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内　　容

原　著

Detection of Antibodies to *Parastrongylus cantonesis* in Human Sera by Gelatin Particle Indirect Agglutination test
Praphathip Eamsobhana, Dorn Watthanakulpanich, Paibulaya Punthuprapasa, Adisakdi Yoolek and Somkuan Suvuttho ····· 1-5

ネズミマラリア *Plasmodium berghei* のスポロゾイトの媒介蚊 *Anopheles stephensi* 唾液腺への侵入：電子顕微鏡による研究（英文）
安藤　勝彦，倉石　慶太，西久保公映，浅見　　哲，Philomene Waidhet-Kouadio，松岡　裕之，鎮西　康雄 ····· 7-12

タイ国北部におけるB型肝炎ウイルス，C型肝炎ウイルスおよび後天性免疫不全ウイルス感染の血清疫学的研究（英文）
Prapan Jutavijittum, Yupa Jiviriyawat, Amnat Yousukh, 鳥山　　寛，板倉　英吾，矢野　右人，林　　茂樹 ····· 13-17

短　報

Evaluation of a Recombinant Protein (rTc24) and Synthetic Peptides in Anti-*Trypanosoma cruzi* Positive Samples from Blood Bank Donors in Chagasic Endemic Areas of Ecuador
Angel G. Guevara, Juan C. Ruiz C., Raymond L. Houghton, Lisa Reynolds, Paul Sleath, Darin Benson, Ali Ouaissi and Ronald H. Guderian ····· 19-22

第39回日本熱帯医学会大会英文抄録

目　次 ····· 23-26
研究奨励賞受賞講演 ····· 27
会長講演 ····· 29-32
特別講演 ····· 33-37
教育講演 ····· 39-46
シンポジウム ····· 47-102
ワークショップ ····· 103-108
学術展示 ····· 109-144

（裏面に続く）

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Jpn. J. Trop. Med. Hyg., Vol. 27, No. 1, 1999, pp. 1 - 5

DETECTION OF ANTIBODIES TO *PARASTRONGYLUS CANTONENSIS* IN HUMAN SERA BY GELATIN PARTICLE INDIRECT AGGLUTINATION TEST

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Abstract: A newly developed agglutination test using gelatin particles as an antigen carrier (GPAT) was compared with a conventional enzyme-linked immunosorbent assay (ELISA) for the detection of *Parastrongylus cantonensis* antibodies in sera from patients. A total of 70 sera was used in the study. Of these, 10 each were from patients with parastrongyliasis, gnathostomiasis, paragonimiasis, cysticercosis, toxocariasis, filariasis and malaria. The control group consisted of 50 serum samples from normal healthy individuals. The mean reciprocal titer of the parastrongyliasis patients group was significantly higher than that of the normal group as well as those of other parasitic infections. The sensitivity and specificity of the GPAT were 100% and 92.4%, respectively. The results of GPAT in detecting *P. cantonensis* antibodies appeared to be closely correlated with those obtained with ELISA. The GPAT, however, is more easy, rapid and cheap; it may also be a test of choice for routine immunodiagnosis of human parastrongyliasis.

Key words: Immunodiagnosis, *Parastrongylus* (=*Angiostrongylus*) *cantonensis*, gelatin particle indirect agglutination test (GPAT), ELISA

INTRODUCTION

Human eosinophilic meningitis or meningoencephalitis caused by *Parastrongylus* (=*Angiostrongylus*) *cantonensis* is endemic throughout Asia and the Pacific Islands (Cross, 1987; Kliks and Palumbo, 1992). The most reliable diagnosis of this parasitic disease is based on the presence of either larvae or juvenile worms in the cerebrospinal fluid (CSF) from the patients. Such diagnosis nevertheless is rare since worms are seldom found in the limited volume of CSF taken for analysis.

A variety of immunological tests based on detecting serum antibodies against *P. cantonensis* has been used to support the diagnosis. These include intradermal test, indirect hemagglutination test, immunodiffusion, immunoelectrophoresis, complement fixation test, ELISA and immunoblot test (Tharavanij, 1979; Ko, 1987; Eamsobhana *et al.*,1997). The enzymatic test system has become more widely used because of its greater sensitivity. The test, nevertheless, requires specific materials, specialized equipment and expensive reagents. Recently, a newly developed agglutination test using gelatin particle as an antigen carrier has been shown to be a sensitive and specific method for the diagnosis of human strongyloidiasis (Sato and Ryumon, 1990), schistosomiasis (Yang *et al.*, 1994; Kobayashi, 1995), Chagas' disease (Yamashita *et al.*, 1994) and opisthorchiasis (Watthanakulpanich *et al.*, 1998). GPAT is technically simple and can be performed rapidly without specialized apparatus or facilities. These make it convenient to use in the diagnosis of many diseases both in the laboratories as well as in the field.

In this study, we attempted to evaluate whether GPAT could be used to detect serum antibodies of parastrongyliasis (=angiostrongyliasis) patients. The results were compared with those of ELISA.

MATERIALS AND METHODS

Antigen preparation

Adult worms of *P. cantonensis* were obtained from the pulmonary vessels of infected Wistar albino rats as

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previously described (Eamsobhana *et al.*, 1997). The worms were homogenized in a small volume of normal saline with a glass tissue grinder. The suspension was sonicated and extracted overnight at 4°C in a refrigerator. Soluble antigens were obtained as supernatant after centrifugation at 4,000 rpm for 15 min. The protein content of the extract was determined using a protein assay kit II (Bio-Rad Labs, USA).

Sera

Serum samples were obtained from five patients with parasitologically confirmed parastrongyliasis (3 with cerebral parastrongyliasis; 2 with ocular parastrongyliasis) and five patients with presumptive parastrongyliasis. The latter group was diagnosed as parastrongyliasis based on clinical symptoms and history of exposure to infection, as well as having high antibody titers as detected by ELISA.

Sixty heterologous sera were collected from patients suffering from other parasitic infections. Of these, 10 sera each were from patients with gnathostomiasis, toxocariasis, filariasis, paragonimiasis, cysticercosis and malaria. All these cases were positive by parasitologic and/or serologic tests for a specific parasite or its products. The normal control group of sera were obtained from 50 healthy adults who were negative for any parasitic infection at the time of blood collection. All serum samples were kept at −20°C until use.

Gelatin particle indirect agglutination test (GPAT)

The GPAT was performed as previously described (Watthanakulpanich, 1998). Briefly, the pre-determined optimal concentration of *P. cantonensis* antigens (50 μg/ml) was conjugated to the artificial gelatin particles (Fujirebio Inc., Tokyo, Japan) treated with 5 μg/ml tannic acid solution. After conjugation of antigens, the gelatin particles were washed 3 times and finally made into a 1% suspension in phosphate buffered saline (PBS), pH 7.0 containing inactivated normal rabbit serum. These coated gelatin particles were then ready for use.

For estimation of agglutination titer, one drop containing 25 μl of the antigen-coated particles suspension was mixed in the U-bottomed micro-wells with an equal volume of test serum in 2-fold serial dilutions. The particles were allowed to settle for at least 3 hr at room temperature and the agglutination patterns in the plates were read according to Campbell *et al.* (1974); particles concentrated in the shape of a compact button in the center of the well indicated a negative result; particles spread out uniformly covering the bottom of the well indicated a positive result. The antibody titer was determined as the highest serum dilution giving a positive agglutination pattern.

Enzyme-linked immunosorbent assay (ELISA)

To evaluate the results of GPAT, the ELISA was also applied for assessment of serum antibodies to *P. cantonensis*.

The ELISA was performed according to the method described by Voller *et al.* (1976) with some modifications. Briefly, wells of microtiter plate (Nunc, Denmark) were sensitized with 100 μl of *P. cantonensis* antigens at a concentration of 5 μg/ml of protein in carbonic buffer solution, pH 9.6. The wells were successively incubated for 2 hr each with 100 μl of blocking solution (2% skim milk in PBS-Tween), serum samples diluted to 1:100 with PBS containing 1% bovine serum albumin and 0.05% Tween 20, and peroxidase-conjugated anti-human immunoglobulins (Dakopatt, Denmark) diluted to 1:1,000 in PBS-Tween. Finally, the wells were incubated for 30 min with the substrate (o-phenylenediamine) solution. The enzymatic reaction was stopped with 50 μl of 2.5 N sulphuric acid and the optical density (OD) was measured at 492 nm with an ELISA reader (SLT Labinstrument, Australia).

The optimal concentration of the antigens and the optimal dilution for patient's serum and conjugate were pre-determined using a chequerboard titration. For each test, a negative, a positive and a PBS-Tween controls were included.

A result was considered positive if the OD value exceeded the mean OD+3SD of the values obtained with the 50 negative sera.

Statistical analysis

Sensitivity and specificity of the tests were determined using the method of Galen (1980). Association between GPAT and ELISA was evaluated using linear correlation and regression after the titers of GPAT were transformed into logarithm ($\log_2$).

RESULTS

The distribution of GPAT titers in 70 patients and 50 uninfected controls is shown in Figure 1. All 10 patient sera with parastrongyliasis showed positive agglutination response at serum titer of 1:32 or more ($\log_2$ reciprocal titer$\geqq$5), whereas negative results were demonstrated at the lowest serum titer of 1:16 ($\log_2$ reciprocal titer$\leqq$4) in all the normal individuals. The

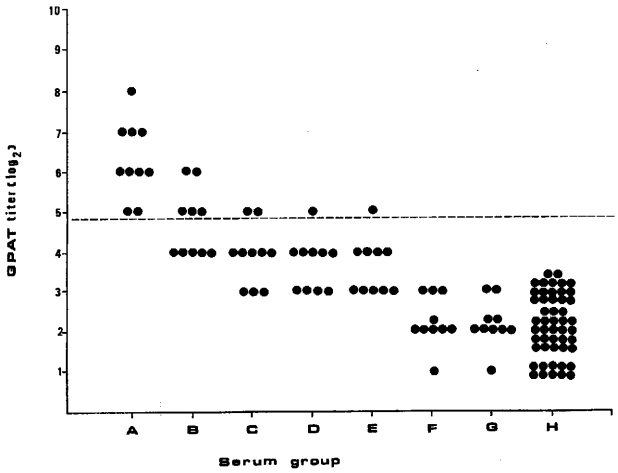

Figure 1 Distribution of GPAT titers (log_2) for detection of serum antibodies against *P. cantonensis* in 10 patients each with parastrongyliasis (A), gnathostomiasis (B), toxocariasis (C), filariasis (D), paragonimiasis (E), cysticercosis (F), and malaria (G) and in 50 normal healthy individuals (H). The dotted line indicates cut-off titer.

log_2 reciprocal titers in the parastrongyliasis patients ranged from 5 to 8, with the majority from 6 to 7.

The mean antibody titer ($\bar{X}\pm SD$) of normal healthy individuals was 3.15 ± 0.58. The mean antibody titer of parastrongyliasis patients group was significantly higher than that of the normal group as well as those of other parasitic infections ($P<0.01$). A cut-off titer for the positive antibody response was then established at $\bar{X}+3SD$ of the healthy group which was at log_2 reciprocal titer of 4.89. The GPAT was positive for all the parastrongyliasis patients but negative for the normal controls.

Of the 60 serum samples from other groups of parasitic infections, 51 (85%) were negative, while 9 (15%) were cross-reactive at the cut-off titer. The cross-reactive serum samples were from patients with gnathostomiasis (5/10), toxocariasis (2/10), filariasis (1/10), and paragonimiasis (1/10). None of the cysticercosis and malaria patients showed cross-reaction. The sensitivity and specificity of the GPAT were 100% and 92.4%, respectively.

The ELISA was carried out using different dilutions of sera from healthy individuals and from patients with parastrongyliasis. The maximum difference in OD values was observed at 1:100 dilution which was used to evaluate all serum samples. The mean OD value ($\bar{X}\pm SD$) of the normal group was 0.253 ± 0.107. The mean plus three standard deviation OD value of the healthy group sera was then taken as the cut-off value, OD> 0.574 indicated positive results.

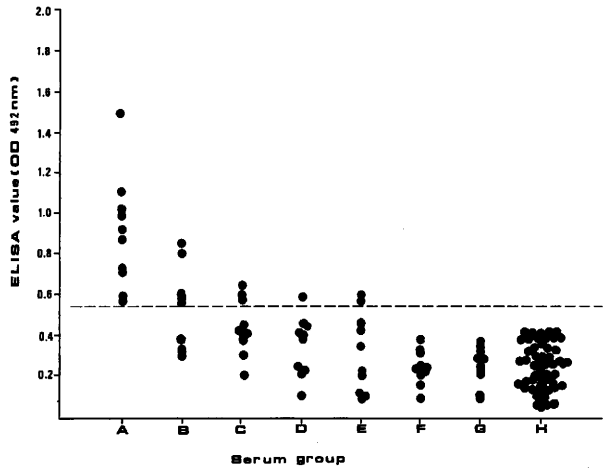

Figure 2 Distribution of optical density values in ELISA for detection of serum antibodies against *P. cantonensis* in 10 patients each with parastrongyliasis (A), gnathostomiasis (B), toxocariasis (C), filariasis (D), paragonimiasis (E), cysticercosis (F), and malaria (G), and in 50 normal healthy individuals (H). The dotted line shows the cut-off value.

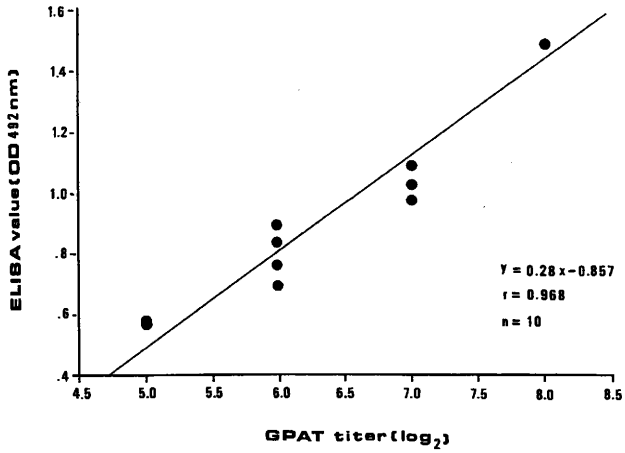

Figure 3 Correlation between GPAT titers (log_2) and ELISA values performed on 10 sera from parastrongyliasis patients.

As shown in Figure 2, all the serum samples from parastrongyliasis cases (10/10) were positive in the ELISA and 48 of 60 sera from patients with other parasitic infections (80%) were negative. Cross-reactions were found with serum samples from gnathostomiasis (6/10), toxocariasis (3/10), filariasis (1/10), and paragonimiasis (2/10). Normal parasite-free individuals were all negative. The sensitivity and specificity of the ELISA were 100% and 90.2%, respectively.

Figure 3 represents the correlation of GPAT titers and ELISA values on the parastrongyliasis patients. A significant correlation was found between the two tests,

the correlation coefficient was 0.968.

DISCUSSION

The definitive diagnosis of parastrongyliasis is made when *Parastrongylus* worms are found in the CSF of patients. Proven cases of human infection showing worms are however rare (Punyagupta, 1979) and the ELISA utilizing either crude or partially purified *Parastrongylus* antigens is currently used as a reliable method for immunodiagnosis of the infection (Welch *et al.*, 1980; Chen, 1986; Yen and Chen, 1991). Although ELISA is sensitive enough for detecting serum antibodies to *P. cantonensis*, it is expensive, laborious and limiting with respect to the length of time to get results.

The recently developed inert gelatin particles are actively being employed as an antigen carrier for various diagnostic kits, and GPAT has already been successfully applied by a number of investigators for immunodiagnosis of various parasitic infections (Sato and Ryumon, 1990; Yamashita *et al.*, 1994; Yang *et al.*, 1994; Kobayashi *et al.*, 1995; Wattanakulpanich *et al.*, 1998). In the present study, we confirmed the findings that GPAT could also be used for immunodiagnosis of human parastrongyliasis. The sensitivity of the present GPAT did not differ from that of the ELISA when the overall rates of positive reactions among patients' sera diagnosed to be parastrongyliasis were compared; in sera of 10 patients with parastrongyliasis, positive antibody response was demonstrated in all of them by the GPAT and ELISA. When such tests were evaluated for specificity by testing sera from healthy individuals presumed to be normal, the result did not show any false-positive reactions in each test. A significant correlation was observed between GPAT and ELISA.

Sera from patients with other parasitic infections were also examined by the GPAT and ELISA to determine the specificity of the tests. Strong positive responses were observed in a few patients with gnathostomiasis. Weak cross-reactive positive reactions also occurred in sera from a few patients with toxocariasis, filariasis and paragonimiasis. The responses, however, were lower in the GPAT. The sera from patients with cysticercosis and malaria showed no cross-reactivity in both tests. The positive responses in gnathostomiasis, toxocariasis, filariasis and paragonimiasis patients, however, seem to correlate with the antigen preparation used rather than the assay itself. By using a more defined antigen, the cross-reaction can be expected to decrease (Ko, 1987).

The present study indicated that the GPAT can be a reliable immunological test for human parastrongyliasis. The indirect agglutination test is technically simple to perform, requires no specialized skill, equipment and facilities, and can be completed within three hours. The advantages of the gelatin particles are their stability and resistance to mechanical agitation. The particles are colored and therefore convenient for reading the setting pattern. Moreover, the particles can be lyophilized for long term storage after sensitization with antigen. Therefore, the GPAT can be performed by the one-step reaction of the preserved antigen-particles with a test serum, thus applicable both in less equipped laboratories as well as in a field survey. Nevertheless, because of the relatively strong cross-reaction with other clinically related parasite, *Gnathostoma spinigerum*, the use of more specific antigenic preparation in the assay will be needed. Experiment on purification of the *P. cantonensis* specific antigen for future use is underway.

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SPOROZOITE INVASION OF *PLASMODIUM BERGHEI*, RODENT MALARIA PARASITE, TO THE SALIVARY GLANDS OF THE VECTOR MOSQUITO, *ANOPHELES STEPHENSI*: AN ELECTRON MICROSCOPIC STUDY

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Abstract: The sporozoite penetration process of a rodent malaria parasite, *Plasmodium berghei*, into the salivary glands of the vector mosquito, *Anopheles stephensi* and sporozoite distribution in the cytoplasm and secretory cavity in the distal region of salivary glands were observed with a scanning electron microscope and a transmission electron microscope. In non-infected mosquitoes, many swellings were observed on the outer surface of the median lobes of salivary glands, whereas many shallow depressions were observed on the lateral lobes. In infected mosquitoes, sporozoites were concentrated on the distal region of median and lateral lobes of salivary glands and penetration occurred from the anterior end into both lobes. Sporozoites were about 10 μm long with one end flat and the other round. Small holes through which sporozoites might have passed were observed on the surface of both median and lateral lobes. A white powder like substance, which might come from the holes, covered the surface of both lobes. Sporozoites invading the cytoplasm of the salivary gland cells were surrounded with vacuoles. These sporozoites invaded the secretory cavity and lodged to form bundles.

Key words: *Plasmodium berghei*, Salivary gland, Sporozoite, Invasion

INTRODUCTION

The salivary glands of mosquitoes play an important role in transmission of mosquito-borne diseases. Therefore, the structures of mosquito salivary glands have been studied by numerous researchers and fine internal structures have also been observed with a transmission electron microscope (TEM) (Wright, 1969; Janzen and Wright, 1971; Barrow *et al.*, 1975). The salivary glands also play an important role in the life cycle of malarial parasites. Sporozoites released from mature oocysts are distributed in the hemolymph of mosqiutoes and actively penetrate the salivary gland cells. It is not known whether the sporozoites move actively or passively in the direction of the salivary glands.

The penetration process of sporozoites into salivary gland cells and biology of sporozoites have been widely studied by transmission electron microscopic observations (Sterling *et al.*, 1973; Posthuma *et al.*, 1989; Golenda *et al.*, 1990; Ponnudurai *et al.*, 1991; Pimenta *et al.*, 1994). In addition, reports on the penetration process of sporozoites into salivary glands using scanning electron microscopy (SEM) have also been published by Sinden (1975) and Meis *et al.* (1992). However their observations did not concentrate on the penetration process of sporozoites so that number of photos (1 sheet of photos by Sinden and 3 sheets of photos by Meis) and descriptions are not sufficient. Especially, there is no discription of the salivary gland lobe and the direction of sporozoite penetration to the lobe is not clear.

In the present study, we show the penetration process of *Plasmodium berghei* sporozoites into median and lateral lobes of the salivary glands of *Anopheles stephensi* by SEM and their distribution in the cytoplasm and secretory cavity in the distal region of salivary glands by TEM.

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Materials and Methods

The parasies, *P. berghei* ANKA strain clone 2.34, were kindly supplied by professor R.E. Sinden, Imperial College, and have been maintained in our laboratory. They were transmitted to BALB/c mice by *A. stephensi* mosquitoes. The blood collected from infected mice (passage-0 mice) was stored at −80°C. This blood was injected intraperitoneally (1×10^7 parasites) into BALB/c mice (passage-1). When the parasitemia of a passage-1 mouse reached 10%, a passage-2 mouse was prepared by injection of 5×10^6 parasites from the passage-1 mouse fresh blood. *A. stephensi* females (4-7 days after emergence) were allowed to feed for 1 hr on a passage-2 mouse 3 days after infection (parasitemia, about 2.0%) at 21°C. Engorged mosquitoes were separated, kept for 1 hr at 21°C to form ookinetes efficiently (Sato *et al.*, 1996) and then maintained at 26°C until dissected.

Live mosquitoes were fixed by 0.1 m*l* injection of 2.5% osmium tetroxide in PBS into the thorax with a capillary tube on 16 days post-feeding. Salivary glands were carefully dissected 2 hrs after injection and specimens for SEM were prepared by standard methods and observed under a JSM-T200 SEM operated at 10 kv. To prepare the specimens for TEM, the salivary glands were dissected from the fresh mosquitoes and prefixed in 1% glutaraldehyde in 0.1 M phosphate buffered saline (PBS) (pH 7.2) for 1 hr. They were washed three times in PBS and were post-fixed with 1% osmium tetroxide in PBS for 2 hrs. After washing with PBS, they were dehydrated in ethanol and acetone, and embedded in Quetol 812. Thin sections were stained with 4% uranyl acetate and lead citrate, and observed under a H-700HX Hitachi TEM operated at 100 kv.

Results and Discussion

We dissected 50 pairs of salivary glands from *A. stephensi* (20 non-infected and 30 infected with malarial parasites). Th salivary glands consisted of paired glands and each gland consisted of one short median lobe and two long lateral lobes. The median lobe was divided into two, proximal and distal regions, while lateral lobes were divided into three, proximal, intermediate and distal regions as described by Wright (1969). The whole body was prefixed by injecting osmium tetroxideto to observe the malarial parasites and the salivary glands together as they are in the mosquito body. This made it difficult to separate whole salivary glands from the other tissues completely for SEM observation without damage. Therefore, we dissected the salivary glands together with the surrounding tisses and mainly observed the distal regions of both the median and lateral lobes by SEM. In some specimens, we could observe the intermediate region.

Many swellings corresponding to salivary gland cells were observed on the outer surface of the distal region of median lobes of both non-infected and infected mosquitoes (Fig. 1). However, these swellings were not so conspicuous in some lobes. Surface morphology of lateral lobes was different from that of median lobes. Many shallow depressions were observed on the outer surface of the distal region of the lateral lobes of both non-infected and infected mosquitoes (Fig. 1). No holes existed on the surface of non-infected median and lateral lobes. Whereas, on the surface of the middle and lateral lobes infected with malarial parasites, small holes through which sporozoites might pass were observed (Fig. 2). In addition, these lobes were covered with a white powder like substance (Figs. 2, 5). We supposed that this powder like substance was saliva which leaked from the small holes because we couldn't observe it on the surface of non-infected lobes. Sinden (1975) observed a sponge like matrix on the surface of salivary glands (lobe and area, not mentioned) in *A. stephensi* infected with *P. yoelii nigeriensis* sporozoites but we didn't observe this structure. The number of sporozoites concentrated on the surface of lobes was different among specimens but usually many sporozoites were present (Figs. 4, 5) except in a few specimens with a few sporozoites (Fig. 3). No sporozoite was observed on the surface of the intermediate region of lateral lobes in this study.

The sporozoites observed on the surface of salivary glands were about 10 μm in length with one end flat and the other round (Fig. 3). Sato (1998) also observed the same morphological features in *P. berghei* sporozoites which emerged from oocysts. We judged from the figure by Knell (1991) that flat and rounds ends were the anterior and posterior ends of the sporozoite, respectively. We observed the round end of the sporozoite outside of the salivary gland. This fact indicated that the sporzozoites penetrate the salivary gland cells from the anterior end (Figs. 2, 5, 6). During the process of penetration into salivary gland cells, sporozoites attach and cross the basal lamina, the plasma membrane of salivary gland cells and then penetrate the cytoplasm. Pimenta *et al.* (1994) observed interaction of *P. gallinaceum* sporozoites with *A. aegypti* salivary glands by TEM and emphasized that the penetration process appeared to involve the formation of a membrane junc-

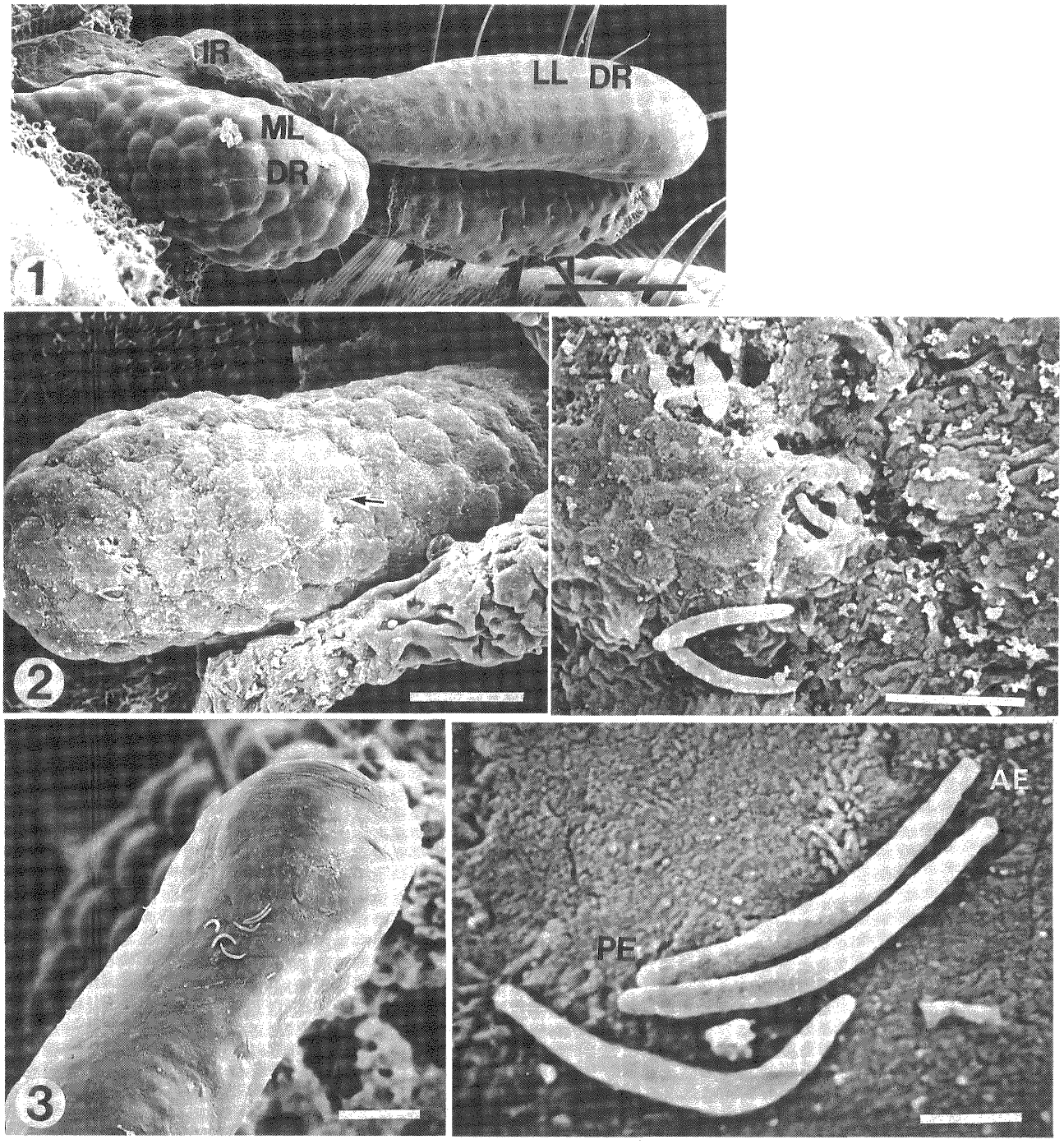


Figure 1 Morphology of the salivary glands of a non-infected mosquito, *A. stephensi*. Many swellings were observed on the surface of the median lobe (ML), while shallow depressions were observed on the surface of the lateral lobe (LL) (bar; 50 μm)

Figure 2 Median lobe infected with *P. berghei*. The surface of the gland was covered with a white powder like substance (arrow) (left, bar; 20 μm) and higher magnification of left micrograph shows that sporozoites were penetrating the gland through small holes (right, bar; 5 μm).

Figure 3 Sporozoites attached to the surface of the median lobe (left, bar; 20 μm). Higher magnification of the sporozoites which were about 10 μm in length with a flat anterior and round posterior end (right, bar; 2 μm).

