 simuliid species, including one new and four newly recorded species. Three of the other known species, previously reported as *Simulium* (*Gomphostilbia*) sp. C, *S.* (*Simulium*) *nitidithorax* Puri, and *S.* (*S.*) *sakishimaense* Takaoka, are newly identified as *S.* (*G.*) *asakoae*, *S.* (*S.*) *tani* and *S.* (*S.*) *fenestratum*, respectively, the former two described from Peninsular Malaysia (Takaoka and Davies, 1995) and the last from Sumatra (Edwards, 1934).

GENUS *SIMULIUM* LATREILLE SUBGENUS *BYSSODON* ENDERLEIN

1. *Simulium* (*Byssodon*) *siripoomense* sp. nov.

The adult female, male and pupal specimens of this species were not available. However this is here described as a new species on the basis of two mature larvae, which show remarkable characters as noted below.

DESCRIPTION. **Female, male** and **pupa**. Unknown. **Mature larva**. Body length 3.3-3.8 mm. Body pale yellowish, somewhat brownish dorsally on posterior abdominal segments. Head capsule pale yellowish with weak positive head pattern on cephalic apotome; cervical sclerites separated and faint. Antenna longer than stem of labral fan, with 3 segments and apical sensillum; proportional lengths of 3 segments from base to tip 1.0 : 1.2 : 1.0. Labral fan with 35 main rays. Mandible (Fig. 1) with comb-teeth decreasing in size from 1st to 3rd; mandibular serrations composed of 1 large and 1 small teeth, without supernumerary serrations. Hypostomium (Fig. 2) with a row of 9 apical teeth; each corner tooth prominent; lateral margin serrate; hypostomal bristles 4 in number lying subparallel to lateral margin on each side. Postgenal cleft (Fig. 3) deep, reaching posterior border of hypostomium. Thoracic cuticle bare. Histoblast of pupal gill with 6 slender filaments in pairs; all filaments tapering toward apex and with minute tubercles on surface. Abdominal cuticle almost bare except sides of anal sclerite of last segment moderately covered with colorless setae; abdominal segments 1-4 each with

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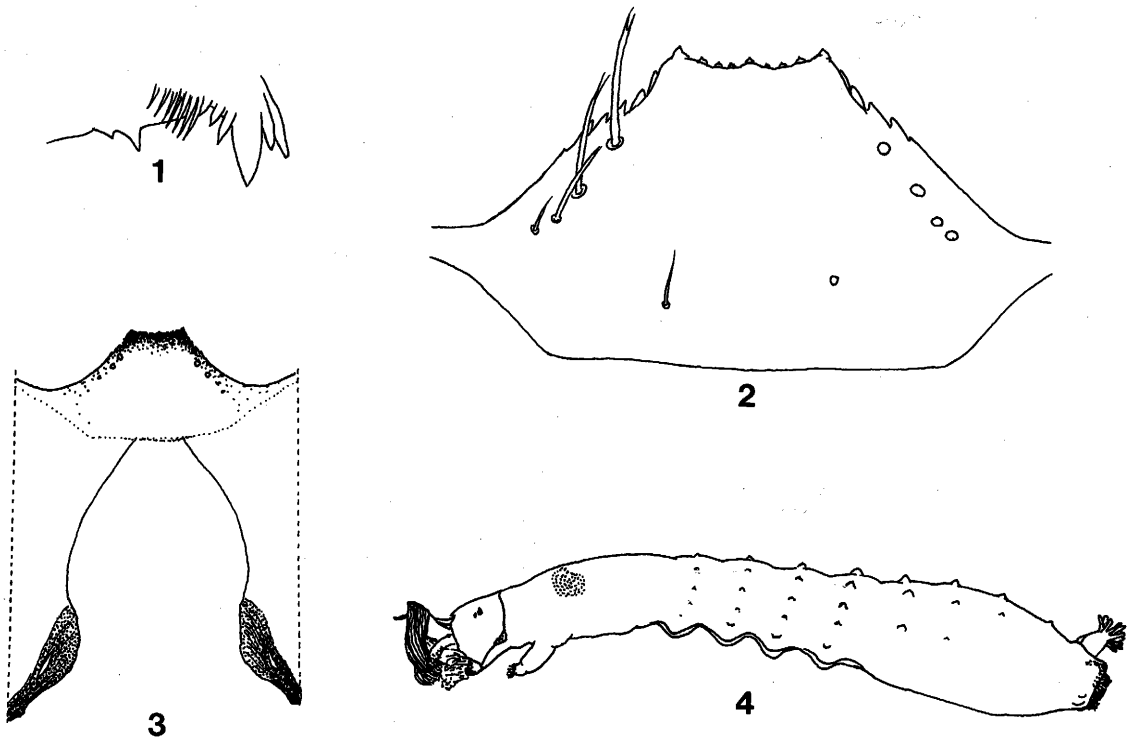

Figs. 1-4. Larva of *Simulium* (*Byssodon*) *siripoomense* sp. nov. 1, apex of mandible; 2, hypostomium; 3, head capsule (ventral view), showing deep postgenal cleft; 4, whole body (lateral view), showing protuberances on the abdomen.

6 pairs of subconical protuberances (1 dorsally, 1 dorsolaterally, 2 laterally, 1 ventrolaterally and 1 ventrally), although those on segment 1 are much less distinct; segments 5 and 6 each with 3 pairs of similar protuberances (1 dorsally, 1 dorsolaterally and 1 laterally); segments 7 and 8 each with 1 or 2 pairs of similar protuberances (1 dorsally and 1 dorsolaterally) (Fig. 4). Rectal papilla compound, each of 3 lobes with 7 or 8 finger-like secondary lobules. Anal sclerite of usual X-shape, with anterior arms ca. 1/2 × as long as posterior arms, broadly sclerotized between arms at base. Last abdominal segment somewhat bulged laterally but without ventral papillae. Posterior circlet with 66 rows of up to 14 hooklets per row.

TYPE SPECIMENS. Holotype mature larva (BMNH), slide-mounted, Siripoom Water Fall, near Chiang Mai, 14.VIII.1989. Paratype 1 mature larva, in alcohol, Ban Tapa, near Chiang Mai, 15.VIII.1989.

ECOLOGICAL NOTES. The holotype larva of this species was collected from a rapidly-flowing mountainous stream which was 3-6 m wide and 0.3-0.5 m deep, together with *S.* (*Himalayum*) *nigrogilvum* Summers, *S.* (*S.*) *rufibasis* Brunetti, *S.* (*S.*) *tani* Takaoka and Davies and *S.* (*S.*) *chamlongi* Takaoka and Suzuki. The water temperature of this stream was 18°C. The paratype larva was collected from a slow-flowing, unshaded stream, 3-4 m wide and 0.1-0.2 m deep. The water temperature was 24°C. This species was caught together with *S.* (*G.*) *asakoae* Takaoka and Davies, *S.* (*G.*) *decuplum* Takaoka and Davies, *S.* (*G.*) *parahiyangum* Takaoka and Sigit, *S.* (*S.*) *nodosum* Puri, *S.* (*S.*) *quinquestriatum* (Shiraki), and *S.* (*S.*) *tani*.

DISTRIBUTION. Thailand.

ETYMOLOGY. The species name *siripoomense* refers to the place name Siripoom from where this species was collected.

REMARKS. This new species is tentatively assigned to the subgenus *Byssodon*, defined by Crosskey (1969), because of its very large postgenal cleft reaching the posterior border of the hypostomium, and subconical prominent protuberances at least ventrally on the abdominal segments 1 to 4, as shown in Figs. 3 and 4. The larva of this species appears to be indistinguishable from that of *S.* (*B.*) *maculatum* (Meigen) and its varieties of the *meridionale*-group (Rubtsov, 1959-1964),

from which it differs by the pupal gill histoblast with six filaments, instead of 22-26.

The subgenus *Byssodon* is widely distributed in the Palaearctic, Nearctic and Ethiopian Regions. In the Oriental Region only one *Byssodon* species, *S.* (*B.*) *languidum*, was described from Sri Lanka (Davies and Györkös, 1988), which showed several characters resembling this new species, including the deep postgenal cleft. However, *S.* (*B.*) *languidum* lacks protuberances on the larval abdomen, and bears three somewhat inflated pupal gill filaments on each side.

The larva of this species resembles that of *S.* (*S.*) *nobile* de Meijere by having the deep postgenal cleft, dorsal protuberances on the abdomen, and the pupal histoblast with six slender filaments, but the latter species lacks ventral and lateral protuberances on the abdomen and has prominent spinous scales on the dorsal surface of the abdomen. By sharing the deep postgenal cleft and dorsal protuberances on the abdomen in the larval stage, this species is also similar to *S.* (*G.*) *parahiyangum* originally described from Java (Takaoka and Sigit, 1992). However, *S.* (*B.*) *siripoomense* differs from the latter by the shape and angle of the main tooth of mandibular serrations, the serrate lateral margins of the hypostomium, the pupal gill histoblast with six filaments and the absence of the ventral papillae.

SUBGENUS *GOMPHOSTILBIA* ENDERLEIN

(A) *batoense*-group

2. *Simulium* (*Gomphostilbia*) *siamense* Takaoka and Suzuki, 1984

Simulium (*Gomphostilbia*) *siamense* Takaoka and Suzuki, 1984: 14-18 (female, male, pupa and mature larva).

SPECIMENS EXAMINED. 2 ♀, 1 pupa and 2 pupal exuviae, Ban Thung Pong, 16.VIII.1989; 1 pupa, 1 pupal exuvia and 1 mature larva, Loaw River, 17.VIII.1989 (both localities near Chiang Mai).

DISTRIBUTION. Thailand.

3. *Simulium* (*Gomphostilbia*) *decuplum* Takaoka and Davies, 1995

Simulium (*Gomphostilbia*) *decuplum* Takaoka and Davies, 1995: 46-50 (female, pupa and mature larva).

SPECIMENS EXAMINED. 1 ♀ and 4 mature larvae, Ban Tapa, 15.VIII.1989; 1 ♂, Huai Kaeo, 15.VIII.1989 (both localities near Chinag Mai).

DISTRIBUTION. Peninsular Malaysia and Thailand (new record).

REMARKS. The female, pupae and mature larvae collected from Thailand are in accordance with the original description given by Takaoka and Davies (1995). This species is readily separated from the other *Gomphostilbia* species from Thailand by the 10 short pupal gill filaments per side. The description of the male of this species will be given elsewhere on the basis of specimens recently collected from Peninsular Malaysia (unpublished data).

4. *Simulium* (*Gomphostilbia*) *parahiyangum* Takaoka and Sigit, 1992

Simulium (*Gomphostilbia*) *parahiyangum* Takaoka and Sigit, 1992 : 135-142 (female, male, pupa and mature larva); Takaoka and Davies, 1995: 50-51.

SPECIMENS EXAMINED. 1 mature larva, Ban Tapa, 15.VIII.1989; 1 mature larva, Hoy Nam Kam, 15.VIII. 1989 (both localities near Chiang Mai).

DISTRIBUTION. Java, Peninsular Malaysia and Thailand (new record).

REMARKS. The morphological characters of the mature larvae collected from Thailand agree well with the original description given by Takaoka and Sigit (1992). This species is readily distinguished from most of other *Gomphostilbia* species in the pupa by the eight short, slender filaments per side, and antennal sheath with nine well marked ridges on the outer margin, each covered with several cone-shaped tubercles, and in the larva by the prominent dorsal protuberances on the abdominal segments 1-5, several dark stout spines on the abdominal segments 6-8, and the deep postgenal cleft reaching the posterior margin of the hypostomium (Takaoka and Sigit, 1992).

(B) *ceylonicum*-group

5. *Simulium* (*Gomphostilbia*) *asakoae* Takaoka and Davies, 1995

Simulium (*Gomphostilbia*) *asakoae* Takaoka and Davies,

1995: 55-60 (female, male, pupa and mature larva); Takaoka, Davies and Dudgeon, 1995: 192-193.
Simulium (*Gomphostilbia*) sp. C Takaoka and Suzuki, 1984: 21-22 (male).

SPECIMENS EXAMINED. 1 ♂, Doi Inthanon, 14.VIII. 1989; 1 ♂, Ban Tapa, 15.VIII.1989; 1 mature larva, Hoy Nam Kam, 15.VIII.1989; 6 ♀, 6 ♂, 1 pupa, 6 pupal exuviae and 10 mature larvae, Ban Thung Pong, 16.VIII. 1989 (all localities near Chiang Mai).

DISTRIBUTION. Peninsular Malaysia, Hong Kong and Thailand.

REMARKS. The male of *S.* (*G.*) sp. C reported from Thailand (Takaoka and Suzuki, 1984) has proved to be the same as that of *S.* (*G.*) *asakoae* described from Peninsular Malaysia (Takaoka and Davies, 1995). It should be however noted that the present reared male specimens slightly differ from those of the Malaysian specimens by having the large eye facets in 15 horizontal rows and in 12 vertical columns (not in 13 horizontal rows and in 10 or 11 vertical columns as in Malaysian specimens and *S.* (*G.*) sp. C).

SUBGENUS *HIMALAYUM* LEWIS

6. *Simulium* (*Himalayum*) *nigrogilvum* Summers, 1911

Simulium (*Himalayum*) *nigrogilvum* Summers, 1911: 586-588 (female); Takaoka and Suzuki, 1984: 22-27 (female, male, pupa and mature larva).

SPECIMENS EXAMINED. 1 ♀, 1 ♂ and 6 mature larvae, Siripoom Water Fall, near Chiang Mai, 14.VIII. 1989.

DISTRIBUTION. Thailand.

SUBGENUS *NEVERMANNIA* ENDERLEIN

(A) *ruficorne*-group

7. *Simulium* (*Nevermannia*) *aureohirtum* Brunetti, 1911

Simulium aureohirtum Brunetti, 1911: 283-288 (male); Edwards, 1934: 134-137 (female, pupa and mature larva).
Simulium (*Eusimulium*) *aureohirtum*: Puri, 1933: 1-7 (female, male, pupa and mature larva); Takaoka, 1976: 170-171; Takaoka, 1979: 382-384 (female, male, pupa and mature larva); Takaoka, 1983: 15-16.
Simulium (*Nevermannia*) *aureohirtum*: Ogata, 1956: 61-62; Ogata, 1966: 129; Takaoka and Roberts, 1988: 194-195; Takaoka and Davies, 1995: 87-88.

SPECIMENS EXAMINED. 11 ♀, 13 ♂, 1 pupal exuvia and 10 mature larvae, Ban Mae Kanin, near Chiang Mai, 16.VIII.1989.

DISTRIBUTION. China, Hong Kong, India, Japan, Java, Peninsular Malaysia, Philippines, Sabah, Sri Lanka, Sulawesi, Sumatra, Thailand and Taiwan.

SUBGENUS *SIMULIUM* LATREILLE

(A) *multistriatum*-group

8. *Simulium* (*Simulium*) *fenestratum* Edwards, 1934

Simulium (*Simulium*) *fenestratum* Edwards, 1934: 110-111 (female, male, pupa and mature larva)
Simulium (*Simulium*) *sakishimaense* (*nec* Takaoka, 1977): Takaoka and Suzuki, 1984: 39-40.

SPECIMENS EXAMINED. 1 ♀, Chiang Dao, 13.VIII. 1989; 1 ♀ and 1 ♂, Hoy Nam Kam, 15.VIII.1989; 1 ♂ and 2 mature larvae, Huai Kaeo, 15.VIII.1989; 1 ♀, Loaw River, 17.VIII.1989; 1 ♂, Doi Steps, 17.VIII.1989; 2 pupal exuviae, Ban Pongyame, 16.VIII.1989 (All localities near Chiang Mai).