AE: anterior end, CP: cytoplasm, DR: distal region, DW: duct wall, GL: gland lumen, IR: intermediate region, LL: lateral lobe, ML: middle lobe, PE: posterior end, S: sporozoite, SC: secretory cavity, V: vacuole

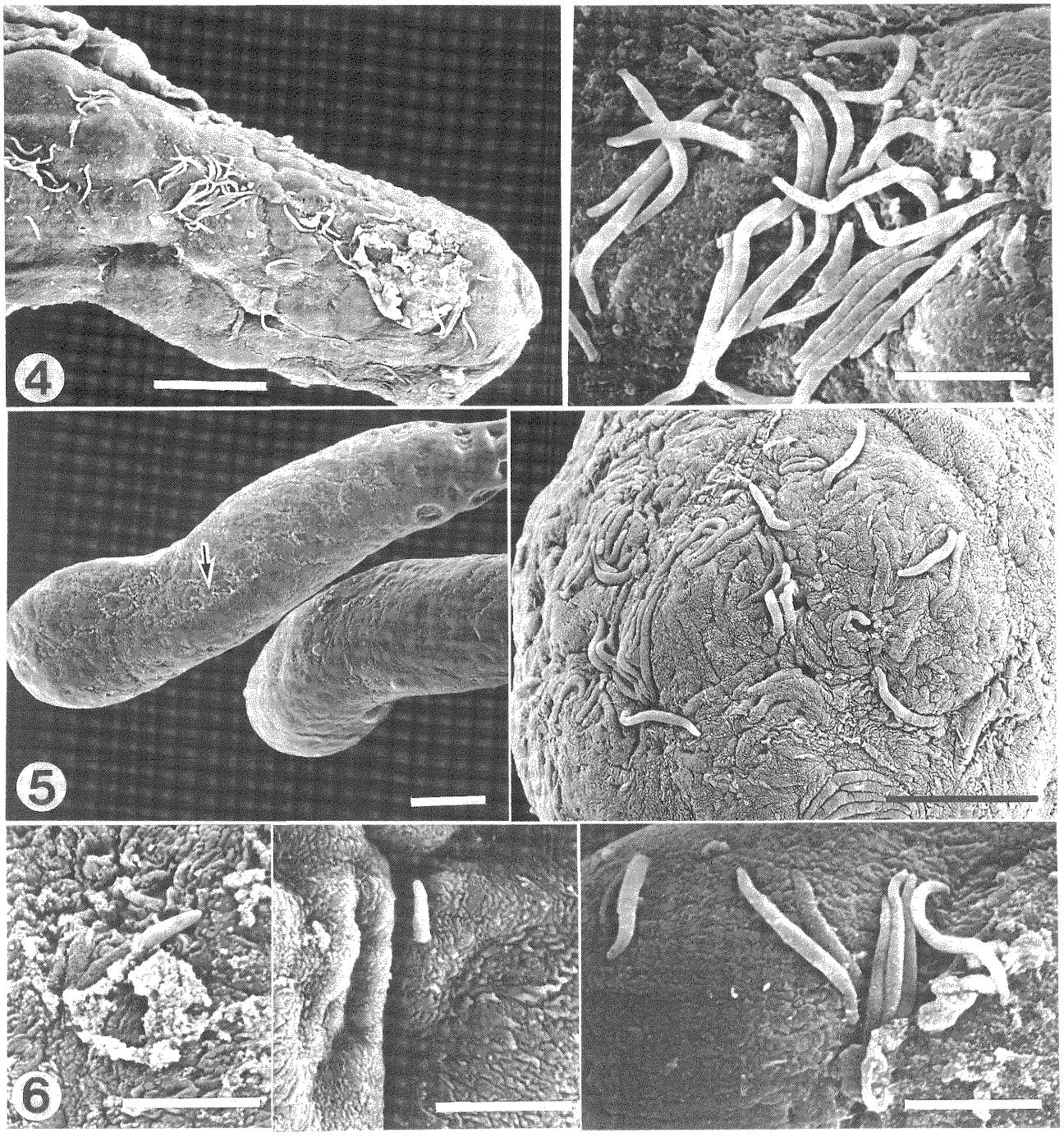
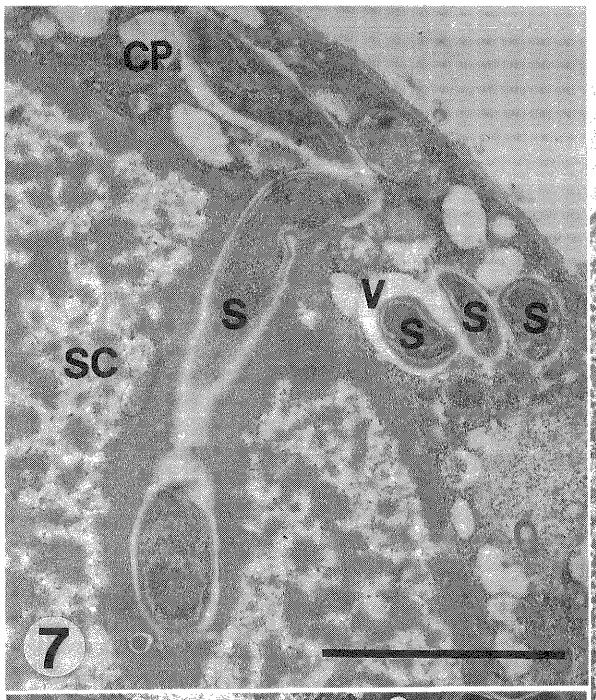
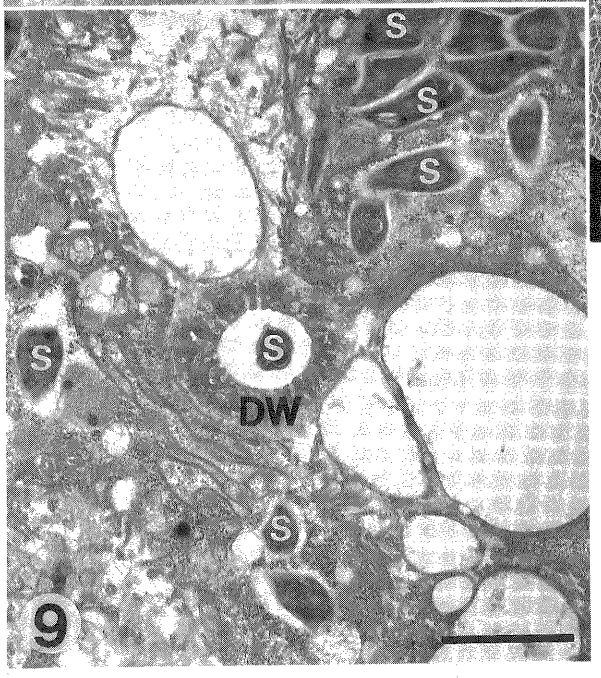


Figure 4 Many sporozoites were concentrated on the surface of median lobes (left, bar; 20 μm). Higher magnification of sporozoites which were penetrating the lobe simultaneously (right, bar; 5 μm).

Figure 5 Lateral lobe infected with *P. berghei* was covered with a white powder like substance (arrow) (left, bar; 20 μm). Higher magnification of bottom of the distal region with many sporozoites attached. Some sporozoites were penetrating the salivary gland (right, bar; 10 μm).

Figure 6 Sporozoites penetrating into the lateral lobes of the salivary gland, anterior end first with posterior end remaining outside (collection of sporozoites not shown in figures 2–5, bar; 5 μm).





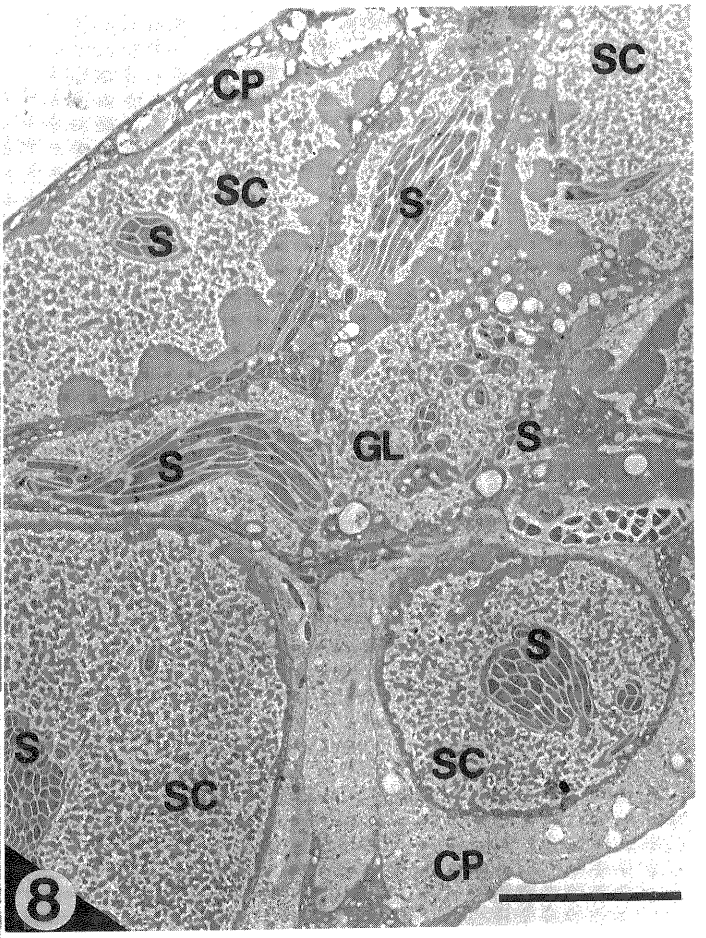


Figure 7 Sporozoites occurred in the vacuole of the cytoplasm in the distal region of the lateral lobe, and a sporozoite was penetrating into the secretory cavity from the cytoplasm (bar; 2 μm).
Figure 8 Sporozoites bundled inside the secretory cavity (bar; 10 μm).
Figure 9 Sporozoite passing through the lumen of salivery duct toward probosis. Thick duct wall of distal region near intermediate region of lateral lobe was observed (bar; 2 μm).

tion between the cell coat of sporozoites and basal lamina of the salivary gland.

Sporozoites were surrounded with vacuoles after penetration into the cytoplasm of salivary gland cells (Fig. 7). Pimenta *et al.* (1944) observed the same phenomenon and explained that these vacuoles appear to be formed by the invagination of the palsma membrane. We observed sporozoite moving just from the cytoplasm to the secretory cavity (Fig. 7). Spopozoites in the secretory cavity became lodged to form bundles (Fig. 8).

A cuticular salivary duct passes through each lobe and salivary gland cells surround the salivary duct. In *Aedes*, the duct continues from the mouth to the end of the distal region of the lobe (Jansen and Wright, 1971), but in *Anopheles*, it ends in the intermediate zone of the distal region (Wright, 1969). The duct wall was not observed at the end of the distal region of lateral lobes (Fig. 8), suggesting that spopozoites in the secretory cavity move to the lumen of the salivary duct more easily at the posterior half of distal region than the anterior half of the distal region. We found a sporozoite moving toward the probosis in the duct lumen with a very thick wall at the distal region near the intermediate region of the lateral lobe (Fig. 9). It is doubtful that sporozoites located around this thick duct could penetrate the duct wall and move toward the probosis.

We observed sporozoites invade from the anterior end into median and lateral lobes of the salivary glands. The anterior end of sporozoites also has an apical complex (Sterling *et al.*, 1973; Sato, 1998). Therefore, apical complex apparatus is also considered to play a role in penetration of sporozoites into salivary glands.

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A SEROEPIDEMIOLOGICAL STUDY ON HEPATITIS B VIRUS, HEPATITIS C VIRUS AND HUMAN IMMUNODEFICIENCY VIRUS INFECTION IN NORTHERN THAILAND

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Abstract: A total of 1,889 voluntarily donated blood from five provinces in northern Thailand were investigated for the prevalences of hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infection. The average positive rates of HBs-Ag, anti-HBs, IgM anti-HBc, anti-HCV and anti-HIV were 8.7, 42.6, 0.5, 1.6 and 1.7%, respectively. The highest positive rates of HBs-Ag, anti-HBs, IgM anti-HCV and anti-HIV were found in the age group under 19 years (13.3%), 40-49 year old group (47.6%), 20-29 year old group (0.6%), 30-39 year old group (2.6%) and 20-29 year old group (2.9%), respectively. We found only two cases that were anti-HIV and HBs-Ag positive. There are no cases that were anti-HIV and IgM anti-HBc positive, and that were anti-HIV and anti-HCV positive. These results suggest that in northern Thailand, most of the HBV infections are due to vertical transmission from mother to child, although HBV infection is a major cause of viral hepatitis, HCV infection will become an important public health problem in the near future and that the prevalence of HIV infection is quite high among sexually active generations. Therefore, there is a need for more medical attention to these blood-borne virus diseases, and we strongly recommend routine blood screening for HCV in every medical institutions in northern Thailand.

Key words: HBV, HCV, HIV, northern Thailand

INTRODUCTION

Although HBV infection is highly endemic in Asia, Africa and Middle and South America, having harmful influences to the population in these countries, the introduction of routine donor blood screening for HBs-Ag has decreased the incidence of HBV infection in many of these countries. In Thailand, HCV infection has become the major cause of post-transfusion and sporadic hepatitis (Tanprasert *et al.*, 1993). The HIV infected population is increasing in northern Thailand and it is also well known that there are high prevalences of HBV and HCV infections among anti-HIV positive populations (Esteban *et al.*, 1989; Botti *et al.*, 1992; Eysler *et al.*, 1993; Bryan *et al.*, 1993; Quan *et al.*, 1993; Yousukh *et al.*, 1996). However the seroepidemiological study of the correlation among these viral infections have not been well established in northern Thailand. It is the purpose of this study to determine the prevalences of HBV, HCV and HIV infections among voluntarily donated bloods and discuss the transmission routes to formulate prevention strategies of these blood-borne virus infections in northern Thailand.

MATERIALS AND METHODS

The total number of 1,889 serum samples were randomly collected from the voluntary blood donors at Chiang Mai University Hospital and Chiang Rai, Lampang, Lamphun and Phayao Provincial Hospitals in northern Thailand (Fig. 1) during June to December, 1995 and stored at −20°C until examination. Sera were

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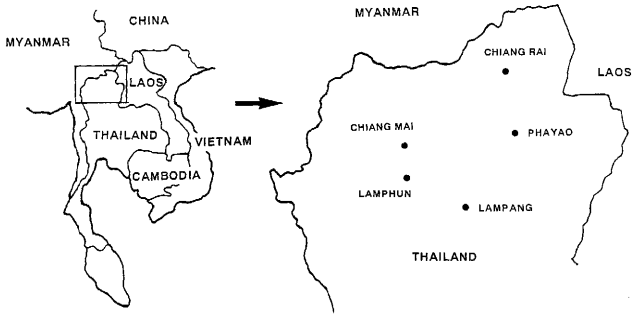

Figure 1 Map of northern Thailand

tested for the presence of HBs-Ag by using *Auszyme Monoclonal* (the third generation EIA kit, Abbott Lab. Ltd., North Chicago, Illinois, USA), anti-HBs by *Ausab EIA* (human-subtypes ad and ay, Abbott Lab. Ltd., North Chicago, Illinois, USA) and the anti-HBs titers of positive samples were calculated and seroprotection was defined as anti-HBs levels≧10 mIU/m*l* (Andre, 1989; Yap *et al.*, 1992), IgM anti-HBc by *Corzyme-M* (γ DNA, Abbott Lab. Ltd., North Chicago, Illinois, USA), anti-HCV by *Abbott HCV EIA 3.0* (c100-3, HC-34, HC-43, NS5, Abbott Lab. Ltd., North Chicago, Illinois, USA) and anti-HIV by *Abbott Recombinant HIV-1/HIV-2* (the third generation EIA kit, Abbott Lab. Ltd., North Chicago, Illinois, USA). The statistical analysis of the results was performed using chi-square test.

RESULTS

Table 1 shows the prevalence of HBs-Ag, anti-HBs, IgM anti-HBc, anti-HCV and anti-HIV in 1,889 voluntary blood donors from five provinces of northern Thailand. The average positive rates are 8.7% for HBs-Ag, 42.6% for anti-HBs, 0.5% for IgM anti-HBc, 1.6% for anti-HCV and 1.7% for anti-HIV. The highest rates of HBs-Ag, anti-HCV and anti-HIV positive are found to be 10.9%, 2.5%, and 2.5% respectively, in Phayao and anti-HBs of 46.4% and IgM anti-HBc of 1.2% in Lamphun.

Table 2 summerizes the age distribution of HBs-Ag, anti-HBs, IgM anti-HBc, anti-HCV and anti-HIV positive among the blood donors. The highest positive rates of HBs-Ag, anti-HBs, IgM anti-HBc, anti-HCV and anti-HIV were found to be 13.3% in the group under 19 years old, 47.6% in the 40-49 year age group, 1.6% in the 20-29 year old group, 2.6% in the 30-39 year old group and 2.9% in the 20-29 year old group, respectively.

The prevalence of HBs-Ag, anti-HBs, IgM anti-HBc, anti-HCV and anti-HIV among male donors is described in Table 3. The average positive rates were 9.8% for HBs-Ag, 44.0% for anti-HBs, 0.4% for IgM anti-HBc, 2.1% for anti-HCV and 1.6% for anti-HIV. The highest positive rate of HBs-Ag of 14.7% was found in the under 19 year old group, anti-HBs of 52.7% in the 30-39 year old group, IgM anti-HBc of 0.9% in the 20-29 year old group, anti-HCV of 3.6% in the 30-39 year old group and anti-HIV of 2.7% in the 20-29 year old group.

The prevalence of HBs-Ag, anti-HBs, IgM anti-HBc, anti-HCV and anti-HIV positive among female donors is represented in Table 4. The average positive rates were 6.5% for HBs-Ag, 39.4% for anti-HBs, 0.8% for IgM anti-HBc, 0.7% for anti-HCV and 1.8% for

Table 1 Prevalence of HBV, HCV and HIV infections in five provinces in northern Thailand

Provinces	Number	HBs-Ag	HBs-Ab	IgM anti-HBc	anti-HCV	anti-HIV
Chiang Mai	390	20 (5.1%)	161 (41.3%)	2 (0.5%)	3 (0.9%)	6 (1.5%)
Chiang Rai	415	42 (10.1%)	167 (40.2%)	3 (0.7%)	7 (1.7%)	8 (1.9%)
Lampang	380	37 (9.7%)	150 (39.5%)	0 (0.0%)	7 (1.8%)	2 (0.6%)
Lamphun	345	27 (7.8%)	160 (46.4%)	4 (1.2%)	5 (1.5%)	7 (2.0%)
Phayao	359	39 (10.9%)	166 (46.2%)	1 (0.3%)	9 (2.5%)	9 (2.5%)
Total	1,889	165 (8.7%)	804 (42.6%)	10 (0.5%)	31 (1.6%)	32 (1.7%)

Table 2 Prevalence of HBV, HCV and HIV infections by age in northern Thailand

Age(years)	Number	HBs-Ag	HBs-Ab	IgM anti-HBc	anti-HCV	anti-HIV
≦19	248	33 (13.3%)	92 (37.1%)	1 (0.4%)	0 (0.0%)	4 (1.6%)
20-29	447	36 (8.1%)	156 (34.9%)	7 (1.6%)	7 (1.6%)	13 (2.9%)
30-39	547	43 (7.9%)	256 (46.8%)	2 (0.4%)	14 (2.6%)	10 (1.8%)
40-49	382	35 (9.2%)	182 (47.6%)	0 (0.0%)	6 (1.6%)	5 (1.3%)
≧50	265	18 (6.8%)	118 (44.5%)	0 (0.0%)	4 (1.5%)	0 (0.0%)
Total	1,889	165 (8.7%)	804 (42.6%)	10 (0.5%)	31 (1.6%)	32 (1.7%)

anti-HIV. The highest rates of HBs-Ag of 8.8% was detected in under 19 year old group, anti-HBs of 48.0% in the 40-49 year old group, IgM anti-HBc of 3.6% in the 20-29 years old group, anti-HCV of 1.8% in the 20-29 year old group and anti-HIV of 3.6% in the 20-29 year old group.

Table 5 demonstrates the anti-HBs levels of 804 anti-HBs positive cases. 727 out of 804 anti-HBs positive cases (90.4%) had the seroprotection level of anti-HBs (≧10 mIU/m*l*) and 77 cases (9.6%) showed the levels less than 10 mIU/m*l*. The highest prevalence (94.9%) of protection levels≧10 mIU/m*l* was found in the 20-29 year old group.

Discussion

According to the classification by WHO (Zuckerman, 1987), Thailand belongs to an intermediate prevalence region group of HBV infection (2-7.9%) and the previous report revealed that the average prevalence of HBs-Ag is 7.4% among the donated blood between 1970 and 1980 in central Thailand (Chainuvati *et al.*, 1990). Our result of 8.7% positive among the voluntary donated blood in 1995 from five provincial areas of norhtern Thailand is similar to the previous report of 7.9% positive in 1991 in Chiang Mai (Yousukh *et al.*, 1996). These findings suggest that the prevalence of HBs-Ag positive in northern Thailand differs little from that of central Thailand. There is a slight difference in the prevalence of HBs-Ag positive between 9.8% in male and 6.5% in female ($p<0.05$) and also in the prevalence of anti-HBs positive between 44.0% in male and 39.4% in female ($p<0.05$). The reason for the higher HBV infection rate in male than female is unclear. However it is suspected that males have higher risk of contacting HBV than females, possibly via sexually transmitted route in Thailand (Tanprasert *et al.*, 1993). The younger age group under 19 years old shows the highest rate of HBs-Ag positivie and the lowest rate is found in the age group over 50 years. Contrary to the rate of HBs-Ag positive, a higher rate of anti-HBs is found among the older age groups. In regard to the protective

Table 3 Prevalence of HBV, HCV and HIV infections among male donors in northern Thailand

Age(years)	Number	HBs-Ag	HBs-Ab	IgM anti-HBc	anti-HCV	anti-HIV
≦19	191	28 (14.7%)	70 (36.6%)	1 (0.5%)	0 (0.0%)	2 (1.0%)
20-29	336	33 (9.8%)	118 (35.1%)	3 (0.9%)	5 (1.5%)	9 (2.7%)
30-39	366	30 (8.2%)	193 (52.7%)	1 (0.3%)	13 (3.6%)	7 (1.9%)
40-49	257	27 (10.5%)	122 (47.5%)	0 (0.0%)	6 (2.3%)	3 (1.2%)
≧50	147	8 (5.6%)	66 (46.5%)	0 (0.0%)	3 (2.1%)	0 (0.0%)
Total	1,292	126 (9.8%)	569 (44.0%)	5 (0.4%)	27 (2.1%)	21 (1.6%)

Table 4 Prevalence of HBV, HCV and HIV infections among female donors in northern Thailand

Age(years)	Number	HBs-Ag	HBs-Ab	IgM anti-HBc	anti-HCV	anti-HIV
≦19	57	5 (8.8%)	22 (38.6%)	0 (0.0%)	0 (0.0%)	2 (3.5%)
20-29	111	3 (2.7%)	38 (34.2%)	4 (3.6%)	2 (1.8%)	4 (3.6%)
30-39	181	13 (7.2%)	63 (34.8%)	1 (0.6%)	1 (0.6%)	3 (1.7%)
40-49	125	8 (6.4%)	60 (48.0%)	0 (0.0%)	0 (0.0%)	2 (1.6%)
≧50	123	10 (8.1%)	52 (42.3%)	0 (0.0%)	1 (0.8%)	0 (0.0%)
Total	597	39 (6.5%)	235 (39.4%)	5 (0.8%)	4 (0.7%)	11 (1.8%)

Table 5 Anti-HBs levels by age and sex in northern Thailand

Age (years)	Male		Female		Total	
	≧10 mIU/m*l*	<10 mIU/m*l*	≧10 mIU/m*l*	<10 mIU/m*l*	≧10 mIU/m*l*	<10 mIU/m*l*
≦19	61 (87.1%)	9 (12.9%)	21 (95.5%)	1 (4.5%)	82 (89.1%)	10 (10.9%)
20-29	112 (94.9%)	6 (5.1%)	36 (94.7%)	2 (1.8%)	148 (94.9%)	8 (5.1%)
30-39	175 (90.7%)	18 (9.3%)	58 (92.1%)	5 (7.9%)	233 (91.0%)	23 (9.0%)
40-49	111 (91.0%)	11 (9.0%)	52 (86.7%)	8 (13.3%)	163 (89.6%)	19 (10.4%)
≧50	57 (86.4%)	9 (13.6%)	44 (84.6%)	8 (15.4%)	101 (85.6%)	17 (14.4%)
Total	516 (90.7%)	53 (9.3%)	211 (89.8%)	24 (10.2%)	727 (90.4%)	77 (9.6%)

anti-HBs level more than 10 mIU/m*l*, the highest prevalence rate was found in the 20-29 year old group and the rate steadily decreased with increasing age. In addition, the rate of IgM anti-HBc positive, which indicates acute or recent HBV infection, usually within six months or less, is 0.5% among the total donated blood. These findings suggest that the main transmission route of HBV infection is a vertical infection during the neonatal period, however there are some occurrences of the horizontal infection of HBV in northern Thailand.

The screening tests for anti-HCV in blood donors using the 2nd generation EIA test, revealed 1.6% of anti-HCV positive in Bangkok (Nuchprayoon *et al.*, 1993), 1.3% in Khon Kaen, north eastern Thailand (Tomanakan, 1994) and 2.4% in Chiang Mai (Hotta *et al.*, 1997). Our results using the 3rd generation EIA test which provides an improved sensitivity for the detection of anti-HCV seroconversion and the identification of HCV infected individuals showed 1.6% of anti-HCV positive among the donated blood. It has been suggested that artificial factors such as reusing syringes for some vaccination, which is contaminated with HCV are the main reasons for the higher prevalence in older people in Japan (Yano, 1992). The fact that the prevalence of HCV infection is the highest in the 30-39 year old group, and no positive case is found in the age group under 19 years old, may indicate that the similar factors play a role in the transmission of HCV among the specified age group in northern Thailand.

Since 1988, Thailand has witnessed an explosive rise in the number of HIV-infected populations (Phanuphak *et al.*, 1995). In particular, prevalence is the highest in the Upper North. Ungchusak *et al.* (1995) reported the result of their serosurveillance for HIV infection of donated blood in Thailand; the median prevalence is 0.77% in northern Thailand; 1.56% in Chiang Rai, 2.3% in Phayao, 1.47% in Chiang Mai, 0.77% in Lampang and 3.24% in Lamphun. Our results are similar to their findings. Sexually active generations in their 20's show higher positive rates of anti-HIV. Phayao and Lamphun have higher rates of HIV infection, 2.5 and 2.0%, respectively, and Lampang shows the lowest rate at 0.6%. The reason for the differences in these geographic features has not been determined. More intensive studies are needed to find the reasons of these differences, especially on geographical and ecological aspects.

It is well known that there is a high prevalence of HBV and HCV infection among HIV positive individuals (Esteban *et al.*, 1989; Botti *et al.*, 1992; Eysler *et al.*, 1993; Bryan *et al.*, 1993; Quan *et al.*, 1993; Yousukh *et al.*, 1996), however, we found only 2 cases that are HBs-Ag and anti-HIV positive and no cases that are IgM anti- HBV and anti-HIV positive, and anti-HCV and anti-HIV positive in the present study. Further investigation is necessary to confirm these findings.

The results of our study suggest as follows; 1) Although there are some occurences of horizontal infection, the transmission route of HBV infection is mainly due to vertical infection in northern Thailand and it is important to prevent HBV infection from HBs-Ag positive mother to infant, 2) Although HCV infection is less prevalent than HBV infection at present, it will become an important public health problem in the near future, and it is strongly recommended to introduce routine screening of anti-HCV in the donated blood, 3) The incidence of HIV infection is quite high in northern Thailand and it will need more medical attention.

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Jpn. J. Trop. Med. Hyg., Vol. 27, No. 1, 1999, pp. 19-22

Short communication

EVALUATION OF A RECOMBINANT PROTEIN (rTc24) AND SYNTHETIC PEPTIDES IN ANTI-*TRYPANOSOMA CRUZI* POSITIVE SAMPLES FROM BLOOD BANK DONORS IN CHAGASIC ENDEMIC AREAS OF ECUADOR

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Chagas' disease, caused by the hemoflagellate *Trypanosoma cruzi*, affects around 17 million people in Central and South America (WHO, 1997). The main route of transmission involves *T. cruzi* infected triatomine insect bites, but other mechanisms of transmission such as blood transfusion or blood derived products have been reported to be responsible for fatal cases of acute Chagas' disease (Villalba *et al.*, 1992). Improved vector control by insecticide spraying and better management of blood banks in endemic areas are essential to reduce the disease transmission. Transfusion-associated transmission is not only a threat in the endemic countries but also in non-endemic areas due to migration of *T. cruzi*-infected individuals (Kirchoff, 1993) and the serological screening of blood banks could reduce the transfusion-associated transmission, avoiding the transportation of contaminated blood with the parasite. Actually, the use of serological screening in disease control is hindered by the cost of the assays and the sensitivity and secificity of the antigens used. Recently, with the development of the DNA technology, highly specific and sensitive recombinant antigens (Taibi *et al.*, 1995), as well as *T. cruzi* specific synthetic peptides, have been useful in the diagnosis of Chagas' disease (Burns Jr. *et al.*, 1992).

In Ecuador, although Chagas' disease has been known to exist since 1927 (Arteaga, 1930), the true prevalence of the disease is still unknown. In the country, blood bank screening for Chagas' disease is mandatory but testing in blood banks located in endemic areas is not regular. Recently, blood donors were found to be positive for anti-*T. cruzi* antibodies in a vector-free region and even in non-endemic areas of the country (Grijalva *et al.*, 1995, 1997). Therefore, the screening of blood bank donors is a necessity in Ecuador.

The present study was aimed to determine the prevalence of anti-*T. cruzi* antibodies in Ecuadorian blood banks from two areas endemic for Chagas' disease. Serum samples from blood donors of the Red Cross Blood Bank of Guayaquil, province of Guayas (n=1,423), and from the Blood Bank of Machala, province of El Oro (n=203), were obtained during the period from March to June 1996 (Figure 1). The samples were randomly collected and no distinction was made between volunteers, paid and frequently members. These samples collected were stored at −20°C until used.

Total lysate obtained from *T. cruzi* epimastigotes

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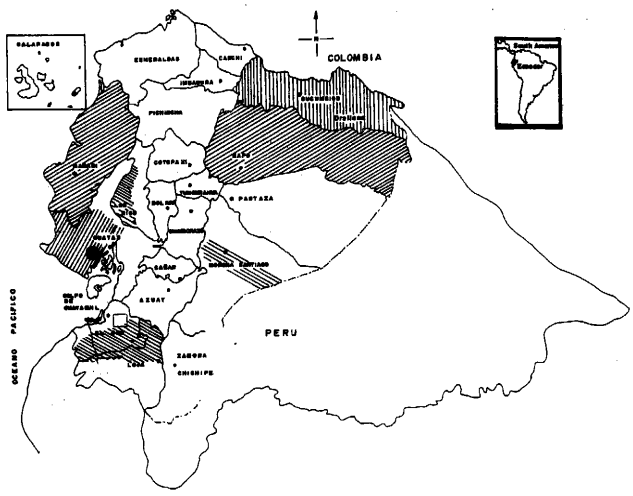

Figure 1 Map showing the Ecuadorian endemic areas for Chagas' disease (////////) and the localities where samples were collected for the study: Guayaquil (●) and Machala (□)

was used for ELISA as described by Chico *et al.* (1997) to test all the sera from blood donors. Briefly, 0.1 μg/ml of crude antigen per well was coated in ELISA plates and incubated overnight at 4°C, non-bound antigen was discarded and 200 μl of blocking buffer was added and incubated for one hour at room temperaure. The plates were washed six times with washing buffer and serum samples, positive and negative controls were diluted 1:50 with washing buffer and 50 μl were added to the corresponding wells and incubated for 30 min at room temperature. Then, wells were washed five more times and 50 μl of protein A horseradish peroxidase (Zymed Laboratories), diluted 1:10,000 in washing buffer was added to the wells and plates were incubated for 30 min. After five additional washes with washing buffer, 100 μl of ABTS [2, 2'-azino-di (3-ethyl-benzthiazoline sulfonate)] in substrate buffer was added (Zymed), the reaction was allowed to develop at room temperature for 30 min in the dark and stopped by adding 100 μl of 5% SDS and the absorbance of each well was read by an ELISA plate reader (Titertek Multiscan) at 405 nm. The "cut off" value was determined by testing 20 samples from healthy individuals from the endemic areas, values higher than the mean absorbance value plus 3 standard deviations [mean+3SD: 0.139+3 (0.041) = 0.262] were considered as positive against total lysate. The positive samples against the total lysate were also assayed by ELISA with two additional antigens: a *T. cruzi* specific synthetic peptide 2/D/E (Houghton *et al.*, 1996) and a recombinant *T. cruzi* protein (rTc24). For the synthetic peptide, 2/D/E, the antigen was diluted to 0.8 μg/ml and 50 μl were coated in microtiter plates, the remaining steps of the ELISA assay was done as described for total lysate, the "cut off" value was calculated and values higher than the mean absorbance plus 3 standard deviations [0.187+3 (0.02) = 0.247] were considered as positives. The ELISA procedure for the rTc24 was performed as described in previous reports (Krautz *et al.*, 1995) with slightly modifications, thus, Immulon I plates (Dynatech Laboratories, USA) were coated with 100 μl of 0.5 μg/ml of rTc24 antigen and incubated with 0.05 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. The unbound material was discarded and plates were blocked with 200 μl of PBS-Tween-BSA solution (PBS pH 7.2, 0.05% Tween 20, 1% BSA) for 2 h at 37°C. After five washes with PBS pH 7.4-0.1% Tween 20 (PBST), 100 μl of human serum diluted 1:100 in PBST and 0.3% BSA were added and plates incubated for 2 h at 37°C. Then, plates were washed five times with PBST and 100μl of a 1:5,000 dilution of peroxidase- conjugated anti-human IgG (γ chain specific) (SANOFI Diagnostics Pasteur, 92430 Marnes-la-Coquette, France) were added to the wells and plates incubated for 1 h at 37°C. After five washes with PBST and an additional wash with PBS, 100 μl of 10 mg o-phenyldiamine dihydrochloride (Sigma, St. Louis, MO 63178 USA) dissolved in 20 ml of citrate buffer pH 5.0 plus 20 μl of H_2O_2 were added to each well and incubated 15 min at 37°C. The reaction was stopped by adding 100 μl of 1 N HCl to each well. The absorbance was measured against the blank wells at 492 nm by mean of an ELISA reader (Titertek Multiscan). The "cut off" value was determined as described in the two previous ELISA tests, values higher than the mean absorbance value plus 3 standard deviations [mean+3DS: 0.183+3 (0.025) = 0.258] were considered as positive against rTc24. In all the ELISA tests described, assays were carried out in duplicate.