DISTRIBUTION. Sumatra and Thailand.

REMARKS. Takaoka and Suzuki (1984), while identifying this species as *S.* (*S.*) *sakishimaense* Takaoka originally described from the Ryukyu Islands, pointed out several characters of the Thailand specimens differing from those of the specimens from the type locality. The most remarkable difference was found in the male hind basitarsus which was mostly darkened except for the basal 1/3 somewhat paler or yellowish in the Thailand specimens but darkened only on the distal 1/2 in the Ryukyu specimens. The difference was also found in the larval stage: the Thailand larvae had more secondary lobules of the rectal papilla (10-15 vs. 5-8 per each lobe). In the original description

of *S.* (*S.*) *fenestratum* (Edwards, 1934) the male hind basitarsus was not observed for its coloration, although the number of secondary lobules of the larval rectal papilla was given as 15-20. Recent collections made in Sumatra by the senior author have revealed that the Thailand specimens are almost the same as those of *S.* (*S.*) *fenestratum* from Sumatra in many characters, including the male and larval characters mentioned above (unpublished data). The male of *S.* (*S.*) *hirtinervis* Edwards described from Peninsular Malaysia (Takaoka and Davies, 1995) has a hind basitarsus similar to *S.* (*S.*) *sakishimaense*. *Simulium* (*S.*) *malayense*, also described from Peninsular Malaysia (Takaoka and Davies, 1995), shows a darker coloration on the male hind basitarsus as in *S.* (*S.*) *fenestratum* according to the reared male specimens recently collected (unpublished data), but differs from the latter by having its cocoon without windows anteriorly.

(B) *nobile*-group

9. *Simulium* (*Simulium*) *nodosum* Puri, 1933

Simulium (*Simulium*) *nodosum* Puri, 1933: 813-817 (female, pupa and mature larva); Takaoka and Suzuki, 1984 : 38 (pupa and mature larva); Datta, 1988: 6 (male); An, 1989: 187; Takaoka, Davies and Dudgeon, 1995: 195.

SPECIMENS EXAMINED. 2 ♀, 2 ♂, 4 pupae and 3 mature larvae, Chiang Dao, 13.VIII.1989; 1 pupa and 3 mature larvae, Hoy Mae Aem, 14.VIII.1989; 3 mature larvae, Ban Tapa, 15.VIII.1989; 1 ♀, 3 ♂, 2 pupae, 1 pupal exuvia and 2 mature larvae, Hoy Nam Kam, 15.VIII.1989; 1 ♀, 1 ♂ and 5 mature larvae, Ban Mae Kanin, 16.VIII.1989; 5 ♀, 9 ♂ and 6 mature larvae, Ban Mae Ha, 16.VIII.1989; 2 pupae, 3 pupal exuviae and 2 mature larvae, Bang Thung Pong, 16.VIII.1989; 1 ♂, 3 pupae, 2 pupal exuviae and 3 mature larvae, Ban Pongyame, 16.VIII.1989; 3 ♀ and 2 ♂, Mae Sa Water Fall, Ban Nie, 16.VIII.1989; 2 ♀ and 9 ♂, Ban Sobpong, 17.VIII.1989; 1 ♀ and 1 mature larva, Loaw River, 17.VIII.1989 (all localities near Chiang Mai).

DISTRIBUTION. China, Hong Kong, India and Thailand.

REMARKS. The reared female and male specimens of this species were obtained for the first time from Thailand. The female characters are almost the same as originally described (Puri, 1933) except the posteriorly produced round robes which are well separated in the middle from each other (their apices not so converged and approximating as originally illustrated). The male genitalia of the Thailand specimens are also the same as illustrated by Datta (1992). The larval characters, such as the dorsal protuberances and chisel-shaped spines on the abdomen present in the Thailand specimens (Takaoka and Suzuki, 1984) but not mentioned in the original description, have been suggested to be present also in the Indian specimens according to Datta (1992).

(C) *striatum*-group

10. *Simulium* (*Simulium*) *chiangmaiense* Takaoka and Suzuki, 1984

Simulium (*Simulium*) *chiangmaiense* Takaoka and Suzuki, 1984: 38-39 (female, male and pupa); An, 1989: 186.

SPECIMENS EXAMINED. 2 ♀ and 5 mature larvae, Chiang Dao, 13.VIII.1989; 1 ♀, Loaw River, 17.VIII.1989 (both localities near Chiang Mai).

DISTRIBUTION. China and Thailand.

11. *Simulium* (*Simulium*) *nakhonense* Takaoka and Suzuki, 1984

Simulium (*Simulium*) *nakhonense* Takaoka and Suzuki, 1984: 33-37 (female, male, pupa and mature larvae); Chen, 1985: 308.

SPECIMENS EXAMINED. 1 pupal exuvia, Hoy Mae Aem, 14.VIII.1989; 1 pupal exuvia, Water Fall, 14.VIII.1989; 1 pupal exuvia, Maeklang Fall, 14.VIII.1989; 1 ♀, Hoy Nam Kam, 15.VIII.1989; 1 ♀ and 3 ♂, Ban Pongyame, 16.VIII.1989; 4 ♀ and 7 ♂, Mae Sa Water Fall, Ban Nie, 16.VIII.1989 (all localities near Chiang Mai). 12 ♀ and 8 ♂, Saeng, 20.VIII.1989; 1 ♂, Nan Long, 20.VIII.1989 (both localities in Nakhon Nayok).

DISTRIBUTION. China and Thailand.

12. *Simulium* (*Simulium*) *quinquestriatum* (Shiraki, 1935)

Stilboplax 5-striatum Shiraki, 1935: 27-33 (female).
Simulium (*Simulium*) *quinquestriatum*: Anonym, 1974: 192; Takaoka, 1977: 205-209 (female, male, pupa and mature larva); Takaoka, 1979: 396-399 (female, male, pupa and mature larva); An, 1989: 187; Takaoka, Davies and Dudgeon, 1995: 195-196.

SPECIMENS EXAMINED. 1 ♀, 3 ♂, 1 pupa, 1 pupal exuvia and 6 mature larvae, Hoy Mae Aem, 14.VIII. 1989; 2 ♀ and 3 mature larvae, Ban Tapa, 15.VIII.1989; 4 ♀ and 2 ♂, Mae Sa Water Fall, Ban Nie, 16.VIII.1989 (all localities near Chiang Mai). 1 pupa and 1 pupal exuvia, Nan Long, in Nakhon Nayok, 20.VIII.1989.

DISTRIBUTION. China, Hong Kong, Japan, Korea, Thailand (new record) and Taiwan.

REMARKS. The female, male, pupal and mature larval specimens collected from Thailand morphologically agree well with the redescription of this species (Takaoka, 1979). This species is distinguished from the other three Thailand species of the *striatum*-group (i.e., *S.* (*S.*) *chiangmaiense, S.* (*S.*) *nakhonense* and *S.* (*S.*) *thailandicum*) by the pupal gill with 10 slender filaments per side, which are all subequal in thickness, and are arranged in 2+3+3+2 filaments.

(D) *tuberosum*-group

13. *Simulium* (*Simulium*) *brevipar* Takaoka and Davies, 1995

Simulium (*Simulium*) *brevipar* Takaoka and Davies, 1995: 132-137 (female, male, pupa and mature larva).

SPECIMENS EXAMINED. 1 ♂ and 1 mature larva, Doi Steps, near Chiang Mai, 17.VIII.1989.

DISTRIBUTION. Peninsular Malaysia and Thailand (new record).

REMARKS. The morphological characters of the male, pupa and mature larvae collected from Thailand are almost the same as those originally described (Takaoka and Davies, 1995). The presence of a pit-like cuticular organ at the base of pupal gill filaments separates this species from all the other simuliid species from Thailand.

14. *Simulium* (*Simulium*) *rufibasis* Brunetti, 1991

Simulium rufibasis Brunetti, 1911: 282-288 (female); Rubtsov, 1959-1964: 554.
Simulium (*Simulium*) *rufibasis*: Puri, 1932: 899-903 (female, male and pupa); Takaoka, 1977: 213-216 (female, male, pupa and mature larva); Takaoka and Suzuki, 1984: 41-42; An, 1989: 187-188.

SPECIMENS EXAMINED. 2 ♂, 2 pupae, 2 pupal exuviae and 1 mature larva, Siripoom Water Fall, near Chiang Mai, 14.VIII.1989.

DISTRIBUTION. China, India, Japan, Korea, Myanmar, Pakistan, Thailand and Taiwan.

15. *Simulium* (*Simulium*) *tani* Takaoka and Davies, 1995

Simulium (*Simulium*) *tani* Takaoka and Davies, 1995: 137-141 (female, male, pupa and mature larva).
Simulium (*Simulium*) *nitidithorax* (*nec* Puri, 1932): Takaoka and Suzuki, 1984: 40-41 (mature larva).

SPECIMENS EXAMINED. 1 pupa and 5 mature larvae, Siripoom Water Fall, 14.VIII.1989; 2 ♀, 1 ♂, 2 pupal exuviae and 2 mature larvae, Water Fall, 14.VIII.1989; 1 ♀, 2 ♂, 1 pupa, 1 pupal exuvia, 10 mature larvae, Ban Tapa, 15.VIII.1989; 3 pupal exuviae, Huai Kaeo, 15.VIII. 1989; 1 ♂ and 5 mature larvae, Doi Stops-Tai, 17.VIII. 1989 (all localities near Chiang Mai).

DISTRIBUTION. Peninsular Malaysia and Thailand.

REMARKS. This species was previously reported from Thailand as *S.* (*S.*) *nitidithorax* Puri originally described from India (Takaoka and Suzuki, 1984). This species closely resembles the latter species, but differs from it by several characters, including the short-stalked pupal gill filaments (not sessile as in *S.* (*S.*) *nitidithorax*), as already discussed by Takaoka and Davies (1995).

(E) *variegatum*-group

16. *Simulium* (*Simulium*) *chamlongi* Takaoka and Suzuki, 1984

Simulium (*Simulium*) *chamlongi* Takaoka and Suzuki, 1984: 27-30 (female, male, pupa and mature larva); An, Zhang and Deng, 1990: 106.

SPECIMENS EXAMINED. 5 ♀, 7 ♂, 5 pupae, 7 pupal exuviae and 3 mature larvae, Siripoom Water Fall, 14. VIII.1989; 10 ♀, 9 ♂, 6 pupae and 4 mature larvae, Water Fall, 14.VIII.1989 (both localities near Chiang Mai).

DISTRIBUTION. China and Thailand.

Appendix. The list of Simuliidae from Thailand

Genus *Simulium* Latreille
Subgenus *Byssodon* Enderlein
meridionale-group
S. (*B.*) *siripoomense* Takaoka and Saito, sp. nov.
Subgenus *Gomphostilbia* Enderlein
batoense-group
S. (*G.*) *decuplum* Takaoka and Davies, 1995
S. (*G.*) *parahiyanagum* Takaoka and Sigit, 1992
S. (*G.*) *siamense* Takaoka and Suzuki, 1984
ceylonicum-group
S. (*G.*) *asakoae* Takaoka and Davies, 1995
[= *S.* (*G.*) sp. C Takaoka and Suzuki, 1984]
S. (*G.*) *inthanonense* Takaoka and Suzuki, 1984
Subgenus *Himalayum* Lewis
S. (*H.*) *nigrogilvum* Summers, 1911
Subgenus *Nevermannia* Enderlein
feuerborni-group
S. (*N.*) sp. B Takaoka and Suzuki, 1984
ruficorne-group
S. (*N.*) *aureohirtum* Brunetti, 1911
vernum-group
S. (*N.*) sp. A Takaoka and Suzuki, 1984
Subgenus *Simulium* Latreille
griseifrons-group
S. (*S.*) *digrammicum* Edwards, 1928
multistriatum-group
S. (*S.*) *fenestratum* Edwards, 1934
[= *S.* (*S.*) *sakishimaense*: Takaoka and Suzuki, 1984 (*nec* Takaoka, 1977)]
nobile-group
S. (*S.*) *nodosum* Puri, 1933
striatum-group
S. (*S.*) *chiangmaiense* Takaoka and Suzuki, 1984
S. (*S.*) *nakhonense* Takaoka and Suzuki, 1984
S. (*S.*) *quinquestriatum* (Shiraki, 1935)
S. (*S.*) *thailandicum* Takaoka and Suzuki, 1984
tuberosum-group
S. (*S.*) *brevipar* Takaoka and Davies, 1995
S. (*S.*) *rufibasis* Brunetti, 1911
S. (*S.*) *tani* Takaoka and Davies, 1995
[= *S.* (*S.*) *nitidithorax*: Takaoka and Suzuki, 1984 (*nec* Puri, 1932)]
S. (*S.*) sp. D Takaoka and Suzuki, 1984
S. (*S.*) sp. E Takaoka and Suzuki, 1984
variegatum-group
S. (*S.*) *barnesi* Takaoka and Suzuki, 1984
S. (*S.*) *chamlongi* Takaoka and Suzuki, 1984

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CASES OF OVALE MALARIA AND MALARIAE MALARIA SUCCESSFULLY TREATED WITH MEFLOQUINE.

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Abstract: Two Japanese with ovale malaria and a Cameroonian with malariae malaria were put on mefloquine and were successfully treated as long as they were observed. Two of the 3 cases entered into this study because the possibility of falciparum malaria was not excluded by the physicians who first examined their blood specimens. These case reports offer a promising alternative to chloroquine in the treatment of ovale and malariae malaria.

INTRODUCTION

The antimalarial drug mefloquine, which was first approved and sold in Switzerland in 1985, has been widely distributed and has become a first-line drug for the treatment of uncomplicated falciparum malaria (Palmer *et al.*, 1993). Although the resistance of *Plasmodium falciparum* to mefloquine is emerging and is posing therapeutic difficulty to a greater extent in the Thai-Cambodian and Thai-Burmese borders, most cases acquired in other areas of the world can be cured with this drug (Kimura, 1996). In addition to its excellent clinical efficacy in falciparum malaria, it was proved to be also effective in vivax malaria (Dixon *et al.*, 1985), sometimes even with a smaller dosage (Harinasuta et al., 1985). Cases of chloroquine-resistant vivax malaria have been reported in some areas (Rieckmann *et al.*, 1989; Schwartz *et al.*, 1991), and mefloquine could be a drug of choice in such settings.

To date, the antimalarial efficacy of mefloquine on ovale or malariae malaria has never been studied extensively (Palmer *et al.*, 1993), because so far both of the two types of malaria can be successfully cured by chloroquine. Recently, we experienced 2 cases of ovale malaria and one case of malariae malaria who were treated successfully with mefloquine. Herein we describe the 3 cases and will discuss about the possible use of mefloquine in the two kinds of malaria.

CASE DESCRIPTION

Patient 1 was a 30-years-old Japanese male who stayed in Equatorial Guinea from June 25 to July 24, 1991. His body weight was 88kg. During and for 4 weeks after the stay, he was on a weakly chloroquine as prophylaxis. On April 19, 1992, he began to have fever reaching up to 40°C and lasting for a few days. On April 22, he visited our hospital and the blood revealed to contain a small number of malaria parasites. Because the species of malaria parasites could not be identified by the first physician, 750 mg of mefloquine base (8.5mg/kg) was administered on April 22, considering the possibility of falciparum malaria. On April 24, his blood had turned negative for malaria parasites and since April 25, his body temperature has been under 37°C (Figure 1).