Using the total lysate as antigen, 15 (1.1%) of the 1,423 sera collected in Guayaquil were positive; of the 203 samples in Machala 14 (6.9%) were positive (Table 1). These 29 positive samples were tested against rTc24 and 21 (72.4%) were positive for the

Table 1 Anti-*T. cruzi* antibodies against total lysate antigen in blood donors

Locality	Number of samples tested	Total positive sera	
		n	(%)
Guayaquil	1,423	15	(1.1)
Machala	203	14	(6.9)
Total	1,626	29	(1.8)

Table 2 Comparison of reactivity of sera from individuals positive for total lysate antigen with two defined antigens, rTc24 and 2/D/E

Locality	*No. of samples positive for* Total lysate n (%)	rTc24 n (%)	2/D/E n (%)
Guayaquil	15	11	14
Machala	14	10	13
Total	29 (100)	21 (72.4)	27 (93.1)

antigen. On the other hand, 27 (93.1%) of the samples were found positives against the 2/D/E *T. cruzi* specific peptide (Table 2).

In Chagas' disease, the present control strategy is dependent upon an efficient vector control program, and with the development of new tools (fumigantion canisters, insecticidal paints) the vector-associated transmission of the disease has been interrupted in some of the endemic countries. However, blood transfusion is considered as a serious danger of *T. cruzi* infection and screening of blood units must be compulsory in endemic areas as well as in non-endemic regions where migration of people infected with *T. cruzi* has been occurred. Our results support the fact that risk of transfusion-associated transmission is real especially in areas of high endemicity (e. g. 6.9% in Machala) and it could be preventable by discarding or treating *T. cruzi*-infected blood. From the results obtained, a regular nationwide program is required in Ecuador. In the current study, we studied serum samples from only two blood banks in two endemic areas but Chagas' disease is described in at least eight provinces of the country and rural-urban migration is important as well.

In relation with the two defined antigens, rTc24 and 2/D/E synthetic peptide tested in this study, the latter is seemed to be more sensitive than rTc24. However, since a negative reaction for rTc24 has been described as a marker of cure of the disease, it is possible to consider that some patients were infected but treated and the positive responses obtained with the total lysate and the peptide could be due to the past infection. Unfortunately, all the positive patients detected in our study are chronic and complete medical records were not available, the only information about treatment comes from personal communications of some of the patients.

Finally, we did not evaluate the negative samples for total lysate since this antigen could be considered enough for screening purposes. However, in order to confirm a Chagas' disease diagnosis at least two positive tests from a panel of three assays must be required. In conclussion, we can not define one or another antigen as the most suitable for diagnosis of *T. cruzi* infection but the use of highly specific recombinant antigens or synthetic peptides should be useful to avoid the use of parasite cultures as required for tests using total lysate as antigen.

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Jpn. J. Trop. Med. Hyg., Vol. 27, No. 1, 1999, pp. 23-144

PROCEEDINGS OF XXXIX ANNUAL MEETINGS OF JAPANESE SOCIETY OF TROPICAL MEDICINE

25-27 November 1998, Naha, Okinawa

President

Shigeo Nonaka

Professor, Department of Dermatology, Faculty of Medicine,
University of the Ryukyus

CONTENTS

Prize Winner's lecture

JSTM (Japanese Society of Tropical Medicine) Young Investigator Award

Epidemiology of influenza virus infections among children with acute respiratory infections (ARI) in Zambia — Mizuta, K.

President's lecture

Cutaneous diseases in Tropical Medicine especially in Okinawa — Nonaka, S.

Special lecture

Onchocerciasis — Tada, I.

Educational lecture

1 Chronic and intensive solar ultraviolet exposure in tropical areas promotes photoaging and skin cancer — Ichihashi, M. *et al.*

2 Immunomodulatory effects of ultraviolet radiation — Horio, T.

Symposium 1: Present and future situation of leishmaniasis research

Introduction — Hashiguchi, Y.

1 Classification and diagnosis of *Leishmania* — Mimori, T.

2 Leishmaniasis in Ecuador, with special reference to its Andean form — Hashiguchi, Y. and Gomez L., E.A.

3 Leishmaniasis in central Eurasia — Matsumoto, Y.

4 Clinical findings of cutaneous leishmaniasis in Ecuador — Hosokawa, A. *et al.*

5 Molecular biology of drug resistance in *Leishmania*: gene amplification and P-glycoproteins — Katakura, K.

6 Expression of heat shock protein correlates with a protective potential against infection with *Leishmania major* — Himeno, K.

Symposium 2: Dengue fever and dengue hemorrhagic fever

1 Clinical feature of dengue haemorrhagic fever — Tateyama, M.

2 Situation and diagnosis on imported dengue fever in Japan — Yamada, K.

3 Breeding habitats of the dengue mosquitoes, *Aedue* (*Stegomyia*) spp. in the coastal, agricultural and mountainous villages in South Sulawesi, Indonesia, 1994-1996 — Miyagi, I. *et al.*

4 Molecular and *in vitro* analysis of eight dengue type 2 viruses isolated from patients exhibiting different disease severities — Mangada, M.N.M. and Igarashi, A.

5 Dengue virus-specific T lymphocyte responses and the role of T lymphocyte in the pathogenesis of dengue hemorrhagic fever — Kurane, I. and Takasaki, T.

6 Construction and characterization of chimeric Japanese encephalitis/dengue type 4 virus Tadano, M.

Symposium 3: How can we effectively control infectious diseases in the Tropics?

Session 1 Immediate reports from current projects in selected areas

1 Parasitic diseases Shimada, M.
2 Leprosy Hatano, K.
3 Malaria-if we can control the disease or what? Kano, S.

Session 2 Global strategies for eradication or effective control of infectious diseases

4 Global surveillance for infectious diseases Nomura, T.
5 Control of infectious diseases control -community approaches in developing countries- Umenai, T.
6 Global strategy for eradicable infectious diseases Arita, I.

Work shop: For meaningful introduction of advanced technology to studies of malaria epidemiology and control

1 Evaluation of the single-step screening method for quick detection of glucose-6-phosphate dehydrogenase (G-6-PD) deficiency in field survey Iwai, K. *et al.*
2 Proguanil polymorphism and efficacy against malaria Kaneko, A. *et al.*
3 A novel gene cloning of ookinete surface protein from *Plasmodium vivax,* and polymorphism in natural parasite isolates Tsuboi, T.
4 Immunogenetic analysis of the patients with falcipurum malaria in Thailand Hirayama, K. *et al.*
5 Antimalarial natural products from *Dichroa febrifuga* (Joh-Zan) Kim, H.-S. *et al.*
6 Expression of catalytic subunits of mitochondrial complex II from *Plasmodium falciparum* in *Escherichia coli* Takeo, S. *et al.*

Panel presentation

1 Comparative analyses of nonstructural protein NS5 of dengue virus type 1 Ishak, H. *et al.*
2 A study on the approach of the use of insecticide aerosol cans combined with active surveillance for dengue control Osaka, K. *et al.*
3 Japanese encephalitis virus recombinant NS5 protein expressed in *Escherichia coli* exhibits RNA-dependent RNA polymerase activity Hasebe, F. *et al.*
4 Characterization of polioviruses isolated from Pakistani children II Hasewaga, A. *et al.*
5 The risk of hepatitis virus infection among Japanese staying in developing countries Hamada, A. *et al.*
6 Prevalence of human herpesvirus 8 (HHV8) in a young Ugandan population Mayama, S. *et al.*
7 Comparison of the cytokine induction levels of verotoxin-producing *Escherichia coli* isolated from human, cattle and swine Zang, H-M. and Yamamoto, T.
8 The isolation frequency of diarrheagenic bacteria in Vientiane, Laos Iwanaga, M. *et al.*
9 Thrombomodulin levels in patients with typhoid fever Ohnishi, K. and Kimura, K.
10 Identification of the receptor for *Aeromonas sobria* hemolysin Wada, A. *et al.*
11 The treatment of leprosy; current situation and problems Namisato, M. *et al.*
12 Role of human polymorphonuclear neutrophils in host defense against infection with *Penicillium marneffei* Kudeken, N. *et al.*
13 Three case report of infection due to *Penicillium marneffei* with AIDS patients in northern Thailand Watanabe, H. *et al.*

14 Study of the salivary gland proteins of malaria vector mosquito, *Anopheles stephensi* Luo, E. *et al.*
15 A functional single-chain Fv directed to a rodent malaria parasite specifically inhibits the development of oocysts in mosquitoes Yoshida, S. *et al.*
16 Application to researches and diagnoses of a newly developed malaria parasite detection method by flow cytometry Saito-Ito, A. *et al.*
17 Influence of *Schistosoma mansoni* infection on susceptibility to *Plasmodium chabaudi* in mice Yoshida, A. *et al.*
18 Detection of subpatent level of malaria parasites by nested polymerase chain reaction (PCR) and its correlation with microscopy in Myanmar patients Mon, H.M. *et al.*
19 Chemotherapeutic malaria control trial with diagnosis kit (ICT-Pf) and single step G6PD deficiency test in the Solomon islands Nagai, N. *et al.*
20 Change in malaria vectors and malaria situation in northern Thailand during 30 years from 1968 Suwonkerd, W. *et al.*
21 Clinical study on fifteen case of imported malaria Nakamoto, A. *et al.*
22 Nine cases of imported malaria in Kyushu district during last one year Ukon, T. et al.
23 Cysteine proteinase gene as a basis for differentiating *Entamoeba histlytica* and *Entamoeba dispar* by PCR Rivera, W.L. *et al.*
24 Bacterial expression of a human monoclonal antibody Fab fragment recognizing an *Entamoeba histlytica* surface antigen Tachibana, H. *et al.*
25 Detection of *Entamoeba histlytica* -specific antigen by sandwich ELISA Cheng, X-J. *et al.*
26 DNA polymerase activity in encysting *Entamoeba invadens* Makioka, A. *et al.*
27 Inhibitory effects of aphidicolin on growth and encystation of *Entamoeba invadens* Kumagai, M. *et al.*
28 Seroepidemiological study of entoamoebiasis using micro-chemiluminescence ELISA in Nepal Tabuchi, K. *et al.*
29 Seroepidemiology of cryptosporidiosis Itoh, M. *et al.*
30 Detection of *Cryptosporidium parvum* DNA in *Apodemus argenteus* in residential quarters by PCR Honda, M. *et al.*
31 Intestinal blockage by carcinoma and *Blastocystis hominis* infection Horiki, N. *et al.*
32 Studies on *Leishmania* species isolated from the great gerbile (*Rhombomys opimus*) in Xinjiang Uygur Autonomus region in the People's Republic of China Sanjoba, C. *et al.*
33 Leishmaniasis in Turkey Ozbel, Y. *et al.*
34 Structure analysis of *Trypanosoma cruzi* trans-sialidase gene family: relation to protein localization and enzyme activity Uemura, H. *et al.*
35 Comparative analysis of cDNA expression profiles between long slender and short stumpy bloodstream forms of *Trypanosoma b. brucei* using fluorescent differential display method Suzuki, T. *et al.*
36 Immunogenetic analysis of Chagas' disease Juares, S. *et al.*
37 Comparison of the polymerase chain reaction with three serological methods for the diagnosis of Chagas' disease Juarez, S. *et al.*
38 Comparative studies on epidemiology of Chagas' disease between Bolivia and northeast region of Brazil Miura, S. *et al.*
39 Study of crossreacting antigenicity between *Ascaris lumbricoides* antigens and human colonic mucosa Ishida, T. *et al.*
40 Immunodiagnostic application of the recombinant *Toxocara canis* antigen for human toxocariasis canis and cati Yamasaki, H. *et al.*
41 *Strongyloides ratti* additive effect of testosterone implantation and carbon injection on

the susceptibility of female mice — Watanabe, K. *et al.*

42 Prevalence of *Strongyloides stercoralis* infection among inhabitants of Yoron Island in Kagoshima Prefecture and clinical study on symptom in patients with strongyloidiasis — Zaha, O. *et al.*

43 Seroepidemioloby of an emerging parasitic disease, neurocysticercosis in Irian Jaya, Indonesia — Ito, A. *et al.*

44 The resistance of treatment and specific IgG4 antibody titer in HLA-DRB1*0901 positive patients with strongyloidiasis — Satoh, M. *et al.*

45 *Strongyloides stercoralis* carriers in Okinawa, Japan — Asato, R. *et al.*

46 A case of genital elephantiasis due to possible filaria infection — Hamamoto, Y. *et al.*

47 Detection of circulating *Wuchereria bancrofti* antigen, filaria specific IgG and IgG4 in chyluria cases in Okinawa — Qiu, X-G. *et al.*

48 Enhancement of IL-10 production by treatment with rIL-3 in *Trichinella spiralis*-infected mice — Korenaga, M. and Hashiguchi, Y.

49 Construction of cDNA library of *Trichinella spiralis* muscle larvae and the gene products — Takahashi, Y. *et al.*

50 Is the *Schistosoma indicum* group monophyletic? — Agatsuma, T. *et al.*

51 Sonographic assessment of both urinary tract and hepatic morbidity due to *Schistosoma haematobium* — Katsumata, T. *et al.*

52 Current status of boar-meat transmission of paragonimiasis in Japan — Kawanaka, M. *et al.*

53 Morphological variations in next generation of *Fasciola gigantica* from Zambia — Terasaki, K. *et al.*

54 A case of cystic echinococcosis in a Jordanian patient: treatment with albendazole — Kimura, M. *et al.*

55 Questionary test and prevalence of intestinal helmintic infections in Burru, Slawesi, Indonesia — Toma, T. *et al.*

56 An epidemiological study of malignant skin tumors in western Kenya and Nagasaki, Japan — Toriyama, K. *et al.*

57 A study of public opinion survey of Japanese residents living in foreign countries regarding their health — Hiroshige, Y. *et al.*

58 A case of *Amblyomma testudinarium* bite — Hara, N. *et al.*

59 System of defense from ultraviolet rays in wild mammals — Ohwatari, N. *et al.*

60 Suppression of the sweat gland sensitivity to acetylcholine applied inotophoretically in tropical Africans compared to temperate Japanese — Lee, J.B. *et al.*

61 Installation and maintenance of protective nets against venomous jellyfish on the beaches of Okinawa — Shinjo, M. and Araki, Y.

62 Molecular characterization of the Japanese encephalitis virus representative immunotype strain JaGAr01 — Mangada, M.N.M. and Tagegami, T.

Prize Winner's lecture

JSTM (Japanese Society of Tropical Medicine)
Young Investigator Award

EPIDEMIOLOGY OF INFLUENZA VIRUS INFECTIONS AMONG CHILDREN WITH ACUTE RESPIRATORY INFECTIONS (ARI) IN ZAMBIA

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A community based study was carried out to define the etiology and epidemiology of viral acute respiratory infections (ARIs) in children. A total of 3,760 throat swab specimens were collected from children with ARI at three heatlh centers in Lusaka, Zambia between June 1993 and September 1995. Specimens were inoculated onto the microplate including HEF, HEp-2, Vero, and MDCK cell line for virus isolation.

A total of 407 viruses such as influenza virus, enterovirus, adenovirus were isolated (isolation rate 10.8%). In 1993, 54 influenza A/H3N2 were isolated between June and November. In 1994, 34 influenza B were isolated between May and July. In 1995, one influenza A/H3N2 was isolated in January and then the same type of 55 influenza viruses were isolated between June and August. These results revealed that influenza virus infections are one of the most important pathogens of ARI in children in the cool dry season (June-August) in Zambia.

Most of the patients with fever have been diagnosed and treated as having a malaria infection in Zambia. However, malaria infections occur mainly in the hot, rainy season (December-April) in Zambia. Our findings indicated that influenza-like illness with fever and/or respiratory symptoms which occur between June and August should be regarded as influenza virus infection rather than malaria infection. This fact also suggested that a differential diagnosis is difficult but important and laboratory based investigation is necessary for a better medical care in developing countries.

Although a worldwide surveillance of influenza virus infection is carried out by World Health Organization (WHO), there is few report from sub-Saharan Afica according to the Weekly Epidemiological Record of WHO. The Virology Laboratory, Lusaka, Zambia, which have reported a surveillance result of influenza virus infections in Central African region since 1993, has been functioning as a WHO National Reference Laboratory for Influenza since 1997.

President's lecture

CUTANEOUS DISEASES IN TROPICAL MEDICINE ESPECIALLY IN OKINAWA

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Abstract: There are tremendous number of diseases with cutaneous manifestations in tropical zone. In the past, filariasis, malaria, and many other parasitic diseases prevailed in Okinawa. However, malaria and filariasis have been completely eradicated to date. Okinawa is located in a subtropical zone. Mean temperature ranges over 20°C from April to Novermber, and high humidity over 80% is one of characteristic climatological features. In tropical climate, metabolic rate, blood flow rate and physical constitution are also quite specific. Mobidity statistics reveals a large difference between Okinawa and mainland Japan. Hypertensive diseases and malignant neoplasms in Okinawa reveal the lowest levels in Japan. Incidence of squamous cell carcinoma of the skin is in a high level in Okinawa. Exposure level of ultraviolet light energy is measured in four points in Japan by the Meteological Agency. The level of ultraviolet light is the highest in Okinawa, compared with those in Sapporo, Tsukuba and Kagoshima. Patients with squamous cell carcinoma on exposed areas showed high percentage in Okinawa. Furthermore, cases of Bowen's disease and malignant melanoma associated with human papilloma virus infection have been reported in Okinawa. In addition to these, there is a trend of high incidence in adult T cell lymphoma, Kaposi's sarcoma, and malignant hemangioendothelioma. Diseases such as multiple piloleiomyoma, pilonidal sinus, and tumors derived from hair follicle are relatively frequent. Hansen's disease is still encoutered in our outpatient clinic of Ryukyu University Hospital.

INTRODUCTION

There are tremendous number of diseases with cutaneous changes in tropical zone. Human skin is affected by environmental factors such as sun light, dust, and high humidity in tropical zone (Nonaka, 1995a, b, c). Okinawa is located in subtropical zone and its climate is different from those in mainland Japan. Skin temperature is higher because of high temperature and high humidity. There is a tendency of hyperhidrosis and hypergrowth of skin flora which are accelerated by these conditions. Therefore, superficial infectious diseases on the skin tend to be highly frequented in Okinawa compared with those in mainland Japan. Furthermore, enegy level of ultraviolet light is high in Okinawa compared with those in Japan proper. It is possible that ultraviolet radiations exert a great influence on carcinogenesis and immunity to infectious diseases. Currently, tremendous infectious and parasitic diseases still exist in developing countries of tropical zone. It is useful to reevaluate the present medical conditions in Okinawa so as to keep the developing countries improves their medical problems. In this paper, it will be discussed whether any differences exist or not in incidence of cutaneous neoplasms and infectious diseases between Okinawa and mainland Japan.

NEOPLASMS (Nonaka *et al.*, 1996)

The number of total cutaneous neoplasms in Ryukyu University Hospital from 1983 to 1994 was 720. The number of actinic keratosis (AK) was 119 cases, that is 16.5% of total neoplasms; and squamous cell carcinoma (SCC) was 132 cases, that is 18.3% (Takamiyagi *et al.*, 1996). The sexual difference in AK showed higher incidence in female (39 cases in male and 80 cases in female, that is, male:female=1:2.1). The average ages of onset were 71.7 years in male and 76.0 years in female. The most frequent area of AK was the face (76.5%), followed by the upper extremities (8.4%), the lip (5.9%), the ear lobes (5.9%) and the scalp (4.2%). The number of SCC in male was 64 cases and those in female was 68 cases. There was no sexual difference in the incidence of SCC. The average age of onset was 68.6 years in male with two cases below 40 years. One case was a 27-year-old male with old burn scars in his childhood, and the other was a 36-year-old male with penile carcinoma. The average age of onset was 76.8 years in female, which means that SCC occurs in higher age in female than in male. The most frequented area of SCC was the face (40.2%), followed by the neck (12.9%), the extremities (12.9%), the trunk (10.5%), the lip (9.8%), the genital regions (6.8%) and others. When

Table 1 Comparison of distributed areas of squamous cell carcinoma

Area	Ryukyu University Hospital[9]	Saitama Medical School[10]	Kyushu University Hospital[11]
Face	40.2%	24.6%	20.2%
Scalp	12.9%	10.5%	13.8%
Lip	9.8%	8.9%	11.7%
Genital region	6.8%	3.6%	2.1%
Extremities	12.9%	41.1%	42.6%

compared with those in Kyushu and Saitama University Hospital, the rate of SCC on the face is larger than those in other two University Hospitals (Table 1). In general, SCC is prevalent in male with outdoor life than in female (Ikeda and Kiyohara, 1988; Wada and Wada, 1989). However, our data showed that the number of female patients with AK was twice as much as that of male in Okinawa (Takamiyagi *et al.*, 1996). On the other hand, there is no sex difference in the incidence of SCC. This difference between AK and SCC should be elucidated in Okinawa. Malignant vascular tumors such as malignant hemangioendothelioma (MHE), Kaposi's sarcoma (KS) are relatively popular in Okinawa. Nine cases of MHE and 8 cases of KS were reported in Okinawa from 1983 to 1995 (Bhutto *et al.*, 1995). Seventeen cases of multiple piloleiomyoma were reported in Okinawa (Takamiyagi *et al.*, 1995). There is relatively a high tendency of this disease because one hundred four cases have been reported in Japan thus far. It is a well known fact that human papilloma virus (HPV) infection has been increasing in the United States and Europe. Some cutaenous tumors such as SCC and Bowen's disease are associated with HPV (Sterling and Kurtz, 1998). Malignant melanoma (Takamiyagi *et*

Table 2 The patients with Hansen's disease observed at Ryukyu University Hospital over the past 16 years (1982-1997)

type / year	I	TT	BT	BB	BL	LL	P.N.	P.L.	Total
1982		7 (50.0)	3 (21.4)	2 (14.3)		2 (14.3)			14
1983	5 (29.4)	8 (47.0)	2 (11.8)	2 (11.8)					17
1984		5(1) (45.4)	2 (18.2)	1 (9.1)	1 (9.1)	2 (18.2)			11(1)
1985		15 (62.5)	4 (16.6)	1 (4.2)	1 (4.2)	3 (12.5)			24
1986	2 (12.5)	7 (48.3)	5 (31.3)		1 (6.2)	1 (6.2)			16
1987		1	2	1	1	1			6
1988		2 (15.4)	6 (46.1)	1 (7.7)		1(1) (7.7)	2 (15.4)	1 (7.7)	13(1)
1989	1	2		1	2				6
1990	1	1	1						3
1991			1	1(1)					2(1)
1992	1			1	2				4
1993			3		1				4
1994	1		3		1				5
1995		1	2	2					5
1996			1		1	2			4
1997			1			(1)			1(1)
Total	11 (8.1)	49(1) (36.3)	36 (26.7)	13(1) (9.6)	11 (8.1)	12(2) (8.9)	2 (1.5)	1 (0.8)	135(4) (100)

( ) : Relapsed cases medicated at other hospitals.

al., 1998), verruciform xanthoma (Kamiyama *et al.,* 1998) and Bowen's disease (Uezato *et al.,* in press-a, b) associated with HPV were reported in Okinawa.

INFECTIONS AND OTHERS

In 1900, 30,000 cases of Hansen's disease were registered in Japan, and 9,458 patients were still registered in 1980 (Kon, 1997). Approximately, five hundreds of new patients were reported in 1955. The reports of new patients had been dramatically reduced since then, and only 18 cases including 6 cases in Okinawa, were reported in 1994. The patients with Hanses's disease observed at Ryukyu University Hospital over the past 16 years (1982 to 1997) were 135 cases (Table 2) (Hosokawa, 1998). Only one new patient in 1997 was observed. The number of indeterminate leprosy (I) was 11 cases (8.1%); tuberculoid leprosy (TT), 49 cases (36.3%); borderline tuberculoid leprosy (BT), 36 cases (26.7%); middle borderline leprosy (BB), 13 cases (9.6%); borderline lepromatous leprosy (BL), 11 cases (8.1%); lepromatous leprosy (LL), 12 cases (8.9%); purely neural type leprosy (PN), 2 cases (1.5%); primary lesion (PL) was one case. Relapsed cases were one case of TT, one case of BB and 2 cases of LL. Epidemiological characteristic in Okinawa is that old age group of the patients are frequent with an increase of high age group. Low immunity of the aged will influence the onset of this disease.

Tinea nigra caused by black colored fungi is a superficial fungal infection generally affecting the skin of palms. The first case of tinea nigra was reported in Okinawa (Nakama, 1988). Since then, there have been many case reports in Japan, especially in Kyushu island. Hyperhumidity and high temperature will be main exacerbating factors of this disease. Atopic dermatitis in Okinawa seems to be different compared with that in mainland Japan, that is, the incidence of atopic dermatitis is relatively smaller than those in other areas (Hagiwara and Nonaka, 1995). It is well-known that atopic dermatitis is clearly correlated with the role of climatological factors: either cold/dry weather leading to skin dryness, or hot/humid weather leading to hyperhidrosis, itch, and secondary infections as aggravating factors. To get a clearer view of this disease, further epidemiologic studies are being awaited.

CONCLUSION

There are many differences in cutaneous diseases between Okinawa and mainland Japan. It will be contributing to tropical medicine to elucidate these differences between the two areas.

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Special lecture

ONCHOCERCIASIS

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1. Global view of onchocerciasis

1) Historical view

The parasite, *Onchocerca volvulus*, was first detected in a Ghanaian by a German missionary in 1890 and was nominated *Filaria volvulus* by Leuckart (1893). No pathogenicity of the parasite was found yet. In 1915, however, R. Robles reported the new distribution of this filarial parasite in Guatemala, Central America, after enormous parasitological, clinical and epidemiological studies of coastal erysipela (Erisipela de la costa). He clarified that *O. volvulus* caused ocular lesion and the denodulization improved the symptoms remarkably. This is the reason that the Government of Guatemala launched denodulization campaign in the endemic areas since 1935. Based on the specimens obtained by Robles and other information on vector blackfly species, Brumpt (1919) proposed *O. caecutiens* n.sp., while this new species was neglected by various parasitologists. Identification of vector insect blackfly by Blacklock in 1926 clarified transmission mechanism and encouraged vector control trials in Africa (Garnham and McMahon, 1947) in particular the Onchocerciasis Control Program (OCP) in late 1960s.

A filaricide drug Diethylcarbamazine (DEC) showed curative effect, while it caused severe accessory reactions in the infected people as seen in Mazzotti reaction. Thus the mass treatment has been considered difficult before the appearance and registration of Ivermectin (Mectizan). Mass treatment by Ivermectin based on the community based system recently accelerated African Program for Onchocerciasis Control (APOC) in Africa and Onchocerciasis Eradication Program of America (OEPA) in Central and South Americas in 1990s.

2) Epidemiological view

In 34 countries in the world, 17.7 million people (17.5 millions in Africa, 30 thousands in Arabia Peninsula and 140 thousands in America) are estimated to be infected and 270 thousands are blind. The estimated global damages by onchocerciasis are 880,000 DALYs. The infections in Volta River basin covered by OCP since 1974 were greatly reduced from one million people to only 10 thousands in 1992. No more transmission occurred among children in the OCP area (WHO, 1995).

3) Pathology

As is called "River blindness", the ocular lesions particularly the damage and loss in visual acuity are the characteristic features of this disease. Onchocercal dermatitis and subcutaneous nodules are the other important lesions. The skin disease (pruritus, papules, excoriations, depigmentation, pachydermia etc.) which accompanies strong itching shares half the global DALYs of onchocerciasis and needs treatments of adult parasites because of its socioeconomic importance. On

Table 1 Global estimates of the population at risk, infected and blind

Region	Population at risk of infection (millions)	Population infected	Number blind as a result of onchocerciasis
Africa			
OCP area:			
Original area	17.6[a]	10,032	17,650
Extensions	6.0	2,230,000	31,700
Non-OCP area	94.5	15,246,800	217,850
African subtotal	118.1	17,486,832	267,200
Arabian peninsula	0.1	30,000	0
Americas	4.7	140,455	750
Total	122.9	17,657,287	267,950

[a] The population given is that which would have been at risk had the OCP not existed (WHO, 1995).

this context, macrofilaricides such as Amocarzine, UMF 078 and etc. are now under clinical trials. The nodules called onchocercomatas show characteristic distribution patterns in each continent: No nodules are found on the head in Africa, while half the nodules are on the head in Central America.

4) Control programs

OCP adopted vector control operations by spraying larvicides such as Temephos (Abate) in the breeding rivers for *Simulium damnosoum* complex in the beginning and then added Ivermectin delivery system. Basically 12 thousand km of river were under weekly spraying of larvicide during rainy season. Rotation of larvicides and biological larvicide (*B. thrungiensis* H-14) were also adopted later. OCP prevented 9 million children born within original OCP area from the risk of onchocercal blindness. Thirty million people are protected from infection and 100 thousands have been prevented from going blind. Thus OCP opened up the way for resettlement in fertile areas along the rivers, previously deserted through fear of disease. OCP activity was scheduled to cease in the year 2002. During the successful achievement of OCP, OEPA (started in 1993) and APOC (started in 1996) were planned and launched in other endemic areas of the world. Both programs rely on the community directed treatment system based on the Ivermectin and supported by Geographic Information System (GIS) for rapid epidemiological mapping.

2. Research of human onchocerciasis on human onchocerciasis by Japanese

No research has ever been performed by Japanese before a joint study of skin test by us (Tada and Figueroa, 1969). In 1970, I visited a plantation Panajabal, located in Sierra Madre Mountains of Guatemala, guided by Dr. H. Figueroa for the first time. The plantation was one of the most highly endemic areas of the disease in America. A blind old man has been walking there. The miserable scene shocked me greatly. Fortunately I was awarded with an opportunity to learn more about onchocerciasis in Ethiopia being dispatched by the ODA agency, OTCA (Overseas Technical Cooperation Agency, Japan), next year together with 2 Japanese entomologists and one parasitologist. We visited several times the endemic areas and obtained

Table 2

Research timeline of human onchocerciasis in Japan

1969	Result of skin test study on onchocerciasis in Guatemala was reported (I. Tada and H. Figueroa)
1970	I. Tada visited an endemic plantation in Guatemala C.A. to see onchocerciasis
1971	Four Japanese stayed in Ethiopia dispatched by Overseas Technical Cooperation Agency (OTCA) for the technical collaboration and study of onchocerciasis (Team leader: T. Ohse)
1973	I. Tada: Brief survey of onchocerciasis in Guatemalan endemic areas dispatched by OTCA. J. Mori requested Japanese Government to support research and control as the Ambassador of Japan
1975	JICA: Dispatch of Basic investigation team to Guatemala (Headed by A. Nakajima) JICA: Dispatch of Project installation team to Guatemala (Headed by S. Hayashi) to exchange Record of Discussion (RD)
1976	JICA-Technical cooperation project of onchocerciasis started (Team leader: H. Takahashi, Chief of JICA Steering Committee: S. Hayashi)
1980	JICA: Three-year extention of research project (Team leader: T. Suzuki)
1981	JICA-Guatemala: International Congress of Onchocerciasis (Guatemala City)
1982	Min. Education, SC, Japan supported a comparative study of onchocerciasis and its transmission between South and Central Americas (1) (Headed by I. Tada)
1983	JICA: Collaboration project expired. Among the Japanese experts, Y. Yamagata later joined OCP
1984	Min. Education, SC, Japan: a comparative study of onchocerciasis and its transmission between South and Central Americas (2)
1985	JICA: Jos University (Nigeria) project dealt with onchocerciasis (Team leader: H. Takahashi)
1986	I. Tada joined WHO Expert Committee on Onchocerciasis (Geneva) Min. Education, SC, Japan: a comparative study of onchocerciasis and its transmission between South and Central Americas (3)
1987	Publishment of 3 volumes of Green books (ed. by I. Tada) dealing with studies supported by Min. Education, SC, Japan
1990s	H. Takaoka: Human infections with zoonotic Onchocerca infections in Japan
1997	I. Tada: Participation to Task Force meetings by TDR/WHO for global onchocerciasis control

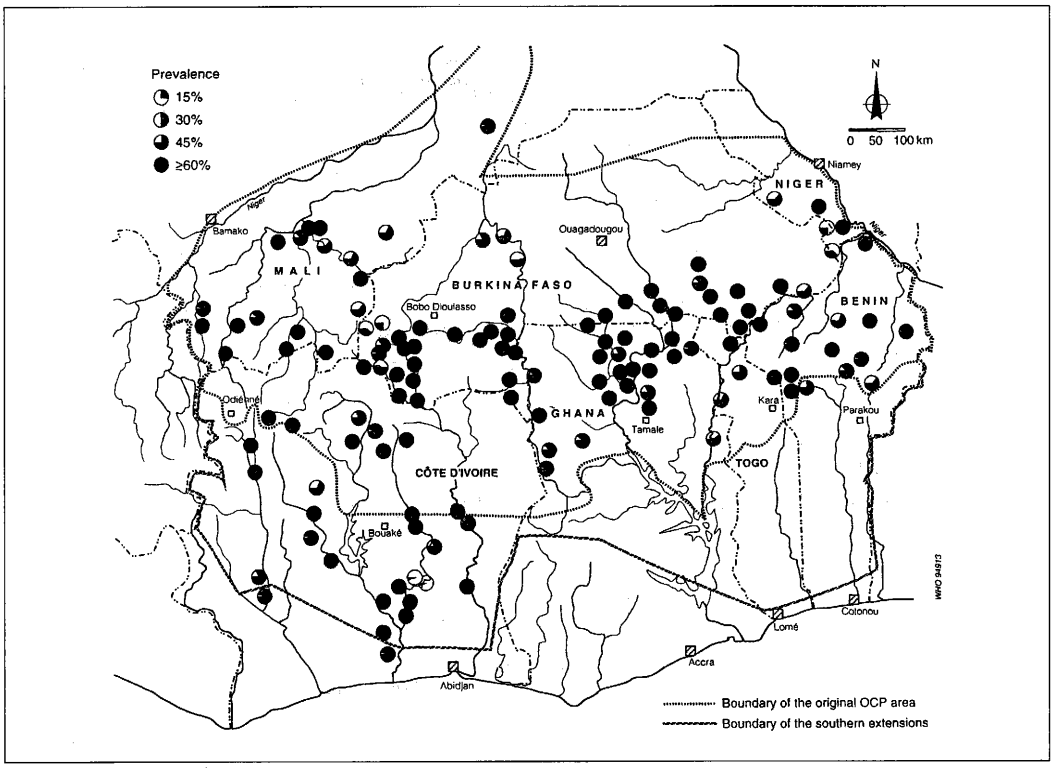

Fig. 1A Pre-control prevalence of skin microfilariae in villages in the original OCP area.

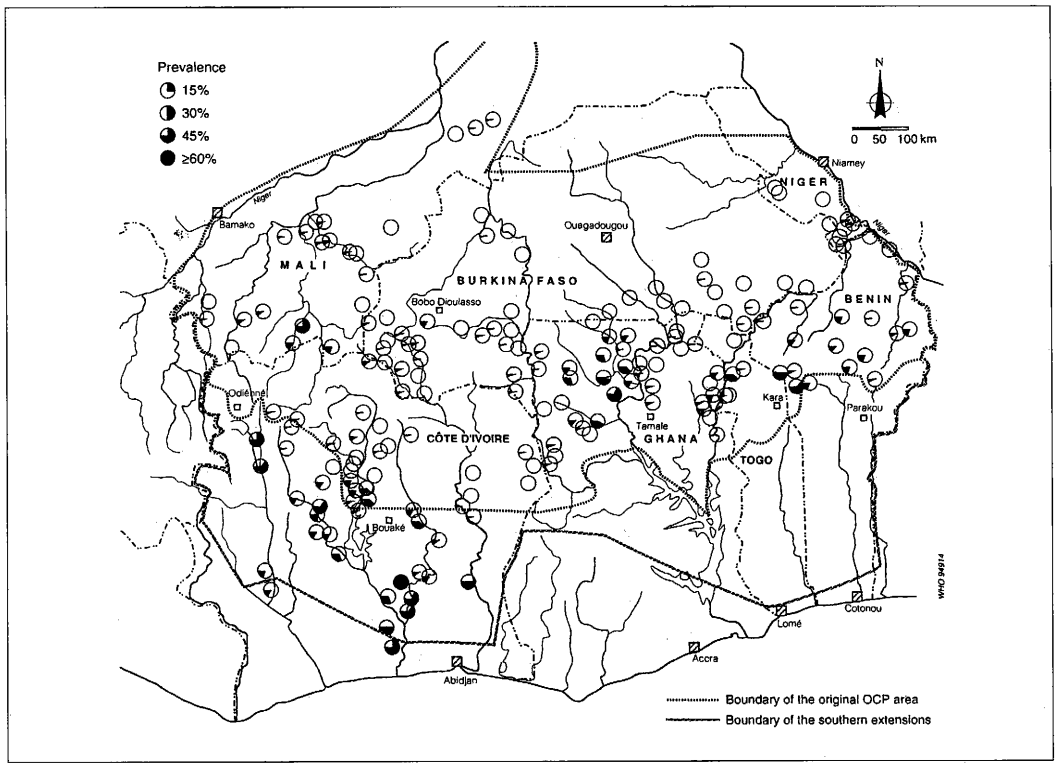

Fig. 1B Prevalence of skin microfilariae in villages in the original OCP area: 1992-1993. (WHO, 1995)

technical experiences and epidemiological data there. In 1973, I was sent to Guatemala for further investigation on onchocerciasis by OTCA. I proposed a report of a brief epidemiological survey to the Governments of Guatemala and Japan asking the survey and control of this disease. Mr. J. Mori, the Ambassador of Japan, requested Japanese government to start a research project for the control of onchocerciasis through JICA (Japan International Cooperation Agency).

1) ODA project for the research of onchocerciasis control

Thus a technical collaboration project started in 1975 (Actually it started in early 1976 because of a big earthquake) and lasted successfully for 8 fiscal years. The majority of the field works were performed in San Vicente Pacaya County. The project yielded various products in terms of technologies in entomological, parasitological, ophthalmologic, and epidemiological fields (Suzuki and Mizutani, 1992). In this project a total of 90 Japanese experts were dispatched and 13 Guatemalan counterparts were awarded with fellowship to visit and learn in Japan. Apart from the scientifically new findings related to onchocerciasis, standardization of various techniques such as immunodiagnosis, inseticide spraying, identification of vector blackflies etc. was intensively promoted. Eventually about 90 scientific papers and a set of technical manuals were produced. Further an international congress of onchocerciasis was convened in 1981 under the auspices of Guatemalan government and JICA with the participation of experts of onchocerciasis control from WHO/OCP (Tada, 1997).

2) Research project on the origin of Central American onchocerciasis

During this ODA project, a question has arisen whether the onchocerciasis in Guatemala was imported by slave trade from West Africa or was autochtonous in continental America (Figueroa, 1963). This has been a matter of arguments in Guatemala and Mexico since when Brumpt proposed *O. caecutiens* as new species. Perforation of the skulls of Pre-Colombian inhabitants suggested its onchocercal origin and Duke and colleagues suggested biological remoteness of *O. volvulus* between South American and African parasite strains. In order to examine this question, my colleagues and I launched a research project to investigate the origin of onchocerciasis in Central America being supported by the grant from the Ministry of Education, Science and Culture, Japan during the period between 1982 and 1987. A total of 12 parasitologists and medical entomologists joined this project. We compared *O. volvulus* and its transmission in Guatemala, Ecuador, Venezuela and Nigeria using cytogenetic, parasitological, entomological, histochemical and morpho-metrical methodologies. For the comparison of *O. volvulus* from Central America (Guatemala) and South America (Venezuela), we adopted the methodologies of Duke *et al.* (1967) and compared bilogical similarity of each strain to vector blackfly by the cross infection. Cytogenetic analysis of the parasite from Central/South America and Africa revealed a decisive conclusion on the identity of the parasite (Hirai *et al.*, 1987). Finally we concluded that Guatemalan *Onchocerca* was biologically quite similar to that of South America and thus considered that it had been imported from Africa by the slave trade, too (Tada, 1983, 1985, 1987),

3) Aftermath

In 1997, I was invited to two Task Force meetings of TDR (Filariasis) and in that occasion I could visit a former endemic village at Burkina Faso, West Africa. Although no more transmission has been occurring in this area due to the long lasting and successful control operations by OCP, I saw a blind old man walking around with stick by himself among actively playing children. Apparently the villagers have come back to once abandoned site as the blackflies completely disappeared. The scene overlapped his counterpart in Guatemala with whom I met 27 years ago. OCP was certainly a successful health program against river-blindness and hence APOC/OEPA also should reveal similar good results.

On the other hand, I would like to indicate a serious fact: After a rush of research on *Onchocerca* and its transmission, very few Japanese scientists or, I might say, only one, are engaging in the study of *Onchocerca* nowadays. Even though we have established the tool and way of control/elimination of onchocerciasis, is research of *Onchocerca* not necessary any more? German scholars belonging to Bernhard-Nocht Institue of Tropical Medicine are still continuously reporting their research activities based on field laboratory in Liberia. They range from morphological to molecular biological findings which are very important to understand parasitism and its treatment in general. On this context, I feel strong necessity to establish and maintain an institution in tropical countries collaborating with local staff. Because Japan lacks adequate number of qualified scientists in infectious and parasitic diseases and tropical medicine.

In the era of emerging and re-emerging infectious diseases, field research is very important to promote research of infections and parasitic diseases.

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Educational lecture

1 CHRONIC AND INTENSIVE SOLAR ULTRAVIOLET EXPOSURE IN TROPICAL AREAS PROMOTES PHOTOAGING AND SKIN CANCER

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Abstract: DNA damage remaining in the cells several hours after a single exposure to ultraviolet radiation may play an important role in the sunburn reaction, and chronic repeated exposures may induce gene mutations related to carcinogenesis, and immuno-suppression by UV may be involved in skin cancer development. Epidemiological studies conducted in people with light skin have shown that sunlight is the most important risk factor for skin cancer, and experimental studies using mice have clarified that ultraviolet B (290-320 nm) radiation is causative of skin cancer. The prevalence and incidence of skin cancer in people having darker skin, however, has not yet been fully studied. Therefore, we examined precancerous lesions and cancer prevalence and incidence in Japan and Indonesia, and analyzed risk and preventive factors for skin cancer in a joint study between Kobe Universtiy and the University of Indonesia, since 1995. Pathology-based cancer registry in Indonesia showed a higher incidence of non- melanoma skin cancer (NMSC) in males (6.62 per 100,000 population) and females (6.54) than in Japan as reported by IARC. The ratio of basal cell carcinoma (BCC) to squamous cell carcinoma (SCC) was about 4:1 for Indonesia and about 2.04:1 for Japan. Further, a case-control study suggests that less education, and outdoor working conditions are risk factors, while diet containing high protein, such as meat and eggs are preventive factors for skin cancer. Incidences of solar keratosis in Kasai city (35°56′N) and at Ie-island (26°42′N) in 1995 were 145.6 and 637.0 per 100,000 population, respectively, indicating that the higher ambient solar radiation induces higher incidence of skin cancer in Japan.

INTRODUCTION

In the early 20th century, sunbathing was shown to be essential to making vitamin D in the skin, which prevents ricket.

Further, sunlight was believed to protect subjects from tuberculosis. Those perceived benefits of sunbathing promoted the public to expose their skin to solar radiation, and a suntan has been regarded as a symbol of health.

However, epidemiological studies on skin cancer in light skined people (Urback *et al.*, 1974) indicated that sunlight is the most harmful cause of photoaging and cancer development of human skin, based on the findings that: (1) people who live in areas with high ambient solar radiation have a higher incidence of skin cancer than people who live in areas of lower solar radiation, (2) people working outside have a higher incidence of skin cancer than people working indoors, and (3) people who spent their lives in strong sunny areas during childhood showed a higher incidence of skin cancer than people who lived in areas with less sunlight, in groups exposed to the similar level of solar light after 10 years of age (Kricker and Armstrong, 1995).

Blue eyes, light coloured hair and light coloured skin that burns easily and tans poorly, have been shown to be primary intrinsic factors for skin cancer.

Experimental studes using hairless mice showed that ultraviolet light B radiation (waveband ranging from 290 nm to 320 nm) is a pivotal causal factor of skin cancer, possibly due to direct and indirect damage of DNA, and suppressive effect on immune system.

Molecular studies on skin cancer cells of humans and other animals indicate that mutations of several genes relevant to cell proliferation, such as p53 and patched, may play pivotal roles in UV-carcinogenesis (Brash *et al.*, 1991; Hahn *et al.* 1996).

1. Sunburn may be caused by DNA damage

Xeroderma pigmentosum patients, develop skin cancers in childhood, have defective DNA repair of UV-induced DNA damage and are easily burned by a small amount of solar radiation with frequent edema and

bullae formation (Ichihashi *et al.* 1982). Japanese skin type I subjects who burn easily and tan poorly have 3-5 times more DNA damage [cyclobutane pyrimidine dimers and (6-4) photoproducts] than do skin type II and III subjects who burn and tan moderately, or who burn rarely and tan heavily, respectively (Ueda *et al.*, 1996). (3) American opossums, who have a photoreactivating enzyme that repairs DNA damage only when the enzyme is activated by visible or UVA light, required about one fouth of UVB dose necessary to induce minimal erythema when the their skin was preexposed to UVB and then kept in the dark compared with animals exposed immediately to visible light irradiation after UVB exposure (Ley, 1985).

These clinical and experimental evidences strongly suggest that DNA damage remaining in the cells several hours after UV exposure may play an important role in sunburn reaction.

2. The role of p53 in UV carcinogenesis

Sunburn cells are now understood to result from an apoptotic process which is controlled at least in part by p53 protein. Ziegler *et al.* (1994) suggested a possible role of wild type p53 protein in the prevention of UV carcinogenesis by increasing apoptosis of UV-irradiated cells which had too much DNA damages to be repaired correctly.

3. Skin cancer epidemiology in Indonesia

The pathology-based cancer registry in Indonesia showed a high incidence of non-melanoma skin cancer (NMSC) in males (6.62 per 100,000 population) and females (6.54), compared with rates in Japan as reported by IARC (Cornain, 1998). Further, the incidence of basal cell carcinoma (BCC) in Indonesia was about 4 times higher than that of squamous cell carcinoma (SCC), whereas the BCC/SCC ratio in Japan was nearly 2: 1, suggesting a role of some unknown environmental and constitutive factors which may cause such differences between these two populations. The incidence of NMSC in Bali in 1996, based on pathology registry, was nearly 19.7 per 100,000 population, suggesting that solar radiation may be an important causative factor of skin cancer for Indonesians with pigmented skin.

A case-control study on skin cancer in Indonesia conducted during 1997 and 1998 suggests that low education and outdoor working conditions are risk factors, while a high protein diet, such as meats and eggs, are preventive factors of skin cancer (Table 1).

Molecular epidemiology is expected to clarify the role of some factors altering the signal pathway from Hedgehog to Smoothened in the future (Fan *et al.*, 1997).

Table 1 Risk factors for skin cancer of Indonesian

Factors	Case		Control		OR (95% CI)
	+	−	+	−	
Gender type	15	32	28	64	1.07 (0.47-2.44)
Education level	41	6	52	40	5.26 (1.89-15.34)
Working outside	20	27	13	79	4.50 (1.84-11.17)
Physical protect	24	23	64	28	0.46 (0.21-1.00)
Smoking	17	30	22	70	1.80 (0.78-4.15)

+ and − in gender means male and female respectively.
+ and − in education means low and high, respectively.

4. Skin cancer and precancer prevalence and incidence of Japanese who live in Kasai city and Ie-island

Studies on skin cancer were initiated in 1992 in Kasai city (35°56′N) and in 1993 at Ie-island (26°42′N). Four cases of NMSC were found in Kasai city (1992-1997) and 11 cases at Ie-island (1993-1997). The number of solar keratosis (SK) found every year in each location was more than 10. The total SK patients were 128 and 130 at Kasai city and Ie-island, respectively. The numbers of NMSC in both areas were too small to calculate the NMSC incidence in Japanese.

The prevalence of SK in Kasai city (Naruse *et al.*, 1997) was between 413.4 and 86.8 per 100,000 population, and showed decreasing trends from 1992 to 1997. The incidence of SK in Kasai city (Suzuki *et al.*, 1997) and at Ie-island in 1995 were 145.6 and 637.0, respectively (Table 2). SK incidence in Ie-island was about 5 times higher than in Kasai city, indicating that the annual UV dose is an important factor of SK development of Japanese. Skin type I subjects who burns easily and tan poorly showed a higher prevalence of SK than skin type III subjects who burn rarely and tan heavily. The SK

Table 2 Incidence of solar keratosis in Kasai city and Ie-island (per 100,000 population)

Year examined	Number of people examined	Patients	Incidence per 100,000
Kasai city			
1993	2,516	16	223.6
1994	2,518	12	171.2
1995	2,622	10	145.0
1996	2,706	5	107.3
1997	2,562	11	99.9
Ie-island			
1995	1,014	15	637.0
1996	1,035	20	625.5
1997	996	20	641.3

Age and sex were standardized to the Japanese population in 1990.

prevalence of subjects having more than 5 seborrheic keratosis was significantly higher than subjects having less than 5, suggesting that numerous seborrheic keratoses may be a risk for SK and possibly for SCC (Table 3).

5. Prevention of photoaging and skin malignancy

UV-induced DNA damage causes UV-specific mutation, such as C → T and CC → TT alterations in oncogenes, tumor suppressor genes and cell cycle regulatory genes, which can result in the development of cancer cells in the epidermis. Tumor formation in the epidermis can be caused by DNA-damage produced directly by UV radiation or indirectly by reactive oxygen species (Hattori *et al.*, 1996), and also by immunosuppression (Fisher and Kripke, 1977).

Protection of the skin from severe sunburn in childhood and adolescence reduces the life-long risk of photoaging and skin cancer.

Skin cancer prevention education is particularly important for school children since they spend a large proportion of their time outdoors.

The Australian, "Slip! Slop! Slap!" guideline, that is; wearing long sleeve clothing, using a sunscreen and wearing a hat, will reduce the risk of skin damage and skin cancer, by protecting skin cells against solar radiation-induced DNA damage and immunosuppression.

Table 3 Prevalence of solar keratosis (per 100,000 population) in Kasai city and Ie-island in relevance to the number of seborrheic keratosis and smoking

Kasai City	Prevalence in the year examined			
	1994	1995	1996	1997
(1) Seborrheic keratosis				
≧6	496.0	207.1	179.1	143.8
0～5	86.6	5.4	38.2	40.5
(2) Smoking				
yes	ND	ND	142.3	139.7
no	ND	ND	163.9	158.4

Ie-island	Prevalence in the year examined		
	1995	1996	1997
(1) Seborrheic keratosis			
	696.1	407.1	379.1
	86.1	75.4	88.2
(2) Smoking			
yes	ND	242.3	264.9
no	ND	263.9	305.0

Sex and age were adjusted to the population in 1990.
ND means "not done".

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2 IMMUNOMODULATORY EFFECTS OF ULTRAVIOLET RADIATION

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Abstract: Sunlight can induce photoallergic reactions both of immediate and delayed types. Endogenous and exogenous chromophores in the skin may be transformed to antigens by light energy. For instance, solar urticaria is induced by IgE-mediated allergic reaction, and drug-induced photoallergic dermatitis is T cell-mediated immunologic reaction. On the other hand, ultraviolet radiation may conversely suppress immunologic reactions, affecting a variety of immuno-competent cells including epidermal Langerhans cell, keratinocyte, T cell, mast cell and vascular endothelial cell. Exposure to UV light inhibits contact sensitization to haptens applied not only to the irradiated skin area but also to the non-irradiated distant skin when the exposure dose is relatively high and/or the application skin area is large. In addition, hapten-specific tolerance develops, which is due to the generation of hapten-specific suppressor T cells. Similar phenomenon can be induced by UVR in delayed type hypersensitivity to microorganisms and tumor immunity. Down regulation of antigen presentation and production of immunosuppressive cytokines may be involved in these UV-induced immunosuppressions.

INTRODUCTION

Recently it is generally accepted that the skin is an important immune organ which is composed of various immuno-competent cells, "skin associated lymphoid tissues (SALT)". These include Langerhans cell, keratinocyte, T lymphocyte, mast cell and vascular endothelial cell. We can easily understand that immunological properties of the skin can be modulated by ultraviolet radiation (UVR), since the skin is an outermost organ which is constantly exposed to the sun. Sunlight can affect the immunologic or allergic reactions of the skin in two different ways. Allergic reactions may be induced by sunlight in selected subjects on the one hand, and immunologic reactions may be suppressed by UVR in all subjects on the other hand. Photoimmunology is a field of research to investigate the effects of light energy on immune system.

1. Photoallergic Reactions

When the skin is exposed to the sunlight, various components in the skin absorb light energy, and some of them are transformed into photoproducts, which may become antigens or haptens. So, we can define that photoallergy is an immunologic reaction in which light energy may play a certain role in the formation of antigens. Endogenous and exogenous substances can be transformed to antigens by sunlight radiation. These light-induced antigens (photoproducts) can develop both immediate and delayed types of photoallergy. Therefore, there may be theoretically at least 4 types of photoallergic reactions; endogenous immediate, exogenous immediate, endogenous delayed, and exogenous delayed photoallergy (Horio, 1984). Immediate photoallergic reaction is clinically solar urticaria. The majority of cases with solar urticaria are induced by endogenous factors (Horio and Minami, 1977). The responsible allergen may be produced by light energy from serum factor. In contrast, exogenous photosensitizers have been rarely identified in immediate type (Horio, 1975). Photoallergic reactions of delayed hypersensitivity can be induced by either topically or systemically applied exogenous drugs and chemicals. The reaction manifests as an eczematous change. The mechanism of photoallergic contact dermatitis is closely similar to that of allergic contact sensitivity. Photoallergic reaction can be experimentally induced in animals using photosensitizing chemicals. The animal model has clarified that photoallergic contact dermatitis is a T cell- mediated immunologic reaction.

2. UVR-Induced Immunosuppression

1) Effects on Tumor Immunity

It is well known that UVR induces skin cancers. The UVR-induced skin tumors in mice do not grow progressively but are rejected when transplanted in normal syngeneic recipient mice. These tumors can only grow when transplanted into an immuno-compromized host, suggesting that the UVR-induced tumors are highly antigenic, recognized as foreign tissues, and are rejected by the immune response of the normal mice. How then do these highly antigenic tumors grow progressively escaping the immune response in the primary UV-irradiated host? It was demonstrated that exposure to subcarcinogenic doses of UVR suppressed the generation of cell-mediated immune reactions by inducing the production of antigen-specific suppressor T cells (Kripke, 1984). This indicates that UVR can impair the

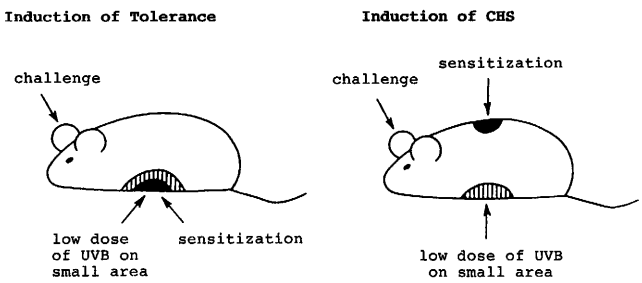

Figure 1 Local immunosuppression.
Topical application of hapten to UV-exposed skin area does not induce contact hypersensitivity, but induces tolerance (left). Hapten application to non-exposed skin can induce hypersensitivity (right).

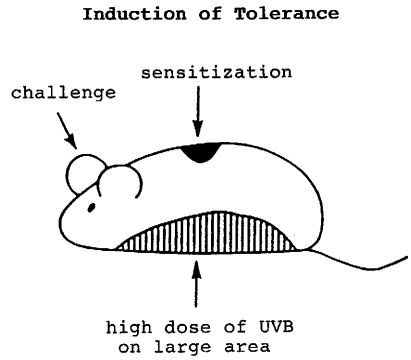

Figure 2 Systemic immunosuppression.
When large area of the skin is exposed to high dose of UVB, hapten application even to non-exposed skin cannot induce contact hypersensitivity.

immunological defense of hosts against tumors.

2) Effects on Contact Hypersensitivity

Based on the experimental evidences described above, UVR has been shown to suppress immune responses to a variety of antigens including sensitizing chemicals (Toews *et al.*, 1980) and microorganisms (Howie *et al.*, 1986; Norval *et al.*, 1994). We have also asked a question how photo(chemo)therapy exerts their beneficial effects on various allergic disorders of the skin (Horio and Okamoto, 1982; Horio and Okamoto, 1983).

The contact hypersensitivity response to antigens applied topically and the delayed type of hypersensitivity response to antigens injected intradermally can be suppressed not only at the irradiated skin (local immunosuppression) but also at a site distant from the irradiation when exposed to higher dose of UVR (systemic immunosuppression) (Figs. 1, 2). Furthermore, these animals, that obtained UV-induced immunosuppression, fail to become sensitized to the subsequent sensitization regimen of the same hapten even through previously unexposed skin. These immunosuppressions may be due to the induction of antigen-specific suppressor T cells. Therefore, the tolerance can be transfered by injecting T cells from UV-exposed mice into naive recipients.

3) Effects on Antigen Presenting Cells

Epidermal Langerhans cell (LC) is an antigen presenting cell which plays the first role in the induction of immunologic reactions in the skin when invaded by antigenic agents. A single exposure of UVB (280-320 nm) alone (Toews *et al.*, 1980) or 8-methoxypsoralen plus UVA (320-400 nm) (PUVA) photochemotherapy (Okamoto and Horio, 1981) depletes LC from the epidermis, and impairs antigen presenting functions. Application of contact allergens to the UV-irradiated, LC-depleted skin results in no induction of hypersensitivity. It is possible that UV-irradiated LC obtains tolerance-inducing ability rather than an activating capacity in the immune system because of impairment of costimulatory signals between LC and T cells. A group of investigators presented experimental evidences that down-regulating antigen presenting cells appear in the skin after UV-irradiation (Baadsgaard *et al.*, 1988).

4) Effects on Lymphocytes

It has been shown that subpopulations of lymphocytes in the periphery blood can be altered after UV-exposure of human body (Morison *et al.*, 1979). In this report, T lymphocytes were more sensitive to UVR than B cells. We indicated that lymphocyte migration towards chemoattractants was inhibited after *in vitro* PUVA treatment (Okamoto *et al.*, 1985), and that IL-2 production by T cells in the spleen was suppressed by *in vivo* PUVA treatment in mice (Okamoto *et al.*, 1987). We also observed that the conversion from $CD45RA^+$ naive T lymphocyte to $CD45RO^+$ memory cell by mitogen stimulation was inhibited after UV-irradiation.

5) Effects on Mast Cells

The mast cell is an essential immunocompetent cell which is involved in IgE-mediated immediate type hypersensitivity such as urticaria. Skin change of urticaria is induced by chemical mediators from mast cells. We have demonstrated that UVB and PUVA irradiation suppressed degranulation and histamine release of mast cells *in vivo* and *in vitro* (Danno *et al.*, 1985, 1988). We confirmed clinically the therapeutic effect of PUVA photochemotherapy on urticaria reac-

tion (Hashimoto and Horio, 1995).

6) Effects on Keratinocytes

The keratinocyte is a major population of the epidermis constituting approximately 90% of the whole epidermal cells. Keratinocytes can produce a variety of cytokines and proinflammatory chemokines. The UVR enhances the production and release of some of these soluble factors such as IL-1, IL-10, TNF-alpha and prostaglandin which can inhibit immunologic reactions (Lugar and Schwarz, 1995). More recently it was demonstrated that immunosuppressive neuropeptides, such as alpha-MSH and calcitonin gene-related peptide, are produced by keratinocytes after UV-exposure (Lugar, 1998).

7) Effects on Vascular Endothelial Cells

Associated with immunologic and inflammatory reactions, various cells infiltrate into the skin from blood vessels. Prior to the extravasation of inflammatory cells, adhesion molecules must be expressed on vascular endothelial cells. Highly expressed adhesion molecules in inflammatory skin are decreased after the resolution of the disease. We observed that the expression of E-selectin and ICAM-1 on cultured endothelial cells by TNF-alpha stimulation was inhibited after UV-exposure (Yamawaki *et al.*, 1996). Therefore, it is possible that UVR may have anti-inflammatory effect at lower dose.

8) Effects on Natural Killer Cells

Natural killer (NK) cell may provide a first line of defense against tumor growth and viral infections. The number and activity of NK cell have been reportedly decreased after UVB and PUVA irradiation (Schacter *et al.*, 1983; Toda *et al.*, 1986).

Conclusion

In the past two decades a new field of research, termed photoimmunology, has been developed, since the immunosuppressive effects of UVR were recognized. Not only UV-induced DNA damages but also alterations of immune regulation are important for skin carcinogenesis, because the immune system contributes to host resistance against tumor growth. UV-induced immunosuppression can also interfere with the response to and clearance of some infectious organisms including bacteria, virus and fungus. From the therapeutic point of view, immunosuppression, in other words, anti-allergic effects may be involved in the beneficial effects of photo(chemo)therapy.

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Symposium 1 PRESENT AND FUTURE SITUATION OF LEISHMANIASIS RESEARCH

INTRODUCTION

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Abstract: In order to know the global situation of leishmansiasis in the world, the transmission and clinical forms were briefly discussed, and the prevalence was also reviewed, mainly based on the reports from World Health Organization (WHO). *Leishmania*/HIV co-infection cases are increasing annually due to different factors, such as human behavioral, environmental and epidemiological changes, especially in southern Europe, Spain, Italy, France and Portugal. The co-infection cases have also been reported from other countries of different continents, Asia, Africa and Central and South America. In such *Leishmania*/HIV co-infection cases, serologic diagnosis is of little use. To overcome the diagnostic problem in HIV-infected patients, an indirect xenodiagnosis of visceral leishmaniasis using laboratory colonized sandflies was recently developed by Spanish workers; the usefulness was shortly discussed in the text as a topic.
Key words: leishmaniasis, global situations, epidemiology, *Leishmania*/HIV co-infections

Transmission and clinical forms. Leishmaniases are caused by different species belonging to the genus *Leishmania*, and the genus divided into 2 subgenera, *Leishmania* and *Viannia*; some 20 species of them are recourded as causative agents of human leishmaniasis in the world. The parasites are haemoflagellate protozoans which are exclusively transmitted by the bite of a tiny 2 to 3 millimetre long female sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Of the 500 phlebotomine species recorded to date, only about 30 of them have been incriminated as vectors of human leishmaniasis in the world. The diseases show a wide range of clinical manifestations depending on *Leishmania* species and/or immunological or physiological conditions of hosts. They are mainly classified into several forms such as visceral (VL), cutaneous (CL), mucocutaneous (MCL), diffuse cutaneous (DCL), and post-kala-azar dermal (PKDL) leishmaniasis. Among these clinical cases, VL is the most serious forms characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anaemia (Bryceson, 1996). If left untreated, the fatality rate of VL cases can be as high as 100% (WHO, 1993). CL forms produce self-healing or non-healing skin ulcers or nodules leaving permanent scars on the exposed skin surface of the body such as the face and the upper and lower extremities, conferring a life-long immunity on the host against the challenge infection with the same species or subspecies of the genus *Leishmania*. Furthermore, MCL, DCL and PKDL forms are usually resistant for the drugs available commercially, and MCL leads to partial or total destruction of the mucous membranes of the nose, mouth and throat cavities and surrounding tissues accompaning with serious secondary infections. The scars and disfigurings caused by leishmaniasis can cause serious social prejudice, and cast out the patient from society (WHO, 1996).