After the start of therapy, the blood smear was examined by a more skilled microscopist. Some of the infected erythrocytes were oval-shaped with fimbriation, contained coarse Schüffner'dots and the parasites themselves were rather small and less amoeboid compared to those seen in vivax malaria. These features are typical of *P. ovale*. The diagnosis of ovale malaria was further substantiated by a species-specific PCR assay which had been developed and extensively used by our group (data not shown, Kimura *et al.*, 1995; Arai *et al.*, 1996). Unfortunately, the blood smears were

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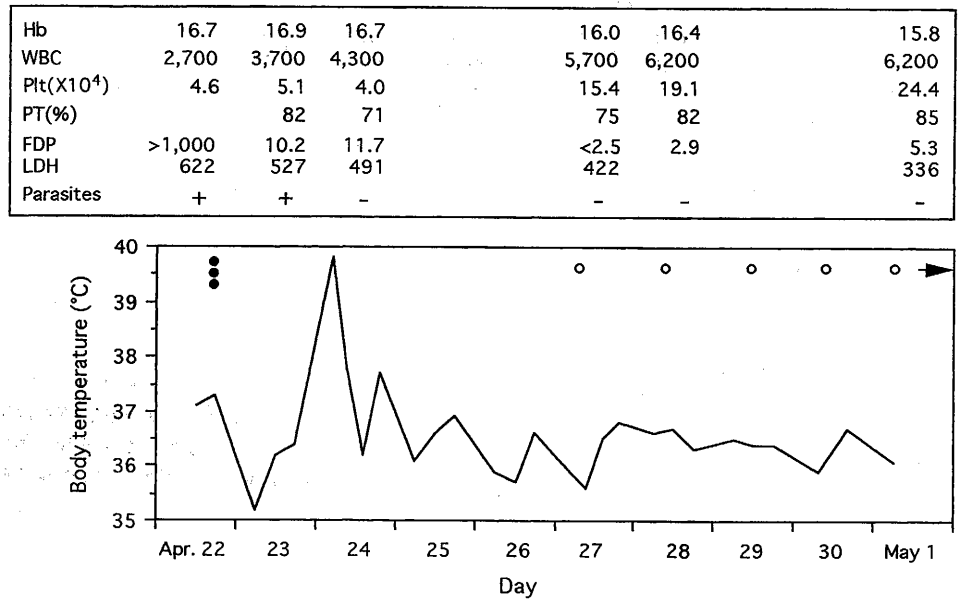

Figure 1 Clinical course of Patient 1 with ovale malaria. A total of 750mg mefloquine base (● =250mg of mefloquine base) was administered followed by a daily dose of 15mg primaquine base (○) for 14 days. Values of Hb are g/dl, those of WBC, Plt ($\times 10^4$) and malaria parasites are per μl blood, and those of FDP and LDH are μg/ml and IU/ml, respectively.

lost and the numbers of parasites were not counted. From April 27, a daily dose of 15mg primaquine base was administered for the following 14 days as a curative therapy. He experienced no recurrence, and no significant subjective or objective adverse reactions were noted during the entire observation period.

Patient 2 was a 46-year-old Japanese male who stayed in Mali from October 20, 1994, to January 17, 1995. His body weight was 65 kg. On November 17, 1994, malaria was diagnosed although he was not informed about the causative species, and was given intravenous quinine for 2 days. For nearly one year after returning to Japan, he was free of any symptoms. On December 30, 1995, January 1 and 3, 1996, he developed high fever with shaking chills. On January 5, he visited our hospital and was diagnosed as ovale malaria (Photo 1a, 124 parasites/μl). This was later substantiated by the results of the PCR assay (data not shown).

On the same and the following day, a total of 1,000mg of mefloquine base (15.4mg/kg) was administered (Figure 2). Fever and the parasites were cleared 6 days and 5 days, respectively, after the treatment. After this successful mefloquine therapy, 15mg daily primaquine base was given for 14 days. No adverse reactions were noted.

Patient 3 was a 29-year-old Cameroonian male who left his country on March 21, 1993. His body weight was 78kg. On April 14, he started to feel feverish although he did not measure the body temperature. On April 20, his fever was up to 38.5°C and he sought medical attention at our hospital. Malaria parasites were detected microscopically by a physician who, however, could not identify their species. On that and the following day, he received a total dose of 1,500mg mefloquine base (19.2mg/kg) divided into two doses 8 hours apart (Figure 3). On April 22 he was no more febrile and on April 26 he had no detectable parasitemia. During the whole observation period until September 1993 when he left for home, he experienced no recurrence, and no adverse effect to mefloquine was observed.

The first blood smear was submitted to a more skilled microscopist who identified it as malariae malaria since band-form trophozoites were evident inside the smaller than normal erythrocytes (Photo 1b). This was also confirmed by the species-specific PCR assay (data not shown).

DISCUSSION

Most of malaria caused by species other than *P. falciparum* had been successfully treated by chloroquine. Recently, however, there appeared reports on vivax malaria that were not cured by the standard course of chloroquine, first from Papua New Guinea (Rieckmann et al., 1989) and then from Indonesia (Schwartz et al.,

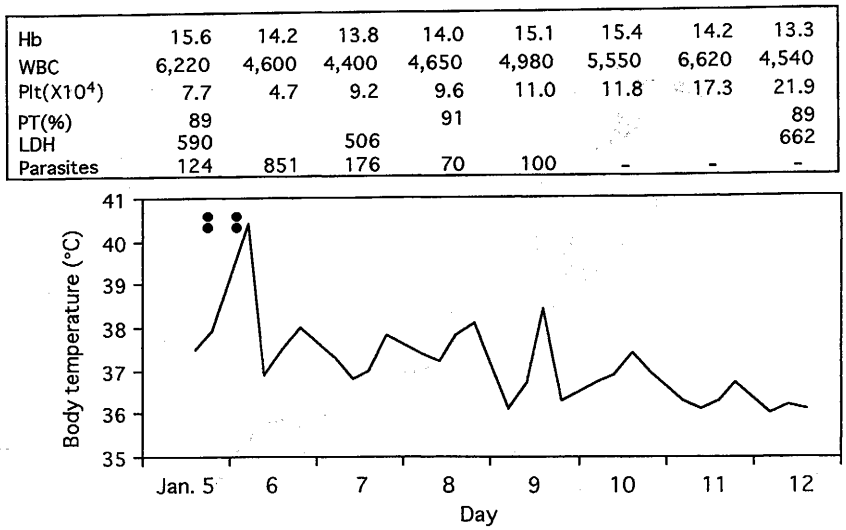

Figure 2 Clinical course of Patient 2 with ovale malaria. A total of 1,000mg mefloquine base (● =250mg of mefloquine base) was administered. Values of Hb are g/dl, those of WBC, Plt ($\times 10^4$) and malaria parasites are per μl blood and those of LDH are IU/ml.

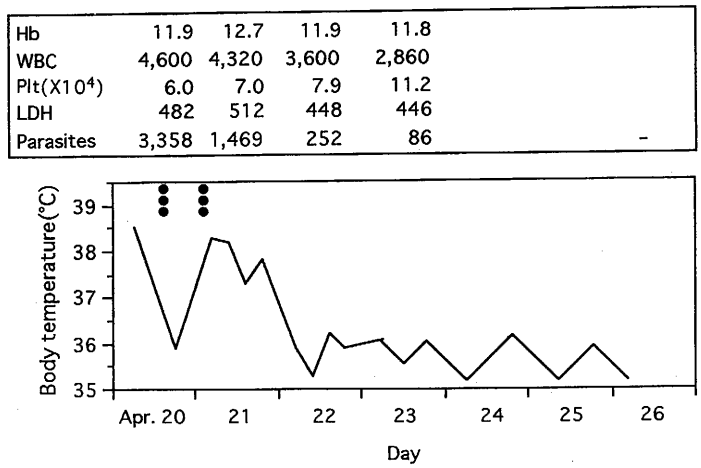

Figure 3 Clinical course of Patient 3 with malariae malaria. A total of 1,500mg mefloquine base (● =250mg of mefloquine base) was administered. Values of Hb are g/dl, those of WBC, Plt ($\times 10^4$) and malaria parasites are per μl blood and those of LDH are IU/ml.