Prevalence. The diseases are endemic in 88 countries on the five continents, with a total of 350 million people at risk, and the diseases afflict at least 12 million people worldwide (WHO, 1996). These numbers are however probably underestimates; figures of 2 million new cases per year, including 1.5 million cases of CL and 500,000 of VL are likely (Desjeux, 1996). Over the last ten years, endemic regions of leishmaniasis have been spreading further afield and there has been a sharp increase in the number of recorded cases of the disease (Hashiguchi, 1996). In Tunisia, for example, 1,300 cases of CL were reported in 1983, then the figure had reached 6,000 in 1991; in the northern States of Brazil, 2,000 cases were reported in 1980, and 9,000 in 1990. Furthermore, in the State of Bihar, India, where 38 out of 42 districts are affected, between 250,000 and 300,000 cases were estimated in 1992—five times the official figure (WHO, 1993); the number of VL cases and the number of deaths reported officially from Bihar during 1985-1992 are shown in Table 1 (Dhanda *et al.*, 1996). Of all VL cases in the world, 90% cases occur in Bangladesh, India, Sudan, and Brazil; 90% of CL occur in Afghanistan, Iran, Saudi Arabia, Syria, Brazil and Peru; and 90% of MCL are reported from Brazil, Bolivia and Peru (WHO, 1993). Thus, leishmaniasis endemic regions and clinical cases are increasing annually without specific measures effective for the control after the first report from Turkey in 1756, about 240 years ago. Since 1993, geographic spread of the disease is also found due to the the factors related mostly to development, including massive rural-urban migration, agro-industrial projects that

Table 1 Number of kala-azar cases and number of death reported from Bihar during 1985-1992

Year	No. of cases	No. of deaths
1985	13,030	35
1986	14,079	46
1987	19.267	77
1988	19,267	121
1989	30,879	475
1990	54,650	544
1991	59,619	843
1992	75,523	1,417

Sourse: Status paper on kala-azar in Bihar, 1993

being non-immune urban dwellers into endemic rural areas, and man-made environmental changes like dams, irrigation systems and wells (WHO, 1996).

***Leishmania*/HIV co-infection.** Over the past few years, AIDS and other immunodepressed conditions have increased the risk of *Leishmania*-infected people developing visceral forms, and in certain areas of the world, especially in southern Europe the risk of co-infection with HIV is rising due to epidemiological changes; of the first 700 *Leishmania*/HIV co-infection cases which have been reported to WHO, 673 (96.1%) were from the regions of southern Europe: Spain (413 cases, 59.0%), Italy (130 cases, 18.6%), France (127 cases, 18.1%), Portugal (3 cases, 0.4%). Of these, 627 (89.6%) were male, 600 (85.7%) were young adults (20-40 years old), and 497 (71.0%) were intravenous drug users (WHO, 1996). In these Mediterranean basin *Leishmania* (*Leishmania*) *infantum* is the causative agent of VL, one of the vectors being *Phlebotomus perniciosus*. Approximately 50% of all VL cases in adults of these endemic areas are associated with HIV infection (Alvar *et al.*, 1992). According to WHO (1996), in the Americas most *Leishmania*/HIV co-infection cases are reported from northeastern Brazil, showing an increase in the risk of overlapping infection of both diseases. In eastern Africa, on the other hand, such a co-infection has been reported from Ethiopia, Kenya, Malawi and Sudan. In these endemic areas, the risk of overlap of leishmaniasis and HIV is increasing due to a number of factors such as mass migration, civil unrest or war, resettllement programmes, and prostiscuity and prostitution in refugees camps.

Diagnosis and treatment: a topic. Serologic diagnosis is of little use in *Leishmania*/HIV co-infected patients, because of abscence of anti-*Leishmania* antibodies in 30% of such cases (Alvar *et al.*, 1989). Therefore, immunologic diagnosis of VL in co-infected patients is difficult, requiring the use of combination of several techniques for confirmation of the disease. Bone marrow aspirate remains the safest and most sensitive techniques, but spleen aspirate and liver biopsy are also used; in some cases search for the parasites can be done in peripheral blood samples (WHO, 1996). Recently, Molina *et al.* (1994) developed an indirect xenodiagnosis of VL in HIV-infected patients using laboratory colonized sandflies. *Phlebotomus perniciosus* females were fed on a membrane-feeding apparatus containing 2 m*l* blood from each patient, with heparin, EDTA, or sodium citrate as anticoagulants; blood samples were kept at 4°C for 6-96 hrs until use. Sandfly guts were dissected and examined for parasites 2-7 days after membrane feeding (Table 2). Among their diagnostic methods used, indirect xenodiagnosis was the only method that proved 100% effective in co-infected patients, confirming it as an alternative in *Leishmania*/HIV co-infected patients. However, its use should be restricted to those cases where the more usual diagnostic techniques have failed and there is a strong suspicion of leishmaniasis in HIV-infected patiens (Molina *et al.*, 1994). As to treatment for co-infected patients, pentavalent antimonials showed a positive response in 83% of cases, with 52% relapsing rate of co-infected patients within a period of one month to three years; the main alternative drugs include pentamidine, amphotericin B and liposomal amphotericin B (WHO, 1996).

Under such a global situation of leishmaniasis in the world, the symposium entitled "leishmnaiasis: its present and future situation" is intended to disclose the underlying problems in the identification of causative *Leishmania* species, diagnosis and clinical manifestations of the disease, epidemiology of the disease in the Old and New World, parasite resistance for drugs, and the role of heat shock proteins (HSP) as an escaping mechanism of the parasite from host defense networks.

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Table 2 Xenodiagnosis of leishmaniasis in HIV-co-infected and non-infected patients: clinical and parasitological data

	Cases											
	1	2	3	4	5	6	7	8	9	10	11	12
Sex	M	M	M	F	M	M	M	M	M	M	M	M
Age (years)	36	33	35	25	38	29	22	28	38	27	22	61
HIV+	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No
Risk factors	IVDU	IVDU	IVDU	IVDU	Hetero	IVDU	IVDU	IVDU	IVDU	IVDU	—	—
CDC HIV group	IVC-2	IVC-1	IVC-1	IVC-1	IVC-1	II	IVC-1	IVD	III	IVC-1	—	—
Associated infections	PTB, OC	CMV	PCP	EC	PTB, EC	OC	EPTB	—	—	PCP	—	—
Fever	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Splenomegaly	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
Leukocytes ($\times 10^6/l$)	2,700	2,400	NA	2,500	4,300	3,600	2,400	1,600	1,900	5,900	3,300	3,100
$CD4^+$ ($10^6/l$)	40	10	68	56	48	51	144	20	148	ND	ND	440
Haemoglobin (g/l)	8.4	8.3	NA	7.2	8.4	9.0	10.1	10	6.5	7.4	13.7	12.4
Platelets ($\times 10^9/l$)	71	164	NA	84	75	98	73	179	131	170	146	116
Diagnostic methods												
indirect xenodiagnostic	+	+	+	+	+	+	+	+	+	+	−	+
Giemsa stain of Blood	−	+	−	+	+	+	−	−	−	+	−	−
Bone-marrow aspirate	−	+	+	+	+	+	−	−	−	+	−	+
Serology (IFAT)	1:160	<1:40	1:80	<1:40	<1:40	<1:40	1:160	1:320	1:40	<1:40	1:5,120	1:1,280
Bone-marrow culture	+	+	+	+	+	+	−	+	−	+	+	+
Peripheral blood monocyte culture	−	+	+	+	−	+	+	+	−	+	−	−
Treatment of leishmaniasis (days)	2	0	0	0	0	0	Relapse	Relapse	Relapse	0	0	0
Anticoagulant used	SC	HE	HE	HE	EDTA	HE	HE	HE	HE	HE	HE	HE
Blood storage at 4°C (h)	6	6	24	6	36	6	24	48	72	24	6	96
Sandfly infections (%)	22.8	37.5	14.2	62.7	92.9	48.3	8	10.7	13.8	53.3	0	50

IVDU, intravenous drug user; Hetero, heterosexual transmission; PTB, pulmonary tuberculosis; OC, oral candidiasis; CMV, cytomegalovirus infection; PCP, *Pneumocystis carinii* pneumonia; EC, oesophageal candidiasis; EPTB, extrapulmonary tuberculosis; NA, not available; ND, not done; IFAT, indirect fluorescent antibody testing; SC, sodium citrate; HE, heparin.

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1 CLASSIFICATION AND DIAGNOSIS OF *LEISHMANIA*

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Species is a category of living things that ranks below a genus. A definition of the word "species" is usually made up of related individuals able to produce fertile offspring in general biology. However, there is no sex in protozoa of the Order Kinetoplastida. Species of *Leishmania* had been mainly classified by the difference of the clinical manifestations in human, geographical distribution and behavior of parasite.

Leishmaniases are widely distributed in tropical and temperate zones, where they present a considerable health problem. According to Desjeux (1993) and WHO report (1998), an estimated 12 million people in 88 countries are affected by these diseases, two million new cases occur each year. The diseases are caused by parasites of the genus *Leishmania* and reveal visceral, cutaneous and mucocutaneous manifestations depending on the parasite species. Therefore, the identification of *Leishmania* species is crucial in choosing the appropriate treatment as well as predicting the prognosis of the patient, and the classification of species is important for epidemiological and public health surveillance.

This paper contains some current topics of the classification and identification of *Leishmania* parasites including molecular biology methods.

Isolation of *Leishmania* parasites

1) Old World visceral leishmaniasis

Visceral leishmaniasis was known as Kala-azar in India, and as infantile or infectious splenic anaemia in the Mediterranean Region in the past. Leishman (1903) reported intracelluar bodies in the viscera of Kala-azar patient of Dum-Dum in Calcata, India, and recognized to be them related to the trypanosomes. Donovan (1903) also discovered the similar bodies from spleen of the patients from India, and sent its sketch to Ross. The parasite was thought to be a piroplasm and was named it *Piroplasma donovani* by Laveran and Mesnil (1903); it was Ross (1903) who amended the name to *Leishmania donovani* in the same year.

2) Old world cutaneous leishmaniasis

Descriptions of cutaneous leishmaniasis are found as Balkh Sore from the first century in Central Asia. Parasites were observed in sections of human skin lesions of Delhi Boil in India, the organsm was thought to be a member of Mycetozoa or "slime fungi" by Cunningham (1885). Wright (1903) reported an organism in the skin of an Armenian child, and named it *Helcosoma tropicum*. Lühe (1906) renamed this organism as *Leishmania tropica*, but he thought it as piroplasms. Rogers (1904) and Nicolle (1908) observed the flagellate stages of the parasites in blood-agar culture media and the organisms were classified under trypanosomatide group. However, the parasites were thought within the genus *Herpetomonas* or *Leptomonas* in trypanosomatide group. Moreover, Wenyon (1926) suggested that *Leishmania* was different from *Herpetomonas* and *Leptomonas* because *Leishmania* adapted to vertebrates, whereas *Herpetomonas* and *Leptomonas* could not essentially infect vertebrate hosts.

3) New world cutaneous and visceral leishmaniases

Lindenberg (1909) and Carini and Paranhos (1909) reported the first cases of autochthonous New world cutaneous leishmaniasis in southern Brazil, it was thought that the parasite was *Leishmania tropica*. Vianna (1911) found the morphological different type of parasite from the patient of disseminated cutaneous leishmaniasis in Brazil; it was named *Leishmania brazilienses* a name later amended to *L. braziliensis* by Matta (1916).

Kirk (1949) had classified *Leishmania* infecting human to the following 3 species; *Leishmania tropica* (Wright, 1903) Lühe, 1906; *Leishmania donovani* (Laveran and Mesnil, 1903) Ross, 1903; *Leishmania braziliensis* Vianna, 1911 emend, Matta, 1916. Thereafter, many subspecies were described within species by the difference of the manifestations in human cases caused by each strain of the parasites. Saf'janova (1982) separated lizards *Leishmania* by the use of the subgeneric name *"Sauroleishmania"* from mammals *Leishmania* as subgenus *"Leishmania"*. Moreover, Lainson and Shaw (1987) divided subgenus *Leishmania* to subgenus *Leishmania* and subgenus *Viannia* by the difference of promastigote localization in gut of the sandfly. Recently, 20 species of *Leishmania* infecting human are described in the WHO report (1990). According this report, 12 species; *aethiopica, amazonensis, chagasi, donovani, garnhami,* and *infantum, killiki, major, mexicana, pifanoi, tropica, venezuelensis* belong to subgenus *Leishmania* group, and 8 species; *braziliensis, guyanensis,*

panamensis, peruviana, lainsoni, naiffi, colombiensis and *shawi* belong to subgenus *Viannia* group.

Classification of *Leishmania*

The classification and identification are done using the following criteria and methods;

A) Clinical features in human and animal infection
B) Morphology by light-microscopy and electron-microscopy
C) Behavior of the parasite in vector (sandfly), host (laboratory animals) and culture media
D) Geographical distribution
E) Host response; Noguchi-Adler test, excreted factor serotyping, immunofluorescent test, indirect haemoagglutination test, cross-immunity trials in host
F) Biochemistry; Enzyme electrophoretic mobilities, protein composition, lipid composition and monoclonal antibody reactivities
G) Molecular biology; Buoyant density of nuclear and kinetoplast DNA, restriction-enzyme cleavage, filter hybridization and polymerase chain reaction (PCR).

The classification of *Leishmania* parasites which infect human had been being based on the clinical features, geographical distributions of the diseases, and the biological characteristics of the parasites in vectors as well as laboratory animals until 1980. These criteria were not sufficient for the classification of *Leishmania* parasite. It is also very difficult to classify *Leishmania* species on the basis of morphological features. In 1980, *Leishmania* standard reference strains were selected by the participants of UNDP/World Bank/WHO Special Programme Workshop on the Biochemical characterization of *Leishmania*, Washington. In the last 2 decades, the system of classification was supplemented by many zymodeme (Kreutzer *et al.*, 1987), serodeme (Grimaldi *et al.*, 1987) and schizodeme (Barker, 1989) analyses. The methods of using isoenzymes, monoclonal antibodies and PCR have been widely used for classifying and identifying field isolates of *Leishmania* comparing the reference strains. To explore the genetic basis of the species and to facilitate the identification of the species, a number of methods have been elaborated. However, the majority of these methodologies are not ideally suited for clinical laboratories and field examinations. We have been performing the diagnosis of leishmaniasis in epidemiological survey in Ecuador, South America since 1983 (Hashiguchi eds., No. 1, 1987; No. 2, 1990; No. 3, 1992; No. 4, 1994; No. 5, 1997; Mimori *et al.*, 1989). We have developed highly specific PCR (polymorphism-specific PCR and arbitrarily primed PCR) panel to enable the identification of the five major *Leishmania* species which cause cutaneous leishmaniases in the New World. PCR diagnosis can be applied to formalin-fixed biopsy and exudate samples from the leishmanial ulcer lesions (Mimori *et al.*, 1998).

However, a few discrepancies exist between the classifications based on clinical manifestations and molecular characterization. Recently, co-infection with *Leishmania* and HIV are increasing in many countries. The symptoms and signs of these co-infected patients are different from classical *Leishmania* infection (WHO, 1998). Therefore, we must also examine the immunologic competence of the human host.

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2 LEISHMANIASIS IN ECUADOR, WITH SPECIAL REFERENCE TO ITS ANDEAN FORM

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Abstract: In this text, New World leishmaniases were briefly reviewed. In addition, a history of the research on Ecuadorian leishmaniasis by the authors' project from 1982 to date was also shortly given. A total of 7 species of the genus *Leishmania* as causative agents of the disease were isolated from humans, sandflies and mammals, and 4 species of *Lutzomyia* and 8 species of mammals were incriminated as probable vectors and reservoirs, respectively, in that country. In this paper, a special emphasis was given to Andean leishmaniasis which was discovered by the authors in 1986 at a small town, Paute, located on the southern part of Ecaudor, near to the Peruvian borders. The disease form is very similar to Peruvian uta especially in clinical features, but the causative agents (*Leishmania* sp.) and vector sandflies (*Lutzomyia* sp.) were completely different from Peruvian ones. Based on the results obtained from our longitudinal surveys on the epidemiology and ecology of the disease in the area, we developed a transmission model of Andean leishmaniasis. From the information collected in our studies, we recommended that measures for vector control should be applied in such an area endemic for the Andean leishmaniasis, during the dry season when the breeding site of sandflies and the transmission site were limited within and/or around rock crevices and animal burrows in the open field located at remote area from Paute town.
Key words: leishmaniasis, transmission, Andean form, uta, Ecuador

In the New World, leishmaniasis is endemic in many areas of Central and South American countries, and it constitutes a significant public health problem in each country. Control of the disease in these regions is complicated by the variety of factors, such as different species of the genus *Leishmania*, diverse clinical forms and epidemiological patterns in each endemic area. In many regions of the New World, two or more *Leishmania* species are sympatric. The species belonging to the subgenus *Viannia*, are known as the causative agents of cutaneous (CL) and mucocutaneous (MCL) leishmaniasis. Among the *Viannia* group, *L.* (*V.*) *braziliensis* shows the most wide range of distribution, affecting many populations and causing CL and MCL. In the New World, almost all of the disease forms of leishmaniasis are zoonoses, and man is usually incidental host of *Leishmania*. In the subgenus *Leishmania* group, *L.* (*L.*) *mexicana* is mainly reported from the Central American countries and from a pat of the northern regions of the South America. The species, *mexicana, amazonesis* and *pifanoi* in this subgenus are important not only as causative agents of CL but also as those of diffuse type of the disease (DCL). *L.* (*L.*) *chagasi* is main or only species which causes visceral leishmaniasis (VL) in the New World with a wide range of distribution from Mexico to Brazil; however, the distribution of *L.* (*L.*) *infantum* which causes VL and/or CL in the Old World is also suspected in Brazil and other South American countries (Momen, H., personal communication).

Leishmaniasis in Ecuador. Ecuador is one of the small countries in South America through which runs the equator; Colombia borders to the north and Peru borders to the southeast. The Andes mountain crosses the country from north to south. It divides the country into three natural regions, the Pacific coastal regions, the Andes and the Amazonian regions. Each geographic region of the country has specific features relating to the transmission factors of leishmaniasis such as terrain, environment and life style of the inhabitans. In that country, leishmaniasis was first reported in 1920 by Valenzuela (Rodriguez, 1974). However, it has remained one of the least studied of Ecuadorian tropical diseases until recently. From 1982 to date, we have studied almost all of the suspectable endemic foci of leishmaniasis, in order to disclose the transmission mechanisms, clinical forms and etc. It was found that leishmaniasis is widespread in most provinces of Ecuador, showing a considerable health problem. The disease occurs in many populations living in rural and mountainous areas on both sides of the Andes, including those living in the Andean plateau. Of the endemic zones, 9 provinces are located in the Paciffic coastal region, 2 are situated in the Andes and 4 in the Amazonean region. As to the causative agents of leishmaniasis in Ecuador, 7 species of the genus *Leishmania*, *L.* (*L.*) *mexicana*, *L.* (*L.*) *amazonensis*, *L.* (*L.*) *major*-like, *L.* (*V.*) *braziliensis*, *L.* (*V.*) *guyanensis*, *L.* (*V.*)

panamensis and *L.* (*V.*) *equatorensis*, were specifically identified based on zymodeme, serodeme, schizodeme and karyodeme analysis. Four species of the genus *Lutzomyia, Lu. trapidoi, Lu. hartmanni, Lu. gomezi* and *Lu. ayacuchensis*, and 8 species of mammals, anteaters, sloths (2), squirrels (2), kinkajous, rats and dogs were incriminated as possible vectors and reservoirs, respectively, in the endemic areas of Ecuadorian lowland and highland areas.

Andean leishmaniasis in Ecuador and other countries. On the Andean plateau and/or highland of Ecaudor, we discovered a new type of leishmaniasis (Hashiguchi *et al.*, 1987; Hashiguchi *et al.*, 1991) caused by *L.* (*L.*) *mexicana* and *L.* (*L.*) *major*-like which are transmitted by sandflies, *Lu. ayacuchensis* (Takaoka *et al.*, 1990). To date, such a type of leishmaniasis was found in three endemic areas of Andean highlands of Ecuador, Paute (2,300 m-2,500 m above sea level), Alausi (2,300 m-2,500 m a.s.l.) and Huigra (1,200 m-1,500 m a.s.l.). Clinically, the disease forms in the country were found to be very similar to those in Peru (Table 1). However, the *Leishmania* parasites and vector sandfly species from Ecuador are completely different from Peru and other Andean countries (Table 2). In Ecuador, the organisms isolated from humans, sandflies and dogs were identified as *L.* (*L.*) *mexicana* by zymodeme, serodeme, schizodeme and karyodeme analysis (Hashiguchi *et al.*, 1991; Katakura *et al.*, 1993). In addition, another species of the genus *Leishmania*, was also isolated from humans living in Paute, Ecuador, and characterized as *L.* (*L.*) *major*-like, by molecular techniques mentioned above. From the results obtained in our survey it was suggested that leishmaniases in the Andean countries would have more complicated epidemiological and ecological features of the disease in different endemic areas than were previously considered.

Transmission of Andean leishmaniasis in Ecuador. In order to disclose a part of transmission mechanism (s) or ecology of Andean leishmaniasis in Ecuador, we made a longitudinal study on natural infections of *Lu. ayacuchensis* with *L.* (*L.*) *mexicana* (Fig. 1), in addition to epidemiological surveys of inhabitants and reservoirs (Gomez and Hashiguchi, 1991; Hashiguchi *et al.*, 1991). Based on these data, we developed a model for *Leishmania* transmission in the area (Paute) endemic for Andean leishmaniasis of Ecuador (Hashiguchi, 1994). From the data and the model developed, it was suggested that transmission cycle of leishmaniasis in the Andes involved variable overlapping of two sets of the biological entities with a degree of overlap governed by micro- or macro-climatic conditions, depending on the seasonal climatic conditions in the area. The first set consisted of three categories of habitats (I ; open field, II; periurban area and III; urban area). Each of these habitats was occupied at the same time by humans and domestic and wild mammals such as dogs and others. The second set consisted of the relationship between the sandfly vectors and the principal reservoir hosts presumed to be rats and other rodents. A high intensity of transmission

Table 1 Clinical and parasitological findings of subjects with Andean leishmaniasis in three towns, Paute, Alausi and Huigra, Ecuador

Locality	Age*	Sex	No. of lesions	Site of lesions	Size of lesion	Duration** (months)	Smear or culture (+/−)	*Leishmania* sp. identified
Paute	6 Y	F	1	face	15×10 mm	14	+	*major*-like
	5 Y	F	1	face	3×3	7	+	*mexicana*
	5 M	M	1	arm	5×5	3	+	*major*-like
	5 M	M	2	face	5×4, 4×3	4	+	*mexicana*
	9 M	M	1	face	2×2	3	+	*mexicana*
Alausi	3 Y	M	3	face	5×5, 3×3, 5×5	24	+	*mexicana*
	5 Y	M	1	face	5×5	3	+	*mexicana*
	7 M	M	1	face	5×5	5	+	*mexicana*
	8 M	F	1	face	2×2	4	+	*mexicana*
Huigra	1 Y	F	2	face	1×1, 1×1	4	+	*mexicana*
	2 Y	F	3	face	3×5, 1×1, 1×1	18	+	*mexicana*
	9 M	M	4	face	5×5, 3×3, 3×3, 3×3	3	+	*mexicana*
	2 Y	M	1	arm	3×2	8	+	*mexicana*
	3 M	F	2	face	2×2, 3×3	1	+	*mexicana*
	1 Y	F	1	face	5×5	5	+	*mexicana*
	1 Y	F	1	face	5×3	9	+	*mexicana*
	6 M	M	1	face	2×3	4	+	*mexicana*

*Y=years, M=months; **duration time of the disease as of the examination.
(Modified from Hashiguchi, 1994)

Table 2 *Leishmania* species causing Andean leishmaniasis, and their vector sandflies and reservoir hosts, reported from different areas of Andean highland

Areas	Altitudes	*Leishmania* spp.	*Lutzomyia* spp.	Mammalian hosts
Ecuador				
Paute	2,300-2,500 m	*L.* (*L.*) *mexicana*	*Lu. ayacuchensis*	human, dog and rat
		L. (*L.*) *major*-like	not determined	human
Alausi	2,300-2,500 m	*L.* (*L.*) *mexicana*	*Lu. ayacuchensis*	human
Huigra	1,200-1,500 m	*L.* (*L.*) *mexicana*	*Lu. ayacuchensis*	human
Peru				
Western slope of the Andes	1,300-2,900 m	*L.* (*V.*) *peruviana*	*Lu. peruensis* *Lu. verrucarum*	human, dog and rat
Venezuela				
Merida	800-1,600 m	*L.* (*L.*) *garnhami*	*Lu. youngi*	human and opossum
Colombia				
Pueblo Rico, Samniego	1,500 m	*L.* (*L.*) *mexicana*	not determined	human

(Modified from Hashiguchi, 1994).

occurred from October to December in open field (habitat I), largely between the wild mammals living in rock crevices and the vector sandflies. During the rainy season from January to February, the sandfly population increased gradually. The new sandfly generations expanded their biting activity towards periurban area (habitat II), and transmission of *Leishmania* parasites to humans by sandfly bites intensified in this season. During March to April there was a high density of infected sandflies and an increased population of wild rodents living peridomiciliary was also found in the periurban area (our unpublished data); sandfly biting activity extended into urban area (habitat III) with maximum overlap of the domestic and wild hosts and the vector sandflies. Therefore, many new cases of human leishmaniasis were observed in this season at habitat III (Hashiguchi *et al.*, 1991). During May to June, the end of the rainy season and the subsequent decrease in humidity caused a rapid decline in the sandfly density; sandflies disappeared from the urban areas (III) of Paute town, though they were still observed in periurban areas (II) and open fields (I). From July to September, because of extremely dry conditions in Andean plateau (lower terrain), sandfly density falled to a minimum. Nevertheless, rock crevices and animal burrows in the open field allowed the breeding of vector sandflies even in this dry season. From these results, it was suggested that in the open fields *Leishmania* transmission occurred mainly between wild mammals and sandflies throughout the year, keeping the life cycle of the parasite in this habitat (I). In periurban areas of Paute town, the time of leishmaniasis transmission

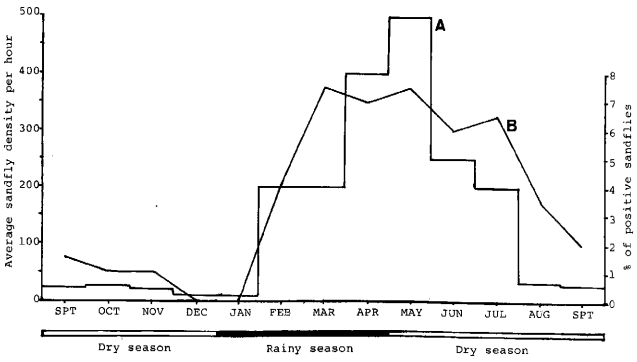

Figure 1 Relationship between sandfly density (A) and natural infection (B) with *L.* (*L.*) *mexicana* in an Andean endemic area, Paute, Department of Azuay, Ecuador. Average density is expressed as the number of sandflies captured per hour in each collection, and percent of those positives for the parasites, calculated from the rate of sandflies infected among the 200 flies dissected in each month. Average temperature and humidity throughout the year ranged from 15°C to 18°C and from 50% to 80%, respectively (Adapted from Gomez and Hashiguchi, 1991).

prolonged to May or June lasting about five or six months. On the other hand, it was very short in urban areas, lasting only two months from March to April with a high incidence. Thus, changes in the incidence and frequency of human leishmaniasis cases in the Andean endemic regions were considered to be the result of migrations of sandflies and reservoir hosts among the three categories (I, II and III) of the habitats. Based on these ecological analysis of Andean leishmaniasis in Ecuador, we recommended that in such

an endemic area the application of control measures should be made during the dry season when the transmission was extremely limited in open field (habitat I), especially within and/or around rock crevices and animal burrows in this area.

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3 LEISHMANIASIS IN CENTRAL EURASIA

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Abstract: The ancient Silk Road crossed over boundless deserts, vast pastures, and high mountains and connected ethnically and culturally diverged areas. It transported not only silk but goods, people, culture and diseases from one end of the Eurasian landmass to the other. Since that time humans living in the sterile lands had to struggle continuously against natural forces to obtain water. When succeeded, it brought about development of great civilization as well as significant changes in ecology of humans, animals, insects, and pathogenic microorganisms. Such ecological changes sometimes resulted in unexpected endemy of a certain type of diseases, such as leishmaniasis, a vector born zoonosis. Leishmaniasis, thus, might be one of the man-made diseases.

Key words: leishmaniasis, *Leishmania*, zoonosis, vector born disease, Central Asia

A vast area of the Central Eurasia has been occupied with deserts. Irrigation might bring wealth to residents and facilitate to socioeconomical development. However it also tremendously changes ecosystem in the irrigated land, sometimes resulting in new unfavorable and unexpected endemy of the diseases including leishmaniasis, a vector-born zoonosis. Leishmaniasis is caused by parasitic protozoa belonging to the genus *Leishmania*, and manifested clinically in different forms; cutaneous, mucocutaneous, and visceral leishmaniasis. This disease is found in the most parts of the world, including the tropical regions of America, Africa and the Indian subcontinents, Mediterranean, Southwest Asia, China and Central Asia (Fig. 1). The parasites are naturally transmitted by different species of blood-sucking sandflies in the genus *Phlebotomus* in the Old World. Leishmaniasis is mostly zoonotic with canines and rodents as major reservoirs. Since 1994 we have performed epidemiological researches on leishmaniasis in some areas along the Silk Road that crosses over the Central Eurasia including Xinjiang Uygur Autonomous Region of China, the Central Asian countries, and Turkey (Fig. 2). Topics and recent problems on leishmaniasis in those countries are discussed in this review.

[Xinjiang Uygur Autonomous Region of the People's Republic of China]

Visceral leishmaniasis, kala-azar, caused by *L. donovani* and *L. infantum* was one of the major parasitic diseases in the vast area of China that lies to the north of the Yangtze River. It has been estimated that 530,000 patients were affected in 1951. As a result of large scale control efforts against kala-azar which include treatment of patients, control of reservoir hosts, mainly dogs, and residual spraying of insecticides, it is now effectively under control in most of the former endemic areas. However, kala-azar has still remained as an important public health problem in the mountainous area of Gansu, Sichuan and Shanxi provinces, where sporadic cases of kala-azar have still occurred and a total of 200-300 cases has been reported annually. In these areas canine visceral leishmaniasis has still been common and the major vector species is *P. chinensis*.