1991). Because the blood concentrations of the drug were high enough to eradicate the usual *P. vivax*, these parasites are considered to be resistant. Thus, the second-line antimalarials for the chloroquine-resistant vivax malaria are of particular importance. In a pre-marketing study performed in Thailand, mefloquine was shown to be effective against vivax malaria (Dixon *et al.*, 1985) even sometimes with a smaller dosage (Harinasuta *et al.*, 1985).

Studies on the efficacy of other antimalarials such as mefloquine against ovale and malariae malaria are limited (Danis et al., 1982; Dixon et al., 1983; Coulaud *et al.*, 1983). Thus, 3 (Coulaud *et al.*, 1983) and 4 (Danis *et al.*, 1982) cases of ovale malaria were reportedly treated with 25mg/kg and 1,000mg, respectively, of mefloquine base. All those reported cases were cured in terms of the acute febrile episodes caused by intraerythrocytic asexual parasites. In one report, one case of malariae malaria was treated with 1,500mg of mefloquine base with the fever clearance of 4 days and the

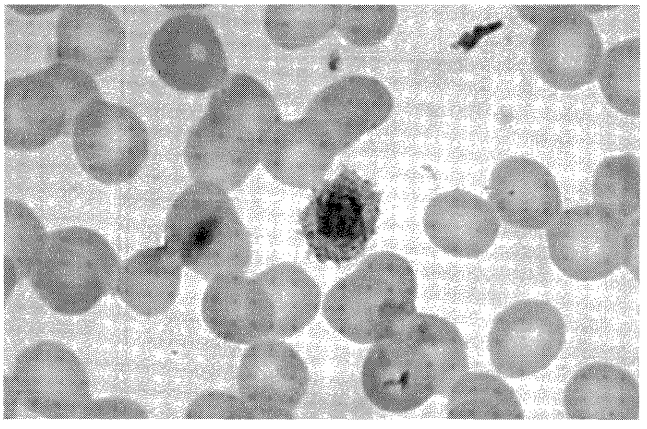

Photo 1a *Plasmodium ovale* seen in the blood of Patient 2. The infected erythrocyte is oval-shaped with fimbriation and coarse Schüffner's stipplings, and the parasite itself is smaller and less amoeboid than *Plasmodium vivax*.

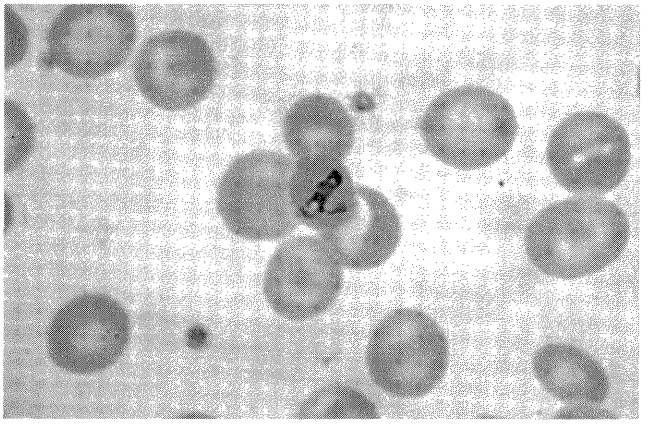

Photo 1b *Plasmodium malariae* seen in the blood of Patient 3. The infected erythrocyte is small-sized without stipplings and contains a band-form trophozoite.

parasite clearance of 7 days (Dixon *et al.*, 1983).

Here we added 3 cases to the literature, 2 with ovale and one with malariae malaria. The former two were treated with low doses, 750mg and 1,000mg, respectively, of mefloquine base, followed by the standard course of primaquine, and the latter with 1,500mg, a high dose. In all cases, fever dissolved in several days. In one of the cases of ovale malaria (Patient 1), the parasites were no more detectable 2 days after the start of therapy. This is consistent with a low number of malaria parasites before the start of therapy although not counted accurately. In the other case of ovale malaria (Patient 2), fever and parasites were cleared 6 days and 5 days, respectively, after the start of therapy. In the malariae malaria (Patient 3) microscopy turned negative 6 days after the start of therapy. Patient 1 was followed up for 3 years, and Patient 3 for 5 months until he left for Cameroon. No recurrence and no adverse reactions were observed in all 3 cases during the entire observation periods. The clinical courses of the patients did not seem to be different from those who were treated with chloroquine. Although more cases should be studied, mefloquine could be a promising substitute for chloroquine in the treatment of ovale and malariae malaria especially when the latter drug is no longer effective due to the emergence of drug resistance in future. Recrudescence of malariae malaria more than several years after chloroquine treatment sometimes poses a diagnostic problem, and is not attributed to the true resistance of the strain. Thus, it is worthwhile to determine whether the recrudescence could be prevented by mefloquine which has a long half-life, although the study requires long-term observation of the treated patients.

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Clinical Report

A CASE REPORT OF AN OVERSEAS-TRAVELER'S DIARRHEA PROBABLY CAUSED BY *CHILOMASTIX MESNILI* INFECTION

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Abstract: Many active flagellates were detected in stool samples of a 22-year-old Japanese male who traveled China and India. Microscopic observation showed that the organisms stained with Wright's solution were pyriform or rounded in shape, measuring 11.7-17.5 μm in length and 7.9-12.2 μm in width. The posterior end was pointed. Three free anterior flagella were located in the front end. A spherical nucleus was situated near the anterior pole. Based on these morphological features we identified the organisms as trophozoites of *Chilomastix mesnili*, a common intestinal protozoon. Although we examined viral, bacterial and parasitic infections other than *C.mesnili*, no pathogen was found. From these results obtained, it was considered to be highly probable that *C.mesnili* infection was the cause of the diarrhea.

INTRODUCTION

Reflecting recent advancement in international interchange and traveling abroad, imported cases with infectious diseases have been increasing in Japan. Annually most of these cases are bacterial diarrhea (Yoh and Honda, 1995). It has been pointed out that Japanese inhabitants in tropical countries have a high risk of infection with intestinal parasites, because of contamination of drinking water or food (Tsukidate *et al.*, 1985). *Chilomastix mesnili* is a species of flagellate protozoa of the digestive tracts and is basically thought to be a harmless commensal. However there is a report suggesting pathogenicity of *C.mesnili* (Mueller, 1959). The prevalence of *C.mesnili* infection ranges from less than 1% to 10% or more in the area of warm climates (Beaver *et al.*, 1984). In this paper, we report a case of a patient with diarrhea possibly caused by *C.mesnili*.

CASE REPORT

The patient was a 22 year-old, male student living in Kochi Prefecture, Japan. He traveled China and India from the end of July to September 1st, 1995. During the stay in India, he had fever on August 28th, followed by diarrhea and nausea. On September 1st, he returned to his home town, Kochi, and visited a physician because of continuing diarrhea. Immediately he was transferred to the Kochi General Hospital of Agricultural Cooperation. The symptom of the patient was diarrhea and nausea. The body temperature was 37.2°C at the time of admission.

Laboratory data are shown in Table 1. C-reactive protein (CRP) was 10.5 mg/dl. Other data of blood chemistry were not remarkable except for a little increase of LDH and uric acid. The urine examination showed a little increment in acetone bodies. The peripheral blood picture was normal. We could not detect antibody against *Entamoeba histolytica* using a hemagglutination test (Japan Lyophilization Laboratory, Tokyo, Japan). Results of fecal examination for bacteria and fungi are shown in Table 2. Feces appeared like mud with mucus. *Escherichia coli* and *Citrobacter amalonaticus*, indigenous intestinal bacteria, were detected by fecal culture. Diarrheagenic *E.coli* was not detected. Serum antibodies against various viruses were examined by neutralization tests (NT) or complement fixation tests (CF) (Table 3). Antibodies against influ-

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Table 1 Laboratory data on September 1st, 1995

1) Blood chemistry		3) Peripheral blood cell counts	
TP	7.8g/dl	RBC	507×10^4/mm³
A/G	1.69	Ht	46.1 %
Glu	95mg/dl	Hb	15.5g/dl
ALP	158IU/l	Plt	18.6×10^4/mm³
T-Cho	116mg/dl	WBC	7200/mm³
γ-GTP	16IU/l	4) Peripheral blood picture	
T-Bil	0.5mg/dl	Band	2.0%
ChE	248mg/dl	Seg	62.0%
Alb	4.9g/dl	Eos	0.0%
Glb	2.9g/dl	Baso	0.0%
GPT	24IU/l	Lymph	22.0%
GOT	26IU/l	Mono	12.0%
LDH	431IU/l	Aty-Lymp	2.0%
CPK	90IU/l	5) Serological test	
BUN	20mg/dl	CRP	10.5mg/dl
Crn	0.8mg/dl	6) Urine analysis	
UA	8.6mg/dl	Pro	20mg/dl
Amy	89IU/l	Glu	0mg/dl
2) Immunoglobulins		Ket	10mg/dl
IgG	1260mg/dl	Occult	(−)
IgA	310mg/dl	7) HA test	
IgM	131mg/dl	*E. histolytica* antibodies	(−)

Table 2 Examination of bacteria and fungi of the fecal sample on September 1st, 1995

Bacteria and fungi	Culture media*	Results
Culture of feces		
E. coli	A	(+)
C. amalonaticus	A	(+)
P. shigelloides	A	(−)
Salmonella spp.	B, C	(−)
Shigella spp.	B, C	(−)
Viblio cholerae	D, E	(−)
Viblio parahaemolyticus	D, E	(−)
Campylobacter	F	(−)
Fungi	G	(−)
Diarrheagenic *E. coli*†		
Enteropathogenic *E. coli* (EPEC)		(−)
Enteroinvasive *E. coli* (EIEC)		(−)
Enterotoxigenic *E. coli* (ETEC)		(−)
Enterohemorrhagic *E. coli* (EHEC)		(−)

* A: BTB agar; B: SSB agar; C: SS agar; D: TCBS agar; E: Viblio agar; F: Skirrow agar; G: Sabouraud agar.
† Agglutination test by serum groups.

Table 3 Serological examinations of antibodies against viruses

Viruses	Methods*	Antibody titers† Sept. 1‡	Sept. 7‡
Influenza A virus	CF	8	8
Influenza B virus	CF	<4	<4
Rotavirus	CF	<4	<4
Enterovirus 72	NT	32	32
Adenovirus 1	NT	64	64
Adenovirus 2	NT	64	64
Adenovirus 3	NT	4	4
Adenovirus 4	NT	<4	4
Adenovirus 7	NT	<4	<4

* CF: complement fixation tests; NT: neutralization test
† 4 or more is positive
‡ Admission (Sept. 1), Discharge (Sept. 7)

enza A virus, enterovirus 72, adenovirus 1, adenovirus 2, adenovirus 3, and adenovirus 4 were detected.

Parasitological examination was made by using fecal samples. In a direct smear specimen of feces many active and motile flagellates were observed under a microscope (Fig. 1). When a fecal sample was diluted tenfold with saline, the number of the organisms was approximately 60 per high power field (400×). The spiral form was recognized in organisms (Fig. 2). The morphology of the flagellates stained with Wright's solution was pyriform and rounded in shape, measuring 11.7 to 17.5 μm long and 7.9 to 12.2 μm wide. Three free

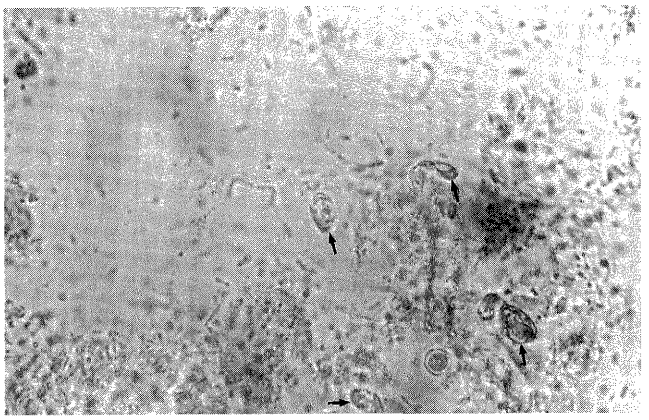

Figure 1 Motile trophozoites with three flagella of *Chilomastix mesnili* (arrows) were observed on a direct smear of fecal specimen from the patient (400×).

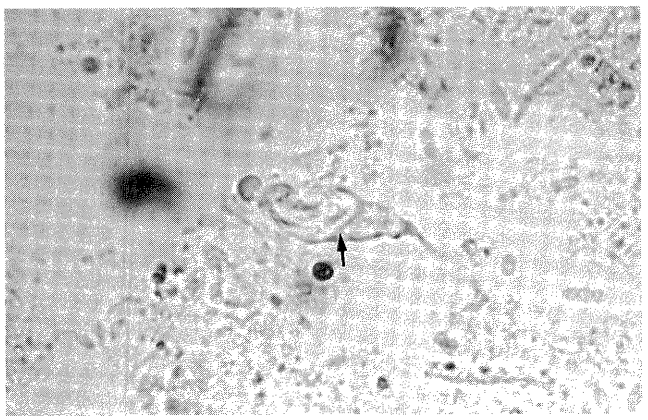

Figure 2 Spiral groove of *C.mesnili* was observed on a direct smear specimen (1000×).

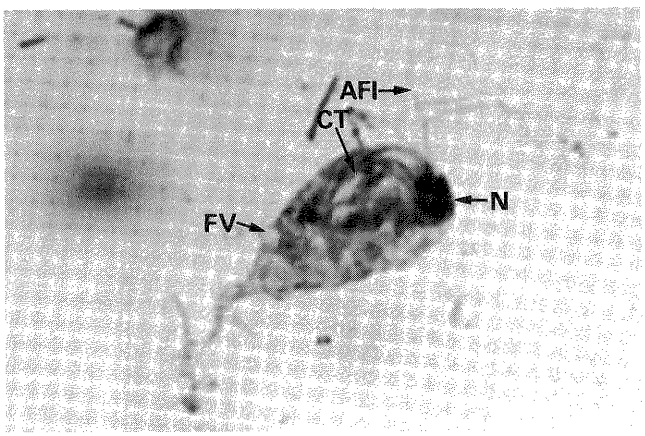

Figure 3 A specimen of *C.mesnili* trophozoite stained with Wright's solution. Various organelles such as flagella (AFI), cytostome (CT), nucleus (N) and food vacuoles (FV) are observed (1000×).

anterior flagella were equal in length and located at the front end. The posterior end was pointed. A spherical nucleus, measuring 2.7 to 4.2 μm, was present near the anterior pole. A well-difined cytostome was seen on one side of the nucleus. The cytoplasm contained numerous food vacuoles. But this flagellates had no undulating membrane and axostyle. Based on these morphological features, we identified the organisms (Fig. 3) as trophozoites of *Chilomastix mesnili* (Wenyon, 1910).

Fecal samples were also examined by formalin-ether sedimentation (MGL method), or were cultured in Tanabe-Chiba medium. Neither protozoa nor helminths except *C.mesnili* were detected by these trials.

The patient was treated with 750 mg/day of metronidazole and 2 g/day of cefmetazole sodium, and 4 g/day of anti-diarrhoics from September 1st (Fig. 4). The parasites were not detected in fecal samples after 4th day of the treatment. No cysts of the parasite were found throughout the clinical course.