In recent years, increases of new infections have been noticed in the desert area of Xinjiang and the western Inner Mongolia (Guan, 1991). In Xinjiang kala-azar has been widely and sporadically distributed over the northwestern fringe of the Tarim Basin, the Turfan-Hami Basin, and the Junggar Basin, mainly affecting children under two years old. The disease is transmitted through the vectors *P. major wui* and *P. alexandri*. These two species of sandflies are extensively distribut-

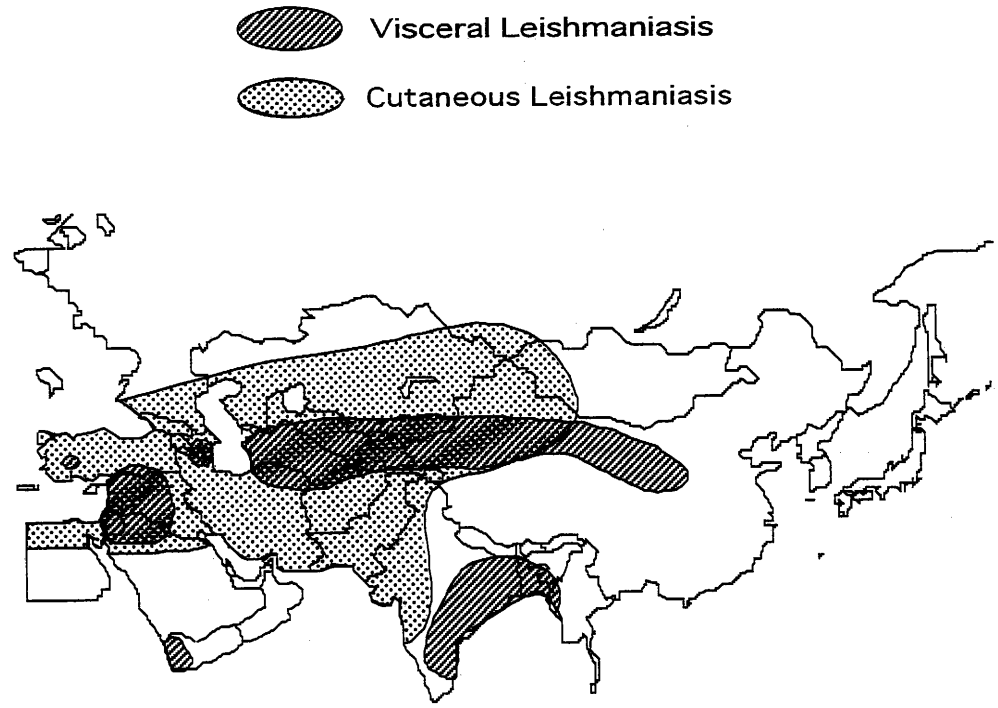

Figure 1 Geographical distribution of cutaneous and visceral leishmaniasis in Central Eurasia.

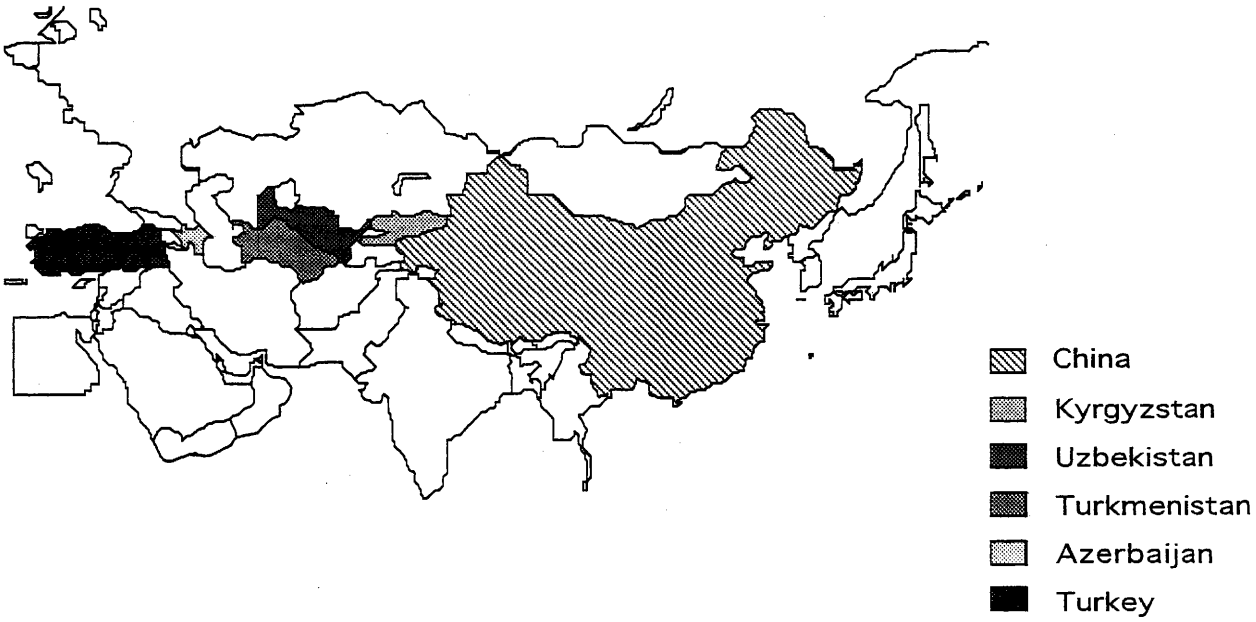

Figure 2 Location of countries which the authors have performed epidemiological researches on leishmaniasis since 1994 and have reviewed in this article.

ed in the field and bite at night. Wild animals are presumably the natural reservoir hosts of the infection. Elaborate examination of rodents, hedgehogs, foxes, dogs, and bats, however, has so far failed to detect any *Leishmania* parasite which causes human visceral leishmaniasis. The control of such zoonotic kala-azar should be difficult. The only available measure against the disease at the present is detection and treatment of the patients.

Recently, cutaneous leishmaniasis has been reported in Karamy located in Junggar Basin. In this region, although *L. major* has not been found, great gerbils,

Rhombomys opimus, are infected with *L. turanica* and *L. gerbilli* with high prevalence (more than 30%). It has been reported that *L. turanica* causes self-healing human skin lesion as proved by human inoculation experiment (Guan, L.-R. *et al.*, 1995). More recently DNA analysis of *Leishmania* parasites from an experimental animal (*Lagurus lagurus*) which was inoculated with biopsy materials from a patient with cutaneous leishmaniasis in Karamy suggested that DNA sequences of a gene from the isolate was highly homologous to that of *L. infantum* (personal communication from Dr. Li-Ren Guan). To determine the etiologic species of *Leishmania* for human cutaneous leishmaniasis in Xinjiang, the further and careful examinations are necessary.

[Central Asian Countries—Azerbaijan, Turkmenistan, Uzbekistan, and Kyrgyzstan]

Leishmania major Yakimov and Schokhor, 1914 was first described for the parasite from a patient with a skin lesion living in Uzbekistan. Because intensive researches have been performed by Russian scientists since that time, the biology and ecology of natural reservoir hosts and vectors have been studied in detail. However, this area has still been endemic of the disease (Strelkova, 1996). Although reexaminations based on modern molecular biological techniques are required on the etiologic agents, four types of leishmaniasis have been distinguished mostly based on epidemiological and clinical features; urban type cutaneous leishmaniasis caused by *L. tropica*, rural type cutaneous leishmaniasis by *L. major*, kala-azar by *L. donovani* and infantile kala-azar by *L. infantum*.

For the rural type cutaneous leishmaniasis, which is distributed most widely among four types of leishmaniasis, the major reservoir is great gerbil, *R. opimus*. In fact, distribution of *L. major* as a pathogenic agent for zoonotic cutaneous leishmaniasis in this region is coincident with the range of *R. opimus* habitat. In *R. opimus* population, three species of *Leishmania, L. major, L. turanica*, and *L. gerbilli* are co-endemic. Among those three *Leishmania* species, only *L. major* is believed to be pathogenic for humans. Although these parasites are easily isolated from Auricula of the great gerbils, no parasites can be detected in visceral organs. In this natural host the parasites are seemed to be maintained without any severe pathological changes. Thus great gerbils are considered to remain as a source of *L. major* infection for a life. Although *L. major* has been isolated from some sandfly species belonging to the genus *Phlebotomus* (*P. papatasi, P. andrejevi*), only *P. papatasi* has been known to transmit the parasites to humans. Visceral leishmaniasis, in which a major reservoir host has been considered to be dogs, is also endemic but sporadic.

Leishmanization, a primitive form of live vaccine against human cutaneous leishmaniasis used in the Ottoman Empire is still going on in Uzbekistan. Under the control of the Samarkand Scientific-Research Institute of Medical Parasitology. Live virulent *L. major* promastigotes grown in culture are mixed with promastigotes lysate, and then inoculated on the arms of human volunteers. This produced a local lesion usually lasting up to a year, after which the person would be immune.

Most of countries in this area became independent in 1991 from the former USSR. Since some confusions after the independence have still remained on taking over the public health affairs, the situation of primary health care is unstable, especially on control of infectious diseases. Establishment of new control projects are in urgent needs.

[Turkey]

Cutaneous leishmaniasis has been known for centuries in Turkey as Beauty Scar, Oriental Sore, Allepo Sore or Annual Sore (Fig. 3). It was highly endemic in whole country before 1950. The number of cases decreased after 1950 with the beginning of the use of insectisides for malaria control, and thereafter increased with relaxation of malaria control programs. Today, because of the development of socioeconomic conditions and the improvement of public hygiene, the disease is effectively under control in most regions except for southeastern part of Turkey where the dis-

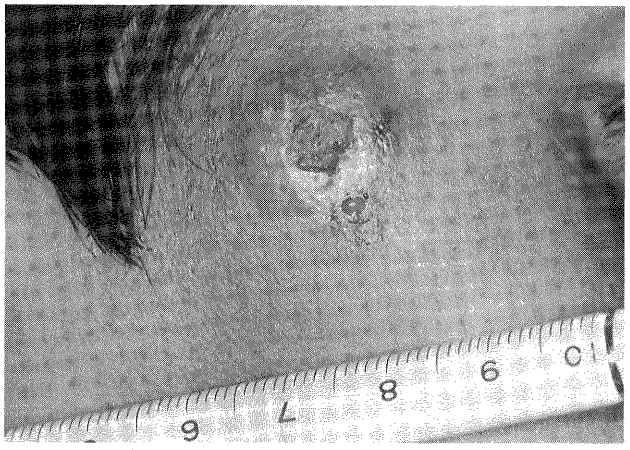

Figure 3 A cutaneous ulcerative lesion of urban type cutaneous leishmaniasis caused by *Leishmania tropica* on the face of a patient in Sanliurfa, Turkey.

ease is still highly endemic.

The southeastern region of Turkey is a hot and dry area. The southeastern anatolia project (GAP) is Turkey's largest ever integrated project. It was launched in 1981, covers a region of about 10% of Turkey's total area, and will affect at least 6 million people, 10% of Turkey's total population. Within this area 22 dums and 19 hydroelectric plants were constructed or are under construction on the Euphrates, the Tigris and their tributaries. The GAP bring about not only the socioeconomical development but also significant migratory changes in human, as well as ecology of animals, insects and parasites. These changes are threatening public health and leading an increase in the prevalence of parasitic diseases including leishmaniasis. According to the reports by the Turkish Ministry of Health, the number of leishmaniasis cases in Sanliurfa, which is the region's main city and has 200,000 population in the urban area, increased from 552 in 1990 to more than 3, 000 in 1996. Hyperendemicity was observed in certain quarters of the city around the river crossing. The type of the disease is urban type cutaneous leishmaniasis, in which the etiologic species is *L. tropica*. In Sanriurfa, elaborate investigations on reservoir hosts have been carried out. So far, however, none of the dogs and small rodents examined were found to be positive serologically and parasitologically suggesting anthroponotic transmission (Aksoy, 1995). Although *P. sergenti* is suspected to be a vector, the parasites have not yet been found from any species of sandflies. Sporadic cutaneous leishmaniasis caused by *L. tropica* has been also reported from Ege region and Mediterranean region.

Human visceral leishmaniasis is seen in western and southern parts of Turkey along the Aegean and Mediterranean coasts affecting mainly infants. Although the most cases are seen sporadically, there is an endemic focus in Manisa district of the Aegean region. The pathogens has been identified as *L. infantum* by isoenzyme and DNA analyses. Although sporadic cases were reported from other regions of Turkey, some of the cases were observed in clusters. Canine visceral leishmaniasis is more endemic in Turkey with the higher prevalence. In a study performed in the Aegean and Mediterranean regions of Turkey, 1.6% of 1,150 dogs were found to be seropositive and in another study, 7% of 478 dogs from 22 villages surrounding Manisa City were found to be seropositive with reaching a maximum positivity of 21% in some villages. The parasites isolated from dogs were also identified as *L. infantum*. There is no report on *Leishmania* promastigotes detected in sandflies, however.

Leishmaniasis is widespread in tropical, subtropical and temperate regions, where different combination of *Leishmania* species, vector sandflies, and reservoir animals exists. Humans are considered to be mostly accidental hosts, although in some places, e.g. India, the disease is anthroponotic. Hence epidemic features of leishmaniasis varied among areas and therefore control measures should be different. Careful and detail epidemiological investigations should be needed in advance to establish effective control measures against this disease.

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4 CLINICAL FINDINGS OF CUTANEOUS LEISHMANIASIS AND THEIR DIFFERENTIAL DIAGNOSIS IN ECUADOR

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Cutaneous leishmaniasis (CL) is a parasitic disease caused by various types of *Leishmania* spp. Man and domestic animals are infected by sandfly, a vector with *Leishmania* parasites.

Clinically, CL is divided into localized, generalized and diffuse types. CL show various cutaneous manifestations, such as papules, nodules, ulcers with elevated borders and erythematous plaques. In generalized cutaneous leishmaniasis (GCL), the eruptions are disseminated throughout the entire body surface (Lazo, 1994). In diffuse cutaneous leishmaniasis (DCL), which is associated with specific immunodeficiency against Leishmania, different clinical manifestations such as nodules, papules and erythematous plaques are observed throughout the entire body surface, with the exception of the scalp, axillary, inguinal, perineal and anal regions. Cutaneous manifestations in leishmaniasis are very similar to those of other infectious and skin diseases. Therefore, differential diagnosis between CL and other diseases including leprosy (Jopling, 1984) and deep mycosis is very important, especially in countries where these diseases are relatively common. In Ecuador, CL, except in its GCL and DLC forms, is clinically divided into highland type (Andean type) and lowland type. The highland type of CL, observed in the highland of the Andes Mountains where the temperature and moisture is relatively low, occures as milialy-to-pea-sized papules resembling insect bites and furuncles on the face and four extremities of children. The inflammation of the lesions is relatively minor compare to that of bacterial infection, although numerous Leishmania parasites are often detected within the lesions. It has been shown that, after a sandfly bite, the lesions gradually increas in size for several weeks. Most of the lesions heals spontaneously and form a small scar within half a year. The lowland type of CL, observed in the lowland with hot and humid forests, shows the above mentioned cutaneous changes, including ulcer with elevated border where induration is palpable at the margin. After the infection, the lesions gradually increase in size and form relatively large and deep ulcers. A portion of each lesion heals spontaneously in about one year and leaves a relatively large scar. The inflammation of the lesion is also minor. Therefore, the clinical symptoms of the lesions such as reddness and pressure pain, are much more minor than those of bacterial infection. As the positive rate of Leishmania parasites of this type is very small, diagnosis in the endemic areas of Ecuador has generally been performed by the history of the present illness and cutaneous changes of the patients. When bacterial infection is coexistent at the lesion site, the ulcers tend to become large and the lesions tend to endure longer. Therefore, disinfection of the lesion is very important for treatment. Though various fungi have been isolated from CL ulcers, their role in the ulceration is still obscure. Because CL lesions of the auricle and nose are likely to cause deformity, it is generally considered that lesions at these sites should be treated aggresively at the early stages of the disease. In the examination for CL in Ecuador, we saw many non-cutaneous leishmaniasis cases diagnosed as CL and treated using meglumine antimonate, Glucantine. In this symposium, we demonstrated mainly ulcerative skin lesions, observed at the Multin y Casa Hospital in Babahoyo city, Province of Los Rios, Ecuador. Those ulcerative skin lesions required differential diagnosis between CL and other diseases, as shown in the Table. Case 1 (No. 1 in the Table), a 44-year-old male. The thumb-sized ulcers on the legs were diagnosed as be ulcus cruris varicosum from the present history and the existence of typical varix on the lower extremities and atrophie blanch at the center of the ulcer. The induration was not palpable at the margin of the ulcer. Case 2 (No. 2), a 60-year-old female. The ulcers on the legs were relatively shallow without the elevated border. In addition, siderosclerosis was observed on the legs. The lesion was diagnosed to be ulcus cruris varicosum. Case 3 (No. 9), a 48-year-old male. The post traumatic lesion without elevated border on the leg was considered to be an ulcer caused by secondary bacterial infection. Case 4 (No. 10), a 17-year-old male. The ulcer on the right leg was diagnosed at the local hospital as CL ulcer. Even though the patient received 19 local injections of meglumine antimonate, he showed no improvement was

Table Cutaneous changes of non-cutaneous leishmaniasis cases observed in an endemic area in Ecuador (Multin y Casa Hospital, Babahoyo city, Province of Los Rios, 1994)

No.	Age	Sex	Diagnonis	Diagnosis performed in the past (mis-diagnosed)	Cutaneous changes	Site of lesions	Treatment performed in the past
1.	44	M	Ulcus cruris varicosum		ulcer, varix stasis dermatitis	rt. leg	
2.	60	F	Ulcus cruris varicosum		ulcer, varix stasis dermatitis	lt. leg	
3.	24	M	Ulcus cruris varicosum	Cutaneous leishmaniasis	ulcer, dark brownish pigmentation, scrach mark	legs	Glucantime (local inj.×5)
4.	63	M	Ulcus cruris varicosum		erosin, dark brownish pigmentation	leg	Glucantime (i.m.×14)
5.	72	F	Ulcus cruris varicosum	Cutaneous leishmaniasis	ulcer, varix induration	legs	Glucantime (i.m.×10)
6.	33	F	Leg ulcer		ulcer		
7.	45	M	Trauma	Skin tumor (excision)	ulcer	leg	
8.	48	M	Trauma		ulcer, dark brownish pigmentation	leg	
9.	17	M	Trauma+ secondary infection	Cutaneous leishmaniasis	ulcer, induration	rt. leg	Glucantime (local inj.×19)
10.	10	M	Impetigo contagiosum		crusted lesion, exudation, erosion	rt. leg	
11.	53	M	Furuncle?		furuncle-like lesion	cheek	
12.	60	M	Chromo-mycosis		verrucous nodule	dorsal aspect of rt. hand	
13.	1	F	contact dermatitis?	Cutaneous leishmaniasis	reddish papules, erythemas	lt. cheeck	Glucantime 2 m*l* (i.m.×5)
14.	20	F		Cutaneous leishmanisis	reddish papules, ulcer	lt. cheeck ~nose	Glucantime (i.m.×15)
15.	40	M	B.C.C		ulcer, black papules	nose	
16.	69	M	B.C.C	Mucocutaneous leishmaniasis	ulcer, black papules nodule	face, oral cavity	Operation, Glucantime (i.m.×30)

rt.; right, lt.; left, im.; intramuscular injection, local inj.; local injection, Glucantime; meglumine antimonate, ×; times of injection of meglumine antimonate. BCC; basal cell carcinoma.

found. This injection caused fibrosis around the lesion which was palpable as to be induration at the margin. Case 5 (No. 11), a 10-year-old male. Pea-sized shallow ulcers without the elevated border appeared on the lower extremities in about one week after the child was bit. Scratch marks were also observed on his lower extremites. The lesions were diagnosed as impetigo contagiosum. Case 6 (No. 13), a 60-year-old male. About one year prior to examination, the patient noticed a rice grain-sized scaly papule on the dorsal aspect of his right hand. The scaly papule gradually increased in size to form a superficial nodule. The nodule was non-pedunculated, with a nearly flat top, and was raised about 1 cm. The surface was rough and irregular with a cauliflower-like appearance. After various examinations, the lesion was determined to be a chromomycosis caused by Fonsecaea pedrosoi (type 4) (Hosokawa, *et al.*, 1997). Case 7 (No. 14), one-year-old female. The reddish papules and erythema were located on the left cheek. The skin lesion was diagnosed to be CL at a local hospital, and she received five, 5 m*l* intramuscular injections of meglumine antimonate for a total of 25 m*l*. Two weeks after the treatment had ended, no improvement was visible. Case 8 (No. 14), a 20-year-old female. The rice-grain-sized reddish papules and shallow ulcers were observed on the left cheek. Even though she received 15 intramuscular injections of meglumine antimonate, no improvement was observed. Case 9 (No.

15), a 40-year-old male. The shallow ulcer and black papules were strongly suspected to be basal cell carcinoma (BCC). Case 10 (No. 16), a 69-year-old male. The black papules and ulcers on the face were suspected to be relapsed BCC lesions. The maxilla was broken, and a metastatic lesion was observed at the oral cavity. At the site of the relapsed lesion, the patient received about 30 intramuscular injections of meglumine antimonate without any improvement. Case 11, a 22-year-old male. This case was observed in other city and not presented in the table. Six years prior to admittance at our clinic, he first noticed discrete papules on his left knee and right cheek; these began to increase in both numbers and size, and to spread over the entire body; papules slowly evolved into infiltrated plaques and nodules without any ulceration. Over the course of about two years, he received different treatments from different physicians, but without improvement; consequently, he decided to visit a dermatological dispensary in Guayaquil. At the dispensary, he was diagnosed with lepromatous leprosy and medicated for about 5 years. Based on the results of our examinations, however, he suffered from DCL (Reyna *et al.*, 1994; Hosokawa *et al.*, 1997). Case 12, a one-year-old female. This case was observed in the highland area of the Andes and not presented in the table. In this case, typical lesions of the highland type of CL, as well as insect bite-like and furuncle-like skin changes on the cheek were observed.

Based on the observations of cutaneous changes described above, special attention should be given to various infectious and non-infectious diseases, including skin carcinomas, for the differential diagnosis at the examination of patients with CL. Therefore, in order to ensure the accuracy of CL diagsosis, it is very important to consider the history of the present illness of the patient and examine the margin of ulcers by palpation; these steps are particularly important in which these diseases are endemic but parasitological and histological examinations are not available.

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5 MOLECULAR BIOLOGY OF DRUG RESISTANCE IN *LEISHMANIA*: GENE AMPLIFICATION AND P-GLYCOPROTEINS

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Drug resistance is a major obstacle to treatment and prevention of many infectious diseases caused by microorganisms, including parasitic protozoa. A better understanding of the molecular mechanisms of drug resistance is important for the design of more rational drugs and for the discovery of new drug targets of protozoan parasites (Borst and Ouellette, 1995; Ullman, 1995). Pentavalent antimonials such as sodium stibogluconate (pentostam) and meglumine antimoniate (glucantime) have been used for treatment of leishmaniasis. However, unresponsiveness to antimonials in leishmaniasis has long been recognized and is now becoming a gig problem in the endemic areas (Olliaro and Bryceson, 1993). Although the therapeutic failure may be explained in part by low immunological competence of the host, the emergence of drug resistant *Leishmania* has been demonstrated by drug sensitivity tests for clinical isolates *in vitro* (Grogl *et al.*, 1992; Faraut-Gambarelli *et al.*, 1997). Since the mode of action of pentavalent antimonials against *Leishmania* is poorly understood, little is known about the molecular mechanisms of antimony resistance in field isolates.

Gene amplification in drug-resistant *Leishmania*

In *Leishmania*, mechanisms of drug resistance have been investigated for promastigotes, usually selected by stepwise increasing concentrations of various drugs *in vitro*. Gene amplification is frequently observed in the drug selected promastigote lines. Overproduction of the target gene product by gene amplification is also known as a mechanism of drug resistance in mammalian cells (Stark and Wahl, 1984). Amplified DNA sequences appear as extrachromosomal circular and linear amplicons in *Leishmania*. The amplicons contain genes encoding enzymes, which are specifically inhibited by given drugs, and genes encoding membrane proteins related to mammalian P-glycoproteins or multidrug resistance (MDR) proteins (Beverley, 1991; Katakura and Ohtomo, 1995; Ouellette and Papadopoulou, 1993; Segovia, 1994) (Table 1).

Selection of wild-type promastigotes for resistance to methotrexate (MTX), an inhibitor of dihydrofolate reductase (DHFR), has lead to the first discovery of DNA amplification in *Leishmania*. The MTX-resistant *L. tropica* contained two different amplified DNA

Table 1 Gene amplification in drug-resistant *Leishmania*

Drugs inducing gene amplification	Sizes of amplified DNA in kb (region name)	Genes	Involvement of gene in drug resistance[a]	References
	Circular			
MTX, CB3717	30(R)	*DHFR-TS*	yes	2), 10)
MTX	36-87(H)	*PTR1*	yes	4), 27), 30)
arsenite	68-87(H)	*pgpA*	yes	20), 28)
tunicamycin	30-63(G)	*NAGT*	yes	21), 25)
DFMO	20?	*ODC*	ND[b]	unpublished
pentostam	100	*orfSbV*	yes	16)
vinblastine	40(V)	*MDR1*	yes	6), 15), 18)
daunomycin	30	*MDR1*	ND	5)
tubercidine	56	*orfTOR*	yes	7)
	Linear			
DFMO	140-230	*ODC*	ND	17), unpublished
mycophenolic acid	280	*IMPDH*	ND	34)
MTX	280-360(H)	*PTR1, pgpA*	ND	27)
arsenite	50	*gsh1*	no	13)
	Unknown			
glucantime	? (H)	*pgpA*	ND	9)

[a]confirmed by transfection
[b]ND, not determined

sequences, the R and H regions, as circular amplicons (Beverly *et al.*, 1984). The 30 kb amplified R circle contained the bifunctional *DHFR-TS* gene. The R region amplification was also observed in *Leishmania* resistant to CB3717, an inhibitor of thymidylate synthase (TS) (Garvey and Santi, 1986). In MTX-resistant *L. tropica*, co-existence of circular (~36 kb) and linear H DNA (280-340 kb) amplicons has been reported (Olmo *et al.*, 1995). Recently, it was suggested that circular amplicons were generated from linear amplicons in MTX-resistant *L. tarentolae* (Grondin *et al.*, 1998). The H DNA contained two different resistant genes, *pgpA* encoding P-glycoprotein A (Ouellette *et al.*, 1990) and *PTR1* encoding pteridine reductase (Papadopoulou *et al.*, 1992; Callahan and Beverley, 1992). The H region amplification was also observed in arsenite-, primaquine- and terbinafine-resistant *Leishmania* species as circular amplicons in a range of 68 to 170 kb in size (Beverley, 1991; Katakura and Chang, 1989; Ouellette *et al.*, 1990). Interestingly, amplified DNA sequences, which were hybridized to the *Leishmania tarentolae pgpA* (*ltpgpA*) gene, was observed in glucantime-resistant lines of *L.* (*V.*) *guyanensis* obtained through the one-step but not through stepwise drug selection (Ferreira-Pinto *et al.*, 1996). In some arsenite-resistant *L. tarentolae*, the *gsh1* gene, which encodes γ-glutamylcysteine synthetase, was amplified as part of a 50 kb linear amplicon or as part of a circular amplicon (Grondin *et al.*, 1997). Amplification of *MDR1* genes on extrachromosomal circular amplicons has been reported in stepwise selected vinblastine-resistant cell lines of *L. enriettii* and *L. amazonensis* (Chow *et al.*, 1993; Gueiros-Filho *et al.*, 1995). An MDR-like gene was also amplified in daunomycin-resistant *L. tropica* as extrachromosomal 30 kb circular amplicons (Chiquero *et al.*, 1998).

Several other drugs also induced amplification of specific loci. In *Leishmania* resistant to tunicamycin, an antibiotic that inhibits *N*-acetylglucosamine-1-phosphate transferase (NAGT), we found that the *NAGT* gene was exclusively amplified as extrachromosomal circular amplicons in a range of 30 to 63 kb in size (Katakura *et al.*, 1991; Liu and Chang, 1992). DL-alpha-difluoromethylornithine (DFMO) is an irreversible inhibitor of ornithine decarboxylase (ODC), and DFMO-resistant *Leishmania* contained extrachromosomal 140 kb linear DNAs, on which amplified copies of the *ODC* gene were located (Hanson *et al.*, 1992). We also found that both circular (~20 kb?) and linear (~230 kb) amplification of the *ODC* gene in DFMO-resistant *L. donovani*, in which linear amplicons appeared at lower drug concentrations and circular elements were generated at higher drug concentrations (unpublished). Mycophenolic acid inhibits the purine metabolism enzyme, inosinic acid dehydrogenase (IMPDH), and the *IMPDH* genes have been amplified as linear amplicons in *L. donovani* lines selected for resistance to mycophenolic acid (Wilson *et al.*, 1991). Toxic inosine analog such as tubercidin and inosine dialdehyde induced amplification of 56 kb region of DNA, on which an open reading frame named as the toxic nucleotide resistance gene (*TOR*) was mapped (Detke, 1997). Finally, circular amplicons were recently reported in *L. tarentolae* resistant to sodium stibogluconate (pentostam), and a novel open reading frame (*orfSbV*) coding for 770 amino acids was identified on the amplicons (Haimeur and Ouellette, 1998).

Recent progress on gene transfection and gene disruption techniques in *Leishmania* allowed us to verify the role of amplified genes in the drug resistance. The amplified gene was isolated and constructed into a expression vector. The vectors were transfected, usually by electroporation, into wild-type promastigotes to see if the gene can confer a drug resistance phenotype to transfected cells. Most of the genes described above were confirmed their involvement in drug resistance by transfection, although the levels of resistance were usually lower than those in drug-resistant cells established by drug selection (Table 1).

P-glycoprotein gene family and multidrug resistance in *Leishmania*

Multidrug resistance in mammalian tumor cells is often mediated by P-glycoprotein or MDR protein, which contains 12 transmembrane and two ATP binding domains, a member of the superfamily of ABC (ATP-binding cassette) transporters (Higgins, 1992). The first class of leishmanial P-glycoprotein gene is the *pgpA* gene, which is structurally more related to the human *MRP* (MDR-related) gene than the *MDR* genes. The *ltpgpA* in *L. tarentolae* and *lmpgpA* in *L. major* were isolated. The *ltpgpA* gene conferred resistance to oxyanions such as arsenite, trivalent antimony (SbIII) and pentavalent antimony (SbV). The *lmpgpA* gene, however, conferred resistance to arsenite and SbIII, but not to SbV (Borst and Ouellette, 1995). Since increased levels of glutathione and trypanothione are important for oxyanion resistance in *Leishmania*, the pgpA protein may recognize metals conjugated to thiols and transport the conjugates into intracellular compartments, displaying metal resistance by sequestration (Grondin *et al.*, 1997, Légaré *et al.*, 1997).

The second class of leishmanial P-glycoprotein gene was isolated as a homologue of the mammalian *MDR1* gene. Human MDR1 protein is known as an ATP-dependent efflux pump that transports various compounds, including steroids, toxic xenobiotics and endogenous metabolites (Gottesman and Pastan, 1993). The *ldmdr1* gene in *L. donovani* is 83% identical to the *lemdr1* gene in *L. enriettii* at the amino acid level. Gene transfection experiments demonstrated that both leishmanial *MDR1* genes can confer a low or middle level of resistance to vinblastine (Henderson *et al.*, 1992; Chow *et al.*, 1993). We have recently isolated *L. amazonensis LaMDR1* and *LaMDR2* genes by employing the PCR-based approach. A genomic library of the *L. amazonensis* DNA was screened with a 408 bp PCR product with primers designed for conserved sequences of ATP-binding motifs of various P-glycoprotein genes. Two open reading frames containing putative two ATP binding domains and 12 transmembrane domains were determined. The *LaMDR1* gene, encoding 1,341 amino acids, is 91% identical to the closely related ldmdr1 protein, revealing less conservation in the C-terminal than in the N-terminal transmembrane domains (Katakura *et al.* in press). The LaMDR1 protein, however, has no potential *N*-glycosylation sites at the extracellular region. Transfection experiments showed that the *LaMDR1* gene conferred resistance to vinblastine, doxorubicin and actinomycin D, but not to puromycin and colchicine. Since the *ldmdr1* gene conferred resistance to both vinblastine and puromycin (Henderson *et al.*, 1992), these results suggested the functional variation of MDR1 proteins among different *Leishmania* species.

On the other hand, the *LaMDR2* gene, encoding 1,267 amino acids, showed 46% and 29% identity to the LaMDR1 and ltpgpA protein, respectively (unpublished). The *LaMDR2* gene could not confer resistance to vinblastine or arsenite, suggesting that the *LaMDR2* gene is a new member of leishmanial *MDR* genes with a novel function.

Possible mechanisms of resistance to pentavalent antimonials

Pentavalent antimony (SbV) is likely to be converted to a more effective trivalent form (SbIII) in host cells. Although interaction of antimony with key sulfhydryl groups of leishmanial components may be a major mechanism of action and/or toxicity, resistance to antimony in *Leishmania* appears to be multifactorial with contributions by several independent mechanisms (Borst and Ouellette, 1995). Efflux systems and high levels of thiols were evident to be involved in antimony and arsenite resistance. The pgpA protein probably contributes to antimony resistance by transporting antimony conjugated to a thiol into an unknown subcellular compartment (Légaré *et al.*, 1997; Grondin *et al.*, 1997). In addition, a novel gene (*orfSbV*), showing no significant homology with sequences present in data banks, was isolated from pentostam-resistant *L. tarentolae*, and was responsible for a low level of resistance to pentostam, glucantime, antimony tartrate (SbIII) and arsenite (Haimeur and Ouellette, 1998). Further understanding of resistance mechanisms in laboratory strains is required to elucidate mechanisms of pentavalent antimony resistance in field strains from patients.

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6 THE ROLE OF HOST-DERIVED HOST-SHOCK PROTEIN IN PROTECTION AGAINST *LEISHMANIA MAJOR* INFECTION IN MICE

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Exposing cells to a variety of stressful conditions such as elevated temperature, chemical intoxication or infection leads to the transcription of a set of genes and, subsequently, to the synthesis of a family of polypeptide known as heat shock proteins (HSPs). These proteins have retained highly conserved animo acid sequences throughout evolution from prokaryotes to eukaryotes. Because HSPs are found commonly as immunogens within numerous microorganisms, these proteins are being studied in detail. However, in infectious states, HSPs are found both in the host and in microorganisms, and they are highly homologous, making it difficult to define their role in infection and immunity. HSPS may be essential for the adaptation of intracellular parasites to the harsh/hostile environment of hosts, and for transformation of microorganisms into infectious forms. The HSPs of parasite also function as immunodominant peptides that can be recognized by host humoral and cellular immune systems. On the other hand, HSPs synthesized by host cells as they respond to stress during certain infections may actually play a role in host defense. Thus, HSPs expressed by either the host or parasites may have the potential to modulate the host-parasite interaction.

We already proposed the ouoline of HSP65 involvement in murine toxoplasmosis. The expression of host-derived HSP65 appears to be crucial in directing the host-immune systems to achieve protective immunity against infection with *Toxoplasma gondii*. Alternatively, highly virulent *Toxoplasma* may escape from host-immune system by exerting a suppressive influence on the ability of $\gamma\delta$ T cells to induce HSP65 or by inhibiting HSP65 expression itself in/on macrophages.

In the present study with murine leishmaniasis, we found that HSP65 expressed in/on host macrophages also plays an important role in host protection. However, the expression mechanism was different from the system with toxoplasmosis as mentioned above.

As the first step, NKT cells instead of $\gamma\delta$ T cells was the primary effecters, which activate *Leishmania*-specific $CD4^+$ T cells by releasing cytokine such as interferon-γ and others. As the second step, those T cells induce the expression of HSP65 in/on host macrophages and this protein may contribute to host defense repairing the host-cell damage with endogenous cytotoxic agents like NO and superoxide.

Finally, $CD4^+$ T cells recognizing host-derived HSP65 activate the macrophages further and completely eliminate the parasites within those macrophages.

Symposium 2 DENGUE FEVER AND DENGUE HEMORRHAGIC FEVER

1 FIVE CASES OF DENGUE VIRUS INFECTION

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Abstract: We experienced five cases of dengue virus infection during the period of 1995-1996. Four were dengue hemorrhagic fever (DHF) and one was dengue fever (DF). Three of the 4 DHF patients were categorized as grade 1 and the other one was graded as grade 2 according to the standards set by WHO. Dengue type 2 virus was isolated from one DHF patient. Fever with other symptoms such as chills, vomiting, headache, general fatigue were presented in all patients. Hemostatic abnormalities were determined in all DHF patients but was absent in the DF case. All the 4 DHF patients were found to have thrombocytopenia (platelets, 12,000-100,000). Grade 2 DHF patient had already developed two different type of antibodies specific for dengue virus, whereas the DF patient had no antibody, thus signifying the severity of grade 2 DHF case.

INTRODUCTION

It is estimated that as many as 100 million dengue virus infections occur annually, principally in tropical and sub tropical areas inhabited by the mosquito vectors, *Aedes aegypti* and *Aedes albopictus*. In Japan we have no endemic dengue virus infection, so only very few physicians have the chance to examine dengue virus infection. With increasing numbers of international travel, physicians should be alert to the possibility of dengue fever when encountering febrile patients returning or coming from endemic countries. This report describes the clinical features and laboratory data from our conducted cases.

PATIENTS AND METHODS

Case records of 5 patients, who were admitted to the medical ward during the period of 1995 to 1996, with symptoms suggesting dengue virus infection were reviewed. A clinical definition of DHF established by the World Health Organization (WHO) is based on the presence of high continuous fever, hemorrhagic manifestations (including at least a positive tourniquet test), hepatomegaly, thrombocytopenia (platelet count $<100,000/mm^3$) and hemoconcentration (hematocrit

Table 1 Clinical features of study subjects at entry, tabulated according to final diagnosis

Case No	DHF1	DHF2	DHF3	DHF4	DF
(Age/Sex)	(35y/M)	(35y/F)	(14y/M)	(17y/F)	(51y/M)
Clinical details					
1. Fever: acute continuous	+	+	+	+	+
2. Hemostatic abnormalities	+	+	+	+	−
Hemorrhage	+	+	+	+	−
PLT ($10^4/mm^3$)	1.2	1.5	11.7	5.5	7.8
Tourniquet test	+	+	+	+	−
Hemoconcentration	+	+	+	+	−
3. Pain	+	+	Unknown	Unknown	+
4. General fatigue	+	+	+	+	+
5. WBC ($/mm^3$)	2,500	6,600	6,100	1,400	5,000

DHF, dengue hemorrhagic fever. DF, dengue fever.

increased by≧20% above baseline value).

RESULTS

Of the 5 subjects, 4 were diagnosed as DHF patients and one as DF patient.

These of the 4 DHF cases were categorized as grade 1 the other as grade 2 (case 1, in Table 1) on the basis of the standards set by WHO. Dengue type 2 virus was isolated from one DHF patient. Clinical details of patients are given in Table 1. Fever with other symptoms such as clills, vomiting, headache, general fatigue were complained by all patients. Hemostatic abnormalities were determined in all DHF patients but was not seen in the DF patient. Thrombocytopenia (platelets ranging from 12,000 to 100,000) was recorded in 4 patients.

DISCUSSION

Host immune responses are important in the pathogenesis of DHF, as the risk for DHF is increased at least 15- fold during secondary dengue virus infections. Further DHF1 had already two different type of antibody for specific dengue virus and has been categorized as grade 2, Therefore, severity may be corelated with the development of antibody as the DF case had no antibody.

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2 LABORATORY DIAGNOSIS OF IMPORTED DENGUE CASES

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Abstract: Dengue fever (DF) and dengue hemorrhagic fever (DHF) are major public health ploblems in tropical and sub-tropical countries. Dengue is not endemic in Japan, but there are imported dengue cases. We have performed laboratory diagnosis of dengue virus infection, using serum samples from suspected cases. We mainly carry out IgM-capture enzyme-linked immunosorbent assay (IgM capture-ELISA) and reverse transcriptase polymerase chain reaction (RT-PCR), along with immunochromatographic test and hemagglutination inhibition (HI) test. Dengue cases can be diagnosed by the combination of serological and molecular techniques.
Key words: dengue, imported case, RT-PCR, IgM capture-ELISA

Dengue virus infections are a major public health ploblem in tropical and sub-tropical countries. Dengue is not endemic in Japan today, but it is occasionally imported by travelers who visit tropical areas and become infected with dengue virus. Serum samples from patients with fever are occasionally brought to our laboratory for diagnosis of dengue virus infection. We examined serum specimens from 293 suspected cases from 1985 to 1998, and approximately half of these cases were confirmed to be infected with dengue viruses.

Laboratory diagnostic methods for dengue virus infection

We carry out IgM-capture enzyme-linked immunosorbent assay (IgM capture-ELISA), reverse transcriptase polymerase chain reaction (RT-PCR), immunochromatographic test and hemagglutination inhibition (HI) assay. We also attempt to isolate viruses using the mosquito cell line, C6/36.

HI test is conducted according to the method of Clarke and Casals (1958) using microtiter plates. IgM capture-ELISA is used to demonstrate dengue IgM in the serum samples. Four prototypes of dengue viruses type 1 (Hawaii), type 2 (New Guinea C), type 3 (H83) and type 4 (H241) are propagated in C6/36 cells for 5 days at 28°C or 31°C, and the supernatant fluids are used as the antigen. Presence of dengue virus-specific IgM and IgG is also examined using commercially available immunochromatographic test (Dengue Rapid Test, Pan-Bio Rty Ltd., Brisbane, Australia), according to the manufacture's instruction.

Reverse transcriptase polymerase chain reaction (RT-PCR) is performed as reported (Morita *et al.*, 1991) with a minor modification. RT and PCR are done sequentially in a single tube. The tubes are set in an oil bath type thermal programmer (Iwaki, Co., Tokyo, Japan) and subjeted to programmed incubation: at 53°C for 10 min for reverse transcription, followed by 30-40 PCR cycles of amplification. PCR products are then subjected to agarose gel electrophoresis. Amplified DNA fragments are visualized by ethidium bromide staining.

Virus isolations are done by inoculating the serum samples onto C6/36 cells in 24-well plates (Linbro, Flow Lab. Limit., Irvine, Scotland). Vero and monkey fetal lung (MFL) cells are used for plaque assays.

Detection of dengue viral genome by RT-PCR

Recent development in molecular biologic techniques, such as PCR, has promised to make rapid diagnosis of dengue virus infection. We have used RT-PCR for diagnosis of dengue virus infection since 1993. Viral genomes are usually detected by PCR in the specimen collected on days 2-5 after onset of fever in our study. Our results are consistant with the report by Morita *et al.* (1994) that viral genome was detected in the specimens by PCR with each dengue type-specific primers until days 3-5 after onset of the disease. Other group detected all four serotypes of dengue viruses by RT-PCR with the universal primers in clinical specimens with high sensitivity (Sudiro *et al.*, 1997). Figure 1 shows the results obtained using serum specimens from the patient NK. The patient was infected with dengue virus in Thailand or Vietnam. Serum specimens were collected on days 3, 7 and 10 after onset of fever. Dengue virus type 1-specific band (lane 1) was obtained using day 3 specimen. Dengue-1 viral genome was not,

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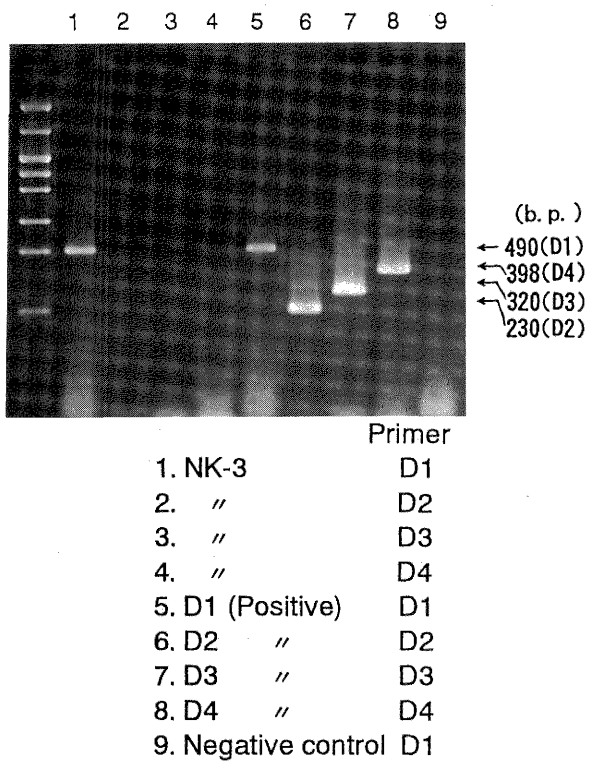

Figure 1 Results of RT-PCR using serum specimens from the patient NK.

however, detected in day 7 and day 10 specimens. The results using dengue virus types 2, 3 and 4-specific primer pairs were all negative.

Isolation of dengue viruses from clinical specimens

We usually attempt to isolate dengue viruses from clinical specimens using C6/36 cells. We put 50 μl of human serum on C6/36 cells and culture at 28°C for 7 days. Presence of viruses in the culture fluids is checked by plaque formation on Vero or MFL cells. We also examine the culture supernatants by RT-PCR. We sometimes detect virus genome in the culture supernatants by PCR when we can not detect dengue virus genome in the original sera. RT-PCR is a useful diagnostic method according to our experience. However, as described by Gubler (1998), it must be emphasized that RT-PCR should not be used as a substitute for virus isolation. The availability of virus isolates is important for characterizing virus strain differences, since the information obtained by isolating viruses is critical for viral surveillance and pathogenesis studies.

Detection of dengue virus-specific IgM by IgM capture-ELISA and a rapid immunochromatographic test

IgM capture- ELISA is the most widely used serologic test for diagnosis of dengue virus infection (Bundo and Igarashi, 1985). Serum samples from 89 suspected cases were examined by serologic tests in our laboratory in 1998, and 44 cases were positive. Dengue virus-specific IgM was detected by IgM capture-ELISA when serum samples were collected 7 days or later.

It has been reported that IgM responses are cross-reactive for 4 dengue serotypes, and that IgM capture-ELISA cannot be used to determine the infecting virus serotype (Gubler, 1998). We examined the relationship between the IgM levels to each of the 4 serotypes, and the dengue serotype determined by RT-PCR. We observed that the levels of IgM tended to be higher to the homologous serotype, compared to the levels to the heterologous serotype. Although IgM capture-ELISA is usually used for diagnosis of dengue virus infection without expecting the determination of serotype, IgM capture-ELISA may provide some information about the infecting serotype. Further studies, however, need to be done concerning serotype specificity of IgM responses determined by IgM capture-ELISA.

A number of commercial test kits for anti-dengue IgM and IgG antibodies have become available in the past few years (Sang *et al.*, 1998b, Kuno, 1998). We have used a commercial immunochromatographic test (PanBio; Australia). The results can be obtained in 5 minutes, and are generally consistent with those obtained by IgM capture-ELISA, as reported by other investigators (Sang *et al.*, 1998a, Vaughn *et al.*, 1998).

Combination of RT-PCR, and IgM capture-ELISA or rapid immunochromatographic test for diagnosis of dengue virus infection

There seem to be quite a number of imported dengue cases in Japan, although exact number of dengue patients is not known. Clinical observation is important for diagnosis; however, laboratory diagnosis is needed to confirm dengue virus infection. According to our experience, most Japanese dengue cases are primary dengue infection in the presence of immunity to Japanese encephalitis virus. Dengue virus genome can be detected in serum samples by RT-PCR until several days after the onset of fever. Detection of IgM by IgM capture-ELISA or a rapid immunochromatographic test provides positive results when serum specimens are obtained on day 7 or later. Thus, we recommend the combination of RT-PCR and IgM-detection for diagnosis. False negative can be avoided by attempting to check the presence of both dengue virus and specific IgM.

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3 BREEDING HABITATS OF THE DENGUE MOSQUITOES, *AEDES* (*STEGOMYIA*) SPP. IN THE COASTAL, AGRICULTURAL AND MOUNTAINOUS VILLAGES IN SOUTH SULAWESI, INDONESIA, 1994-1996

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The breeding habitats of the dengue vector mosquito, *Aedes aegypti* and *A. albopictus* were studied using larval collection method inside and outside houses in 2 coastal (Pancana and Lalolang), 1 plain (Lompo Riaja), 2 hill (Doi Doi and Bette) and 1 mount (Harapan) villages of Barru, (total population 150,000 in 52 villages) South Sulawesi, Indonesia from July 1994 to August 1995. And also seasonal appearances of these species were studied using ovitraps in and around houses in Ujungpandang and Barru, throughout the year from March 1995 to 1996.

A total of 615 houses in the 6 villages was examined and *Ae. aegypti* was the dominant species, being abundant indoors especially in the coastal areas. *Ae. albopictus* was breeding primarily in outdoor containers in the hill and mountain areas. Earthen jar was the most common breeding habitat of *Ae. aegypti* in all villages surveyed. Drum can was the most common outdoor breeding habitat of *Ae. albopictus* in the hill and mountain areas. Larval indices (house, container and Breteau) for the two species were high especially in coastal, but apparently low in hill and mount villages. The house index ranged from 8.3% in Harapan to 82.0% in Pancana, with a mean index of 59.5% for the 6 villages. Like the house index, the container and Breteau indices ranged from 2.6% and 8.3 in Harapan to 53.4% and 174.1 in Pancana, with the mean indices of 29.5% and 113.7 respectively.

The number of *Aedes* mosquito eggs, particularly in outdoor ovitraps, increased at the onset of the rainy season (November) in Barru, but increased mid rainy season (January) in Ujungpandang. In the dry season (June-October), the number of eggs decreased in Barru, but not in Ujungpandang. *Ae. aegypti* was dominant in both indoor and outdoor ovitraps in Barru in the dry season, while *Ae. albopictus* was dominant in both indoor and outdoor ovitraps throughout the year in Ujungpandang.

The high Breteau indices of *Ae. aegypti* suggests that this species may play an important role in the transmission of dengue hemorrhagic fever in coastal and plain villages, Barru where epidemics of the fever occur occasionally.

4 MOLECULAR AND *IN VITRO* ANALYSIS OF EIGHT DENGUE TYPE 2 VIRUSES ISOLATED FROM PATIENTS EXHIBITING DIFFERENT DISEASE SEVERITIES

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Potential genetic determinants of dengue virulence were studied by sequencing the entire genomes of eight dengue 2 virus strains isolated from patients exhibiting different disease severities during an epidemic season in northeastern Thailand in 1993. The isolates came from one dengue shock syndrome (ThNH-7/93), three dengue hemorrhagic fever and four dengue fever patients. Phylogenetic analysis showed that the isolates belonged to the Southeast Asian genotype. The 3' noncoding regions showed distinctive secondary structures with

one specific structure for the isolate ThNH-7/93. Analysis of the predicted polyprotein showed several amino acid changes scattered mostly in the non-structrual region. Of 30 positions with amino acid changes, 7 were unique to the isolate ThNH-7/93 and 3 of those led to radical alterations in amino acid character. Several amino acid changes coincided with previous studies relating genome sequence and virulence. Minimal changes in computer predicted protein secondary structures were observed. Infective particles in the inoculum for all isolates were approximately equal as measure by focus formation on BHK-21 cells, but this did not correlate with the number of plaques formed on LLC-MK2 cells. Isolates from patients that experienced secondary infections were shown to have significantly larger plaques than the isolates from primary infection patients.

5 DENGUE VIRUS-SPECIFIC T LYMPHOCYTE RESPONSES AND THE ROLE OF T LYMPHOCYTES IN THE PATHOGENESIS OF DENGUE HEMORRHAGIC FEVER

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Abstract: Pathogenesis of dengue hemorrhagic fever is one of the main subjects in dengue virus research. We hypothesize that rapid increase in the levels of cytokines and chemical mediators induces malfunction of vascular endothelial cells, and leads to plasma leakage and shock. The process may be initiated by antibody-dependent enhancement of dengue virus infection, and triggered by serotype-cross-reactive T lymphocytes.

Dengue viruses are the members of the family *Flaviviridae* and there are four serotypes, dengue virus types 1, 2, 3 and 4. The genome of dengue viruses consists of a single-stranded RNA nearly 11 Kb in length which is plus-stranded and infectious (Rice *et al.*, 1986). Dengue virus genome codes for three structural proteins, capsid (C), preM which is a precursor to membrane (M), and envelope (E), and seven non-structural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (Henchal and Putnak, 1990).

Dengue virus infections cause dengue fever (DF) and dengue hemorrhagic fever (DHF). Analysis of human T lymphocyte responses in dengue virus infection is important for understanding the mechanisms of recovery from dengue virus infection and the pathogenesis of DHF. It is especially important to determine the proteins recognized by dengue virus-specific T lymphocytes. This knowledge would also be useful for the development of dengue vaccines that induce protective immunity, but do not induce immune responses that may lead to immunopathology of DHF.

Dengue virus-specific human CD4$^+$CD8$^+$ T lymphocytes

Dengue virus infection induces dengue virus-specific CD4$^+$ T lymphocytes in humans. Dengue virus-specific CD4$^+$ T lymphocytes include dengue serotype-specific, dengue serotype-cross-reactive and flavivirus-cross-reactive T lymphocytes (Kurane *et al.*, 1989; Green *et al.*, 1993). Dengue virus-specific CD4$^+$ T lymphocytes are mainly Th 1 type and produce IFN-γ and IL-2. Proteins recognized by dengue virus-specific CD4$^+$ T lymphocytes have been determined using dengue virus-specific T lymphocyte clones. CD4$^+$ cytotoxic T lymphocyte (CTL) clones recognized NS3 protein (Kurane *et al.*, 1991a). These clones included serotype-specific, serotype-cross-reactive and flavivirus-cross-reactive CD4$^+$ CTL clones. Dengue virus-specific, human CD4$^+$ CTL clones established from other donors' PBMC recognized E, C and NS 1, 2a proteins (Livingston *et al.*, 1994; Gagnon *et al.*, 1996).

Dengue virus specific-human CD8$^+$CD4$^-$ T lymphocytes

Dengue virus-specific CD8$^+$ T lymphocytes also include dengue serotype-specific and dengue serotype-cross-reactive T lymphocytes. Dengue virus-specific CD8$^+$ T lymphocytes are cytotoxic. CD8$^+$ CTL were demonstrated in the PBMC of 7 of 8 donors who had received monovalent, live-attenuated experimental vaccines of the 4 dengue serotypes (Mathew *et al.*, 1996). NS3, NS1, 2a and E proteins were recognized by CD8$^+$ CTL from 6, 5 and 3 donors, respectively. CTL of these 7 donors recognized either NS3 or NS1, 2a. CTL from one donor recognized prM protein. CTL that recognized NS3 were mainly serotype-cross-reactive, while those which recognized NS1, 2a and E proteins were mainly serotype-specific.

Epidemiological and immunological studies that suggest immunopathology of DHF

Following primary infection, life-long immunity develops to protect against repeated infection by the homologous serotype of dengue viruses; however, protective immunity to the heterologous serotype is short-lived. It is known that antibodies to dengue viruses have ability to augment dengue virus infection of Fcγ receptor-positive cells such as monocytes (Kurane *et al.*, 1991b). On the basis of the epidemiological and laboratory studies, it has been hypothesized that antibodies to

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dengue viruses and other serotype-cross-reactive immune responses may contribute to the pathogenesis of DHF (Halstead, 1988).

We found that the levels of T cell activation markers, i.e. soluble IL-2 receptors, soluble CD4 and soluble CD8 were elevated in the serum samples of patients with DHF (Kurane *et al.*, 1991c). This suggests that T lymphocytes are highly activated in patients with DHF.

Possible immunopathological mechanisms of DHF

In secondary infection with dengue virus of a different serotype from that which caused primary infection, serotype-cross-reactive, non-neutralizing antibodies increase the number of dengue virus-infected monocytes by forming dengue virus-antibody complexes. Serotype-cross-reactive $CD4^+$ T lymphocytes are activated, and produce IFN-γ and IL-2. Increase in the number of dengue virus-infected monocytes and augmented expression of HLA class I and class II by IFN-γ facilitate the recognition of the epitopes on infected cells by dengue virus-specific T lymphocytes, and results in very high levels of T cell activation. Marked T cell activation results in the production of much higher levels of lymphokines.

IFN-γ-activated monocytes may release various kinds of cytokines upon infection with dengue viruses, or dengue virus-infected monocytes may release high levels of cytokines and chemical mediators as a result of lysis by dengue virus-specific $CD8^+$ and $CD4^+$ CTL and/or as a result of contact with virus-specific T lymphocytes. Furthermore, the production of cytokines and chemical mediators is induced by other cytokines. Therefore, once various cytokines are produced, the complex network further increases the production of cytokines and chemical mediators. These cycles could result in high levels of cytokines and chemical mediators in a short period of time. Cytokines not only induce the production of other cytokines, but also have synergistic effects.

We hypothesize that rapid increase in the levels of cytokines and chemical mediators induces malfunction of vascular endothelial cells, and leads to plasma leakage and shock (Kurane and Ennis, 1992, 1994). These processes appear to be initiated by antibody-dependent enhancement of dengue virus infection of monocytes, and are triggered by serotype-cross-reactive T lymphocytes. It is known, however, that some DHF patients do not have pre-existing dengue virus antibody and develop DHF during primary infection. Therefore, other factors such as virulence of dengue virus strains or genetic background of the hosts probably also contribute to the pathogenesis of DHF.

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6 CONSTRUCTION AND CHARACTERIZATION OF CHIMERIC JAPANESE ENCEPHALITIS/DENGUE TYPE 4 VIRUS

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The pre-membrane (prM) and the envelope (E) genes of a full-length cDNA clone of dengue type 4 (DEN4) virus (strain 814669) were replaced with the corresponding genes of a Japanese encephalitis (JE) virus (strain JaOH0566). An RNA transcript prepared from chimeric cDNA was used to transfect permissive mosquito cells (C6/36). The chimeric virus was recovered at five weeks after transfection, despite only a few viral-antigen positive cells were detectedat a week after transfection.

The viral proteins of the chimera were identified by PAP staining of infected cells with a panel of monoclonal antibodies (MAbs). The prM and E proteins derived from JE virus were detected, together with core (C) protein derived from DEN4 virus. However E proteins of DEN4 virus and non-structural proteins (NS1, NS3 and NS5) of JE virus were not detected. The chimera contained the antigenic epitope(s) that was (were) reactive with a panel of neutralizing Mabs by neutralization test.

Genomic RNA of the chimera was sequenced through the structural protein genes (E, prM/M and C) and the 5' non-cording region (NCR). Two cDNA clones (# 3 and # 7) from genome of chimera, original cDNA souse and intermediate clone of a chimeric cDNA were examined. A deletion between nts 76 and 81 in 5' NCR was observed in the all cDNA clones. An amino acid substitution of leucine for arginine at amino acid position 472 (Leu472Arg) of E protein was found in cDNA clones (# 3 and # 7) of chimera and intermediate cDNA clone, but not in original cDNA souse. A nucleotide substitution (G86C) and an amino acid substitution (Phe133Leu) have occurred during transfection of RNA and producing progeny virus, but no changes were detectedin C protein gene of chimera. The cDNA clone # 3 carried an amino acid change (His689Leu), but not in the clone # 7. On the other hand, clone # 7 has an amino acid change (Ala660Thr), but not in the clone # 3.

The growth property of the chimera was examined. The chimera was restricted in replication in Vero cells at 37°C, but grew to low titer in same cell line at 32°C and in C6/36 cells at 28°C. Also, the chimera exhibited mouse neurovirulence that significantly reduced compared with parental JE virus. Significantly, the chimera lacked neuroinvasiveness after intraperitoneal inoculation of Balb/c or C3H/he mice, but produced antibody by injection of infected cell lysate.

Symposium 3 HOW CAN WE CONTROL TROPICAL INFECTIOUS DISEASES IN THE TROPICS?

Session 1 Immediate reports from current project in selected areas 1 —PARASITIC DISEASES—

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Since most of parasitic infection do not cause life-threatening diseases, they are usually considered less problematic than bacterial or viral infections both for individual health and for public health. The DALY loss due to parasitic diseases other than Malaria in sub-Saharan Africa is only 1.8% of total loss which is much smaller than expected (World Bank, 1995).

When people living in a rural area in Guatemala, where parasitic diseases were prevalent, were asked what the most important disease is in the area, only 6.2% of the people provided parasitic diseases as most problematic ones in their village. This may be one of the factors which leads to the low participation rate in a control project of parasitic diseases.

Is it so unimportant to control of parasitic diseases?

For the control of parasitic diseases, non-specific control measures, which sometimes are almost neglected in the control of other infectious diseases, have been taken as a main control measure. Although non-specific measures such as safe water supply, health education and construction of toilets are very much effective also on the control of other infectious diseases, the impact is not necessarily taken into account. It must be recognized that the control of parasitic diseases with very low DALY loss also reduces the DALY loss by other infectious diseases.

The control of infectious diseases by non-specific measure should be revived.

2 LEPROSY

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INTRODUCTION

Is it really true to say that leprosy is the tropical disease ? Many ambiguities still remain in its etiology, and it is difficult to positively say that this disease has the characteristic factors that are only seen in the tropics. Actually, it is the disease of poverty, rather than of the tropics. However, nowadays the high prevalence rate of this disease is recognized in the developing countries in the tropics.

At the 44th WHO General Conference held in May, 1991, a resolution was made to attain the global elimination of leprosy as a public health problem by the year 2000. In another words, this resolution is meant to reduce the prevalence rate of leprosy under one per ten thousands population, and to recognize that this public health problem is solved. This was the epoch-making event. Since then, much efforts have been made and, statistically the leprosy prevalence rate has drastically fallen down. Furthermore, the WHO report of 1998 states that "Today, we can be confident that elimination (of leprosy) is within our reach" and "Viewed over all, the leprosy situation in the world gives every reason for satisfaction". Aside from the minor details, this analysis sounds essentially optimistic.

This paper is going to introduce the statistics of WHO report, as well as the leprosy condition in Bangladesh, and tries to discuss the possibilities of leprosy control in the tropics.

Characteristics of Leprosy

The definition of leprosy is as follows; "A chronic disabling disease of man, mainly attacking the nerves and skin, caused by the *Mycobacterium leprae*, mildly communicable, diagnosable, curably and of good prognosis, if recognized early and treated properly".

In leprosy, at its early stage, a patient is not much disturbed by any particular symptoms, and this is one of the diseases which is difficult to detect by the patient himself. It is especially vulnerable for the poor who are more concerned about their daily meal than their own health, making this tendency worse. For the early detection of the disease, the social education is very important for those people.

Historically and world wide, leprosy is one of the diseases which has been recognized with its strong prejudice and discrimination. Without wiping this stigma away, the early detection or elimination is not possible.

Another characteristic which makes the control difficult lies on leprosy inducing *M. leprae* itself: Its generation time is extremely long. Whereas the generation time of *M. tuberculosis* is 18 hours, *M. leprae* takes 12 days. The long incubation period makes eradication of the disease difficult. The artificial culture of the *M. leprae* has been impossible so far, and this is also one of the characteristics which makes the research hard.

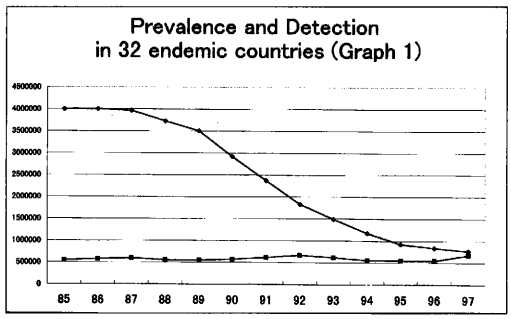
Since the past, trials have been made to eradicate leprosy. Four noticeable turning points in the history of leprosy control can be described as follows;

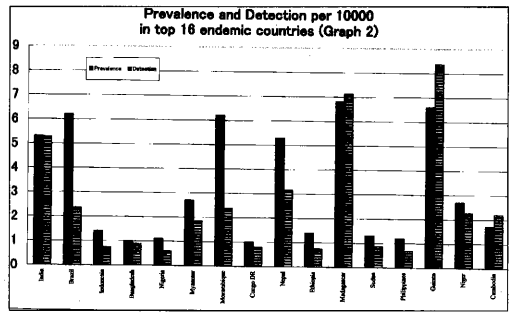
1. Discovery of *M. leprae*. This event precedes that of *M. tuberculosis* and many other bacteria.
2. Discovery of the first effective drug (Promin) in 1943. Discovery of this drug is not much behind in time from the discovery of the effective drugs of other infectious diseases.
3. In 1981, multi drug therapy (MDT) was recommended by the proposal of WHO's expert committee. This meeting was actually intended to discuss on DDS resistant control, but it played a significant role in expanding the MDT as worldwide standard therapy. Also, we cannot ignore the simplification of the classification which was recommended at the same time. It made the work possible for the field workers.
4. The epochal declaration by WHO in 1991, and the worldwide actions which follow, have been very effective, holding up the goal of leprosy elimination.