Discussion

Chilomastix mesnili has a cosmopolitan distribution. The infection occurs by swallowing the cysts of the parasite (Kreier,1978). *C.mesnili* infection is known to be endemic in many tropical areas where sanitation and personal hygiene are bad (Clarke *et al.*, 1974; Chacin-Bonilla *et al.*,1993). It has been thought generally that *C. mesnili* is a normal inhabitant of the cecal region of the large intestine, where the trophozoites live on enteric bacteria in the lumen of the glands, and that *C.mesnili* is a harmless commensal and is not responsible for symptoms (Beaver *et al.*, 1984). However, infections with *C. mesnili* have been observed in diarrhea of children

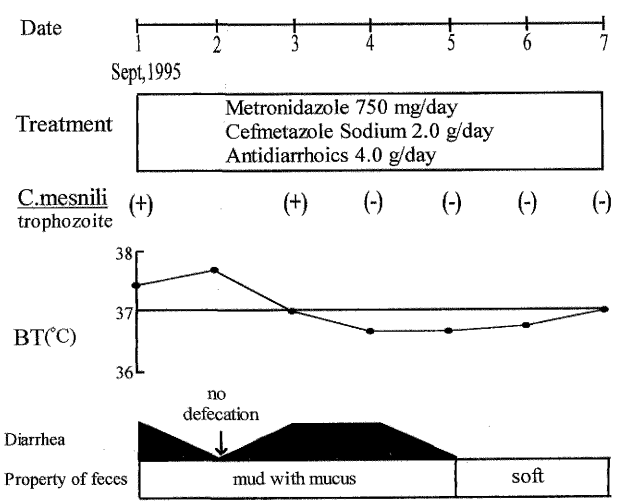

Figure 4 Clinical course of the patient.

(Červa and Větrovská, 1958), homosexual patients (Peters *et al.*, 1986), HIV (human immunodeficiency virus) positive populations (Mendez *et al.*, 1994) and a visitor from the United States who stayed in a developing country (Mueller, 1959). This suggests that *C. mesnili* might be pathogenic to immunocompromised, or less resistant hosts.

In the present case, pathogenic intestinal bacteria, helminths and protozoa were not detected except for *C. mesnili*. Serum antibodies against viruses were detected by complement fixation tests and neutralization tests. A slight increase of titers was seen against adenovirus 4 during hospitalization. We can not exclude the possibility that diarrhea in this case was caused by the viral infection. However, it should be noticed that the score of titration was the lowest level at the recovery stage (Table 3). No cysts were found in fecal samples throughout the present observation. It is possible that the metronidazole acted effective by on the trophozoites. In the literature, a similar case report was mede by Mueller (1959).

In conclusion, we reported that the *C.mesnili* infection accompanied the severe diarrhea in the Japanese patient who possibly did not have immunity to the intestinal protozoa. When the international interchange of personnel and the imported cases of diarrhea have been increasing, *C.mesnili* should be considered as one of the possible pathogens for so-called traveler's diarrhea.

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Research Note

INFLUENCES OF THE PREVENTIVE USE OF ANTIFILARIAL DRUGS ON THE TRANSMISSION OF *DIROFILARIA IMMITIS* IN HOUSE-DOGS IN NAGASAKI CITY, JAPAN

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Keywords: *Dirofilria immitis*, antifilarial drugs, preventive use, house-dogs.

The microfilarial prevalence of *Dirofilaria immitis* in house-dogs in the southern and northern parts of Nagasaki City decreased for 27 years from 1968 to 1994 (Oda *et al.*, 1993, 1995). Oda *et al.* (1994a, 1996) reported that the decrease in the number of the main vector mosquito, *Culex pipiens pallens*, in parallel with the expansion of public sewage system and the increase of indoor-kept dogs were related to the reduction of the prevalence. The rate of dogs that was given the antifilarial drug against microfilaria (Mf) of *D. immitis* was estimated not to be important to the reduction, because the rate was found not high and about similar in questionnaire surveys in 1989 and 1993 (Oda *et al.*, 1994b). However, Oka *et al.* (1988) reported that the Mf prevalence of *D. immitis* in house-dogs examined in a domestic animal hospital has been decreasing gradually for 30 years since 1956 in Tama district in Tokyo, and the increase of dog-owners using the antifilarial

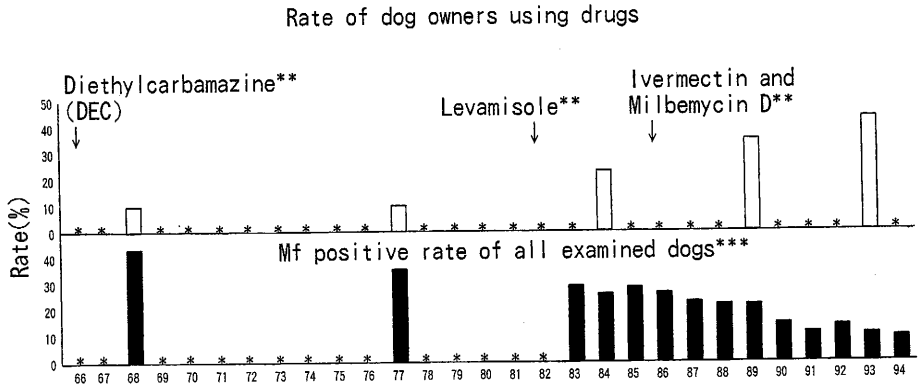

Figure 1 Annual changes in rate of dog owners using antifilarial drugs (upper) and Mf positive rate of *Dirofilaria immitis* in house-dogs (lower) in Nagasaki City.
*Data not available.
**Data on new drugs are cited from Ohishi (1990).
***Cited from Oda *et al.*, 1995.

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Table 1 Comparison of positive rates between dogs with and without use of antifilarial drug*

Year	Dogs with drug-use		Dogs without drug-use	
	No. examined	No. positive (%)	No. examined	No. positive (%)
1989	72	15(20.8)	134	25(18.7)
1993	90	5(5.6)	111	12(10.8)

*Dogs with or without drug-use were determined according to questionnaire survey. (Oda *et al.*, 1994b).

drugs and of small-sized dogs kept indoors became important to the decrease of Mf prevalence. We reexamined whether or not the use of the drug by dog-owners contributed to the reduction of the Mf positive rate during recent 27 years in Nagasaki City.

Fig. 1 shows the year-to year change in the rate of dog-owners using antifilarial drugs in relation to the appearance of the drugs and the Mf positive rate of dogs in Nagasaki City. The rate of dog-owners was utilized from a record of a joint meeting of veterinarians in 1983, question to 43 dog-owners at blood examination for *Dirofilaria* Mf in 1984 and questionnaire surveys in 1989 and 1993. Diethylcarbamazine became commercially available in 1966. During the decade starting from 1966, the rate of dog-owners who used the drugs in Nagasaki City was very low (it was assumed to be about 10% or less), and the Mf positive rate of dogs did not change markedly during the same period. Levamisole became commercially available in 1982, and ivermectin and milbemycin D in 1986. The rate of dog-owners using the drugs increased from 1984, and Mf positive rate of dogs began to decrease from around 1983. To make clear a relationship between these rates, we compared the Mf positive rates between dogs with drug-use and those without drug-use according to questionnaire surveys in 1989 and 1993 (Table 1). Positive rates were not significantly different between dogs with drug-use and those without drug-use. Therefore, decrease in Mf positive rate does not seem to be related to the increase in the rate of dog-owners who used drug. This may be due to the low rate of dog-owners using drugs for a sufficient period to allow complete elimination of *D. immitis* infection in house-dogs, because only about 20 % of the drug-using owners used these antifilarial drugs regularly, according to questionnaire surveys (Oda *et al.*, 1994b).

On the other hand, Oka *et al.* (1988) showed that the infection rate of *D. immitis* in house-dogs examined in Tokyo was 33. 3 % in 1956 to 1957, and this rate decreased gradually to 13. 6 % in 1984 to 1985. Oka *et al.* (1988) speculated that the increase in the rate of drug-using owners as well as the increase of the small-sized dogs kept indoors played important roles in the reduction of the Mf infection rate. The microfilarial prevalence was reduced both in Tokyo and in Nagasaki, but the relative importance of the factors reducing the transmission of *D. immitis* seemed to be different in the two cities.

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