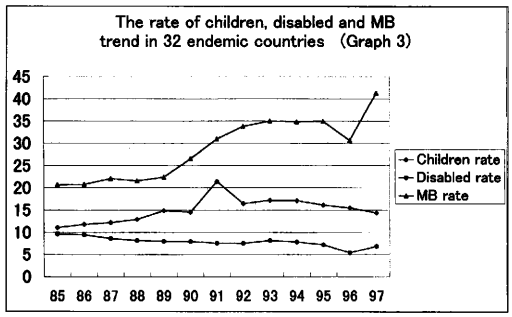
Leprosy in the World

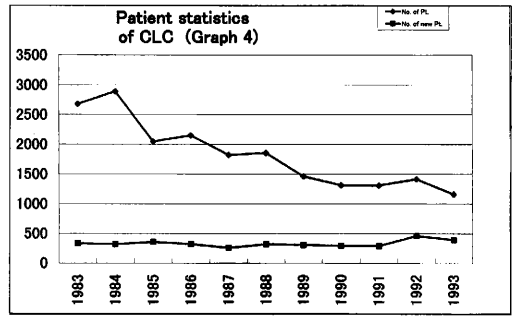
First, I want to introduce some statistics from WHO report. Graph 1 shows changes in the number of patients from 1985 to 1997 in 32 endemic countries. It is a joy to notice that the number of patients drops down from four million to less than one million.

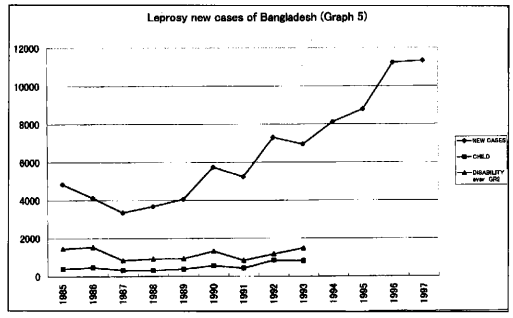
However, we must pay attention to that there was a change in definition of leprosy patient who can be counted for statistics. On the PB cases, no change has been added to the definition. But in the MB cases, the











definition of 1982 states a patient "more than two years and until bacterial test becomes negative". By this definition, many MB cases took more than two years for the treatment. Sometimes, it took six years. In the beginning of 1990s, by the instruction of WHO, the term is limited to "two years". The patient who has completed this period was no longer counted as a "patient". It is necessary to analyze actually how the number of patient decreased by this change of definition.

Graph 1 also shows change of number of newly discovered patients. For thirtten years, there is almost no changes. Much efforts surely have been made during this period for the early detection, and the fact is that, locally looking, some area does show distinctive improvement. However, looking around all over the world, it is difficult to see the distinctive decrease of new patients.

Graph 2 shows the comparison of number of patients and new patients per ten thousands population in the top 16 endemic countries. The rate of MB in these countries varies from 31.7% to 80.6%, and an analysis seems to be needed. Anyhow, there seems to be some contradictions which can not be understood between the number of newly discovered patients and prevalence.

Graph 3 shows changes of the MB rate of newly discovered patients in 32 countries, disabled rate of degree 2 by WHO disability table, and the rate of children during 13 years. In this graph, changes are shown by the rate, but essentially, as there is no change in the number of newly detected patients, changes are of the actual number.

Three points can be pulled out from these graphs

1) MB rate is increasing. Looking locally, MB rate increases as elimination proceeds. It may well said that this is a good trend. But it is necessary to keep in mind that definition of MB was changed by WHO during 13 years from "over than B.I.2" to "more than B.I.1". Also, it is likely that a field worker who experienced recurrent case becomes to feel psychological pressure and, being afraid of making the same mistakes, he tends to sift the diagnosis to more MB side.

2) Disability rate of WHO 2 degree seems to be surely decreasing. The effort for the early detection must be effective on this.

3) The children's rate does not decrease. In leprosy where the incubation period is long and the incidence rate can be hardly known, the children's rate itself can be the index of incidence rate. It is likely to think that no decline in the rate implicitly states some serious situation.

From the field in Bangladesh

From January 1985 to April 1994, I was engaged in a NGO leprosy project in south-east of Bangladesh where there are five district, first half of the term as the only medical doctor in the project and in later half as a senior medical officer given all responsibilities of the project, and training a native doctor at the same time. From what I experienced there, I would like to talk about the leprosy control work.

The area where our team covered extended as far as 28 thousands square kilometers where there lived more than ten million people. Our leprosy project with ten field workers was the only project in the area, and there was no countermeasure of the government.

Graph 4 indicates number of patients in our project. As MDT started in 1984, number of patients who were receiving treatment gradually decreased, but there is no signs of decrease in the number of newly discovered patients. Meanwhile we worked energetically to train volunteers, to share information to medical institutions, and to campaign leprosy education. However, in this vast area, with the small project as ours, we could not improve the condition up to where we could satisfy, that is to see the sign of an interception of the infections. By the time when I was to leave the project, we trained new field workers, and we were able to set up a new field control system, separated from the leprosy hospital. We needed this system to respond to the National Leprosy Elimination Project which, at last, started in fall 1993. By this time, the number of our field workers were about 50. But still we felt inadequate to do the control work in this large area.

The National Leprosy Elimination Project started in 1993 in Bangladesh as the very first National Leprosy Project. First it was necessary to divide the control area between government and NGO. As the government scheme, they decided to send a worker per one thana (small administrative area) hospital. Among 489 thanas in the nation, 57% of area was under government direct control. It was an urgent need to educate about 300 workers in a short time and to arrange them in the country. The government intended to achieve the control work in 5 years, and they hoped eagerly to send workers quickly throughout the nation. At first, they planned to do the training in 3 months, but because of lack of facilities and manpower for training, the plan was shortened to one month. After that, each candidate was alone assigned to the thana hospital. This elimination project started anyhow with a lot of uncertainties about workers' knowledge, skill, and motivation. Meantime, the NGO side worked together to educate about 20 students at once who were sent from projects all over the country, and in four months, they were to be ready to go back to each place assigned. They were to work a little longer with their senior workers for more experiences.

Though there was some unevenness over the region, this was the first control casting-net on all over Bangladesh. Graph 5 shows changes of number of newly detected patients in Bangladesh. A drastic increase can be seen just before and after 1994. This is the very effect of the National Leprosy Elimination Project which government and NGO jointly worked, each taking 57% and 43% of the land in the nation. This action can be highly evaluated. As a result of this effort, for the first time in history, prevalence reached a level of one person per ten thousands population. However, because this number coincides with the number of WHO goal, it was said that this plan would be ended successfully. Only when the prevalence increases more, and after that incidence decreases, if prevalence again comes down under the target point, then we will be able to consider that the plan have reached the goal.

Merits and demerits of WHO program

After the resolution at WHO conference in 1991, big efforts were paid under the leadership of WHO. It has brought great advancement. No one denies on this matter. At the same time, the resolution enabled to heighten international interest for leprosy elimination, and to accommodate necessary fund. Also, by the resolution, priority for the leprosy control was raised in the developing countries. Also it worked to encourage government's participation.

It was the WHO's expert committee held in 1981 that prepared WHO's resolution and enabled for it to pass. By this proposal, MDT was standardized, and the simplification of the classification enabled native field workers to work more effectively. As the result, leprosy control has become world-wide. These are the big steps in the work of leprosy control.

However, with the year of 2000 just ahead, there are a few things which weigh on my mind for the world wide

leprosy control. What I am concerned most is that, ironically, in many areas, leprosy elimination statistically seems to achieve its goal by the year of 2000. WHO confronts with many other problems also, and they seem to wish to draw the leprosy project as it attained its goal and a success.

I do appreciate the merits of WHO work, but wonder if the figures reported are the true reflection of the fact. A WHO report of the Leprosy Elimination Campaign (LEC) is told as follows; in India, during the concentrated LEC done in relatively short period, two million thirty thousands people were presented to a specialist with their suspicious symptoms, and four hundred twenty three thousands were diagnosed as leprosy. Also, in three towns in Myanmar where there were 330 leprosy patients and 227 newly detected patients in 1996, LEC was carried out for two months, and 311 new cases were found. This is truly the fruit of much efforts, but at the same time, this result seems to imply that as much cases are still left undiagnosed.

Also, my friends at work in the field report many stories. For example, in Niger, an area of two million people has been left out from the control work, or in Uttar Pradesh in India, 1,900 cases were found in 1997 in a small project carried only by catholic sisters without a doctor.

Coming to know these incidents, I feel that, with the target year of 2000 near at hand, unless information on what are left out in the leprosy control are correctly reported, there will be much problems later.

SOME ISSUES ON LEPROSY CONTROL AND QUESTIONS FOR THE FUTURE

As stated so far, still there are many problems left in order to conquer leprosy. As an imminent issue, it is said that in the year of 2000, leprosy control will be integrated as the object of usual local health system in the area where the elimination goal is achieved. But as stated before, in the early stage, leprosy itself does not give much discomfort for a patient, and hence it does not give a motivation to him to receive treatment voluntary. Also, it is only after the peripheral nerve damage progresses when anyone can diagnose, and at that point, he would not go and take treatment willingly. Moreover, because of discrimination in a society, he would be afraid to be diagnosed. For these reasons, there is a great possibility that this disease is likely to be neglected if one does not have a positive attitude to detect it. Many cases have been reported in which, by the local specialist's efforts, prevalence in that area increased. Would a local health worker who is very busily occupied can make this steady effort for early diagnosis ? More efforts will be needed to succeed in the integration of leprosy work, but who will take the responsibility for this efforts ?

Moreover in the international society, once the announcement is made that we are reaching the goal, interest for the control may be lost. If we are to face the era when acquiring of manpower and financial support is difficult, the distribution of free treatment drug may also become impossible.

In 21st century, when WHO has finished all the special efforts that have been made, who on the earth will be able to take a positive attitude for the leprosy control in the world, and who will take the leadership ? These issues are still enveloped in a fog. But some system in taking leadership must exist in order to carry out elimination, and eventually true eradication.

The most dreadful thing is that etiology of leprosy still leaves many uncertainties. Moreover, the present leprosy control rely too much on the effectiveness of MDT that have been observed, and at some point, there is an atmosphere that every problem has been solved.

Is the source of infection only limited to a man ? Is MDT presently used really all right as it is ? What is the mechanism of recurrent and how do we identify the risk group ? What is the mechanism of the tissue degeneration ? Is there any means to prevent and alleviate the peripheral nerve damage ? Above all things, is prevalence really decreasing in the world ? What is the method of leprosy control that can be effective to decrease incidence ?

Without answering these many basic questions, and without clarifying the etiology of leprosy itself, eradication of leprosy does not seem possible.

SUMMARY

The great advance has been made in the world leprosy control under the leadership of WHO. At many places, the goal of elimination has been achieved.

Despite of above fact, there seems no clear evidence that prevalence is decreasing in the world.

After the year of 2000, in order to link elimination with eradication, there are still many hurdles for us to jump over, such as of leadership, integration of leprosy control to the usual health system, and above all, elucidation of etiology which still has much obscurities.

ACKNOWLEDGMENT

I would like to give thanks to Professor S. Nonaka, the president of the Japanese Society of Tropical Medicine, also I wish to extend my thanks to the Chairman of symposium, Dr. N. Ishikawa and Dr. I. Arita for giving me a chance to present the situation of leprosy.

3 MALARIA—IF WE CAN CONTROL THE DISEASE

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Abstract: The 39th Annual Meeting of Japanese Society of Tropical Medicine (President of the meeting was Professor Shigeo Nonaka, Ryukyu University) held in Okinawa, focused on the control strategy of some tropical infectious diseases, and attempted to give chance of discussion in an important symposium organized and chaired by Dr. Isao Arita, Chairman of Agency for Cooperation in International Health, Dr. Nobukatsu Ishikawa, Vice-Director of the Research Institute of Tuberculosis, and Dr. Akira Igarashi, Professor of Institute of Tropical Medicine, Nagasaki University. This report summarizes one of the presentations in the syrposium spoken by the author on world malaria situation, emerging trends of malaria, as well as the prospect for the global malaria control into the 21st century.

INTRODUCTION

Malaria has been with man for thousands of years, however it was only towards the end of the last century that Dr. Charles Louis Alphonse Laveran discovered the malaria parasite and Sir Ronald Ross demonstrated that this parasite was transmitted by mosquito bites. Following these Nobel Prize-awarded discoveries, many researchers explored and proved how malaria was interwoven into the fabric of life in an extraordinarily varied and rich way (Litsios, 1996). Then, time had come to prepare a definitive statement concerning the principles and practice of malaria eradication by World Health Organization (WHO) after the 6th session of the WHO Expert Committee on Malaria held in Athens, June 1956. Despite a global effort aimed at its eradication, lasting practically from 1955 to 1969 and costing hundreds of millions of dollars, malaria was still with the people in tropical and sub-tropical areas. Dr. Marcolino Candau, Director-General of WHO at the time, said in January 1969, "The Organization had reached, where the malaria eradication program was concerned, a crucial moment in its history. It should be realistic and, while counting its success, it should not fail to recognize its failures" (Litsios, 1996). Now, malaria remains the most important of the tropical diseases, and in fact, it is re-emerging to areas where it has been once eradicated. Furthermore, controlling malaria is proving to be more and more difficult with increasing levels of drug and insecticide resistance of parasites and mosquitoes. It would be foolhardy for us to predict when and how malaria will be conquered.

WORLD EPIDEMIOLOGICAL SITUATION

Now, WHO's estimates of populations at risk within totalling 100 endemic countries and territories are 2,300 million in number or 41% of the world population. The incidence of malaria is 300-500 million clinical cases annually, and 1.5-2.7 million people die of malaria each year, including approximately 1 million deaths among children under 5 years of age (WHO, 1997). Severe malaria caused by *P. falciparum* (*P. f.*) is striking particularly young children, non-immune adults, and women during their first pregnancies.

The problem of the emergence of drug-resistant malaria and its worldwide expansion continued to intensify. Among the countries where falciparum malaria is endemic, only those in Central America and Egypt have not recorded chloroquine resistant *P. f.* malaria (WHO, 1997). Resistance to sulfadoxine/pyrimethamine is widespread in South-East Asia and South America. Mefloquine resistance is also widely reported in South-East Asia, and particularly in Thailand (Karbwang and Wernsdorfer, 1993). Multi-drug resistance including quinine resistance is a matter of great problem. Consequently, in certain areas, artemisinin and its derivatives are coming to be used for first-line treatment. Resistance of vivax malaria strains to chloroquine has also been confirmed in some Asian countries (WHO, 1997). Primaquine resistance of vivax strains in the hepatic stage is another topic which makes it hard to avoid the relapse of vivax malaria. Worsening drug resistance makes policy decisions on the use of antimalarial drugs increasingly difficult.

Emerging Trends

In fact, an expansion of the geographical areas susceptible to malaria transmission and a widespread increase of potential malaria risk are to be expected as the climate changes. As it is known, malaria incidence is determined by a variety of factors, particularly the abundance of anopheline mosquitoes, human behavior, and the presence of malaria parasites. Anthropogenic climate change directly affects the behavior and geographical distribution of the malaria mosquitoes and the life cycle of the parasite, and thus change the incidence of the disease. Indirectly, climate change could also have an effect on vegetation thus the availability of breeding sites. The large-scale migration and resettlement of populations from malaria endemic areas into receptive areas, a movement induced by rural impoverishment influenced by climatic change, will play an important role in the dynamics of the disease.

The re-emergence of malaria will also be associated with socio-economic development and expanding infrastructure, population growth and urbanization, natural disaster or civil unrest, and effectiveness of control measures (Aikawa *et al.*, 1998). Increased risk of malaria due to various environmental changes may seriously affect human health in the coming century worldwide.

The Burden of Malaria

Malaria was one of the very first diseases to be characterized as a "social disease". Sir Ronald Ross in 1911 proposed the calculation of the economic loss to the community caused by malaria, based on mortality and morbidity and "the local values of human life and labor" (Najera and Hempel, 1996). For example, estimates focus on days lost from work or output foregone, the loss of wages during sickness plus cost of treatments. World Bank (1993) measured the global burden of disease in units of disability-adjusted life years (DALYs), and estimated that 2.6% of the total world DALYs was from malaria. Of course, a man cannot be considered as an exchange value, nevertheless, calculations are thought to be necessary to justify funds. Because public health action against malaria often consisted of the implementation of large scale program which required the use of considerable public funds, the justification is needed for their political approval having either been fully based on economic arguments.

The burden on society was first measured in economic terms, such as lost productivity, and later, coming to include more general social values, such as learing ability and impact on education. It would be very difficult now to weigh the cost/effectiveness of the malaria control and its socio-economic impact as the social structure becomes more and more complicated.

Malaria Summit

The worsening malaria problems, as summarized above, led the WHO to convene a Ministerial Conference on Malaria in Amsterdam in October, 1992. Health leaders from 102 countries, as well as representatives from UN agencies and NGOs joined the conference, which endorsed a Global Malaria Control Strategy (GMCS). The goal of the GMCS was to prevent mortality and to reduce morbidity and social and economic loss due to disease through the progressive improvement and strengthening of local and national capabilities. The four basic technical elements of the strategy are:

- -to provide early diagnosis and prompt treatment;
- -to plan and implement selective and sustainable preventive measures including vector control;
- -to detect early, contain or prevent epidemics; and
- -to strengthen local capacities in basic and applied research to permit and promote the regular assessment of a country's malaria situation, in particular the ecological, social and economic determinants of the disease.

The Ministerial Conference was concluded by the adoption of a World Declaration on the Control of Malaria. The two major global objectives of the declaration was that, by the year 1997, at least 90% of countries affected by malaria implement appropriate malaria control programs, and that, by the year 2000, malaria mortality will have been reduced by at least 20% compared to 1995 in at least 75% of affected countries. To achieve this goal, it was estimated that WHO will require a total of just over 40 million US dollars through the period 1993-1999 in addition to its regular budget allocation to malaria control (WHO, 1993). In fact, WHO allocated extra funds for malaria (10 million US dollars between 1996 and 1997 and the same amount again from 1997 to 1998) to help countries develop plans for tackling malaria, training staff and initiating more effective action.

Roll Back Malaria

World malaria situation was proposed in discussion among the leaders of the G8 nations in Birmingham, UK,

1998, and remained as one of the issues on the agenda as part of the G8 development focus. The summit discussions, 3 days after Gro Harlem Brundtland's election, enabled the leaders of the G8 countries to offer strong support for the new WHO initiative, which is called the "Roll Back Malaria (RBM)" initiative, announced on 13 May 1998 by Brundtland herself as new director general of WHO. There was also a strong political commitment in affected countries (African Initiative on Malaria), and now, the research community (Multilateral Initiative on Malaria) is mobilized and the private sector (Medicines for Malaria Venture) is showing interest. RBM is not another attempt to eradicate malaria; instead, the aim will be to halve malaria-associated morlality by 2010 and again by 2015. The greatest emphasis of the initiative is to strengthen health services, so that effective treatment and prevention strategies are accessible to all who need them. It will apply every existing tool more effectively, building on experiences of the past—failures and successes alike (Nabarro and Tayler, 1998).

RBM was backed up by a financial commitment of £60 million by the British government to kick-start. Now, it is reported that the budget justification of RBM (July 1988-December 1999) is US$ 7 million for Direct Country Support, US$ 6 million for Resource Support Networks, US$ 4.5 million for Global R&D, US$ 1,870 thousand for Core Staff Support, and US$ 500 thousand for Operational Support (travel, consultants, meeting), totalling US$ 19.87 million (WHO, 1998). To achieve the above described goal, RBM will not be a "one-time" project, and indeed, sustained effort and financing will be required over the next two decades.

JAPAN'S ROLE

Japan was once a malaria endemic country. Indigenous malaria cases were observed even in Hokkaido in the late 19th century. The numbers of malaria cases gradually decreased; 200 thousand cases in 1903, but 13 thousand in 1945. When the second world war ended, about 430 thousand soldiers and civilians who returned to Japan from the war zones suffered from recurrent malaria. To counter this situation, malaria control programs were implemented across the country. As a result, along with subsequent economic development, the last indigenous malaria case in the main land of Japan was reported in 1959. Today, only imported malaria case (which exceeds 100 cases per year though) are reported. In Japan, malaria control measures were integrated into public health activities, such as health education and hygiene control, which employed the method of community participation and community empowerment. Along with the community, public institutions, parasitologists, and private organizations were involved in a coordinated manner (Aikawa *et al.*, 1998).

In recognizing the major role which parasite control can play in advancing public health and hygiene, the former prime misister, Mr. Ryutaro Hashimoto, pointed out its importance, and stressed the need for international cooperation in this area during the G8 Summit held in Denver, 1997. Upon the heels of this initiative was formed a working group on parasite control by the Government of Japan. Subsequently, teams of the working group members were dispatched to the developed countries as well as developing countries, in order to accumulate much information on how Japan can play a role in the global parasite control. As a result, a report entitled "The Global Parasite Control for the 21st Century" was published and distributed (Aikawa *et al.*, 1998). This report presented Prime Minister Hashimoto and the Japanese Government with important ideas to meet with the preparation for the next Summit held in Birmingham, 1998.

Recent Japan's overseas malaria control projects through Japan International Cooperation Agency are:

Solomon Islands Primary Health Care Promotion Project, Sep. 1991-Aug. 1996; Laos-Japan WHO Public Hygiene Promotion Project, Oct. 1992-Sep. 1997; Malawi Public Hygiene Project, Sep. 1994-Aug. 1999; and Zimbabwe Infectious Disease Control Project, Jul. 1996-Jun. 2001.

We believe that, if Japanese experiences and techniques are employed, and if the G8 and other developed countries join forces in organizing suitable malaria control programs according to the specific conditions of endemic countries, more effective control strategies will be by all means feasible.

ACKNOWLEDGMENTS

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Session 2 Global strategies for eradication or effective control of infectious diseases

4 GROBAL SURVEILLANCE AND NEW LAW REGARDING THE PREVENTION OF INFECTIOUS DISEASES AND MEDICAL TREATMENT GIVEN TO PATIENTS WITH INFECTIOUS DISEASES

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One hendred years have passed since the Infectious Diseases Prevention Law was established in 1897. Over the past century, medical science and treatment methods have progressed, hygiene standards have improved, and residents have become more aware of health and hygine issues. The situation has improved accordingly and incidents such as the death of over 10,000 people from cholera no longer occur. On the other hand, the prevalence of *E. coli* become social problem in Japan in 1997, while at the same time a new infectious diseases called Ebola hemorrhagic fever which had not been known previously, appeareded outside Japan. It is conceivable that such a disease could be brought into Japan, given the sheer volume of international exchange today. Also, despite the common belief that malaria would disappear in the near future it has reappeared and has once again become a problematic infectious disease. By their reappearance in society, infectious diseases are threatening people in a new way. Given the circumstances, we propose the establishment of a new law in order to promote comprehensive measures to prevent infectious diseases and improved medical care. The new law is to become effective on April 1, 1999, except for certain provisions.

One of the most important factor in new law is diseases surveillance. If a doctor diagnoses a patient as having an infectious disease notify the prefectural governor of information specified in Ministry of Health and Welfare. This surveillance system is composed of two parts. The one is to require the information of all patients diagnosed by the doctors. The number of infectious diseases in this system is about 40. The other is sentinel surveillance including about 30 infectious diseases. Furthermore, groval surveillance is essential for infectious diseases control measures. So under the new law, the national government is required to establish a system to promote the collection on infectious diseases, and make efforts to secure global cooperation.

5 CONTROL OF INFECTIOUS DISEASES —COMMUNITY APPROACHES IN DEVELOPING COUNTRIES—

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INTRODUCTION

Environmental changes such as climate change have a big influence on the emergence and reemergence of infectious diseases. Recent studies have identified an upward trend in global temperatures and now estimate a global mean temperature increase of 1-2°C by the year 2050.

How to meet the challenge of controling infectious diseases caused by global environmental changes is the most urgent question to be seriously explored, particularly taking into consideration of the mobilization and participation of people at the grass root level who make up almost 70% of the total population in the world.

Epidemiology

Concerns have been increasing about the relationship between environmental changes and the spread of infectious diseases in the world. For example, recent studies indicated that a global mean temperature increase of 1-2°C would enable mosquitoes to extend their range to new geographical areas, leading to increased epidemics of mosquito-borne diseases such as malaria and dengue fever.

It is reported that a temperature rise of 1-2°C by the year 2050 could result in an increase in the population at risk for Malaria, presently estimated at 2-4 billion people in the world of 45% to 60%. Similarly, in case of dengue, which currently threatens 1.8 billion people, infects 50 million people and causes 25,000 deaths annually, it is indicated that a temperature rise of 1-2°C could result in an increase in the at risk population of several hundred million, resulting in 20,000-30,000 more dengue deaths a year in 2050 (Table 1).

Table 1 Climate change* and risk of infectious disease

Disease	1998	2050
Malaria		
population at risk	2.4 billion	3.4-3.8 billion
Annual deaths	2-3 million	3.4-5 million
Dengue		
population at risk	1.8 billion	Over 2 billion
Annual deaths	25,000	30,000

*A temperature rise of 1-2°C by 2050

Over the past century, the average sea surface temperature has increased approximately 0.7°C and marine growth of algae has been observed responding to localized temperature increases in nutrient-replete waters. Zooplankton, which feed on algae, can serve as reservoirs for *Vibrio cholerae* and other enteric pathogens, particularly gram-negative rods. Large coastal blooms may have contributed to the recent multi-epicentered cholera pandemic in Latin America.

Control

For the control of infectious diseases, both preventive and curative measures are necessary. Preventive measures include areas such as environmental protection, vector control, immunizations education, behavioral changes, safe water and sanitation. For curative measures, development of simple and low-cost diagnosis methods and effective and low-cost anti-microbial drugs are important.

Development and implementation of even single one of the above measures is not easy. For example, effective policy and measures have not yet been accepted globally for the control of CO_2 emissions which has resulted in a global temperature rise.

Another example is a chronic shortage of financial and human resources for the control of the infectious diseases, particularly at the grass root level in many disease endemic countries. To cope with these problems, several efforts have been taken at the grass root level in developing countries.

Table 2 Disease vector control linked with income generating schemes

1) Composite Fish Culture -Ponds free from aquatic vegetation -Preventing vector mosquito breeding -Gain was Rs 5.8 million
2) Vector Control Linked with Agricultural Development Programme -Getting rid of noxious aquatic weeds -Growth alternative aquatic weeds (nitrogen rich and non -vector mosquito breeding) as a source of green manure for agricultural purposes -Rs 100 thousand for the community

Table 3 Health revenue in one commune in Vietnam

(in Million VND)

Source	1991	1992	1993	1994	1995	1996(1/2)	Total
Regular government allocation	0	0	0	0	11.30	11.30	22.60
User fees	0	3.40	5.60	6.20	7.40	n/a	22.60
Drug revolving fund	n/a	17.50	21.90	34.60	55.00	26.30	155.30
Community regular contribution	0	4.80	5.60	3.00	3.20	1.60	18.20
Donors	0	0	0	0	0	0	0
Others	0	0	0	0	0	0	0
Total	0	25.70	33.10	43.80	76.90	39.20	218.70

Community Approach

Infectious diseases have been endemic in many developing countries. In these countries 70%-80% of people live in rural areas where they have long suffered from disease as well as a shortage of resources to cope with problems caused by disease. Among many efforts so far taken for the control of infectious disease at the grass root level in these countries, several approaches have drawn attention. One was the control of Malaria and Filariasis by people in the rural community in Kerala State in India where vector control was linked to income generation such as fish culture and agricultural development (Table 2).

Another example was the development of community health fund project through the introduction of an essential drug revolving system to improve health services including community infectious disease control at a rural community in Vietnam (Table 3).

During the several years of the project, the success of community health fund project encouraged community people to challenge Japanese encephalitis and dengue control.

These examples provide an important precedent for people living in rural areas of developing countries to initiate and develop infectious disease control activities themselves.

CONCLUSION

By facing unprecedented changes taking place in the global climate and the consequent possibility of the introduction and dissemination of many infectious diseases, efforts to cope with these problems have been initiated both globally and locally.

Encouraging people to participate in these efforts at the grass roots level is quite important and several approaches have been proven to be workable.

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6 GLOBAL STRATEGY FOR ERADICABLE INFECTIOUS DISEASES

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Abstract: Initiation of a global eradication programme of selected infectious disease may be one of the major health activities of international interest in the 21st century. The article reviewed the current topics in this regard and discussed the conditions with which further eradication may succeed with reference to the experiences in smallpox and polio eradication programmes.

The 1980's WHO declaration of smallpox eradication dictated an important lesson, namely "humankind can eradicate a severe disease by mobilising world resources and modern technology" (Arita, 1979). The characteristics of eradicable diseases would be described as ① no animal reservoir ② no persistent infection ③ availability of effective control measures and ④ world interest for allowing mobilisation of world resources.

The lesson together with eradication efforts of other diseases was studied by two conferences, first the Dahlem Workshop in Berlin, in March 1997 and secondly, Conference of Eradication of Communicable Diseases in Atlanta in Feb. 1998. The latter Conference suggested diseases-eradicable as well as diseases of which transmission could be suppressed with effective control to the minimum so that the diseases will be no more threat to the community (Table 1), (Personal communication, Walter Dowdle). To me, effective control of lymphatic filariasis is of particular interest as it was prevalent in southern part of Japan there just four decades ago, but it now disappeared with effective surveillance and drug treatment.

Table 1 CDC Conference, February 1998

	Eradication	Effective Control
Bacterial Disease		
Congenital syphilis	−	+
Trachoma	−	+
Hib	−	+
Parasitic Diseases		
Dracunculiases	+	−
Onchocerciasis	−	+
Lymphatic filariasis	−	+
Chagas	−	+
Viral Diseases		
Polio	+	−
Measles	+	−
Rubella	+	−
Hepatitis A	+	−
Hepatitis B	−	+

Source: Walter Dowdle's personal comunication

These reviews have been reflections of recent and new interest of world community to promote the world consensus, namely if a disease is potentially eradicable, should we not proceed with implementation of such programmes.

Operational strategy for eradication should meet with certain conditions. It should last within 10-15 years with globally coordinated efforts in all the continents (Arita, 1998). Global surveillance should be the key that reveals effective method of stopping the transmission within above mentioned length of time. Otherwise, it can not be cost-effective, which is the major advantage of eradication effort. In principle the execution of global eradication should be financed by "additional" global resources, not obtaining the support from that of ongoing health programmes. These considerations lead to the necessity for intensive mobilisation of world resources for eradication, should it start.

The intensified smallpox eradication was launched in 1967 and succeeded to interrupt the transmission in three endemic continents, within 10 years, namely in Latin America in 1971, in Asian subcontinent in 1975 and in Africa in 1977. Global polio eradication was initiated in 1988. The transmission stopped in Americas in 1991 and East Asia in 1997. Global eradication is planned to be completed in 2000. In both programmes, the mobilisations of world resources have been adequate although polio eradication further requires resources since major foci still remain in Asian subcontinent as well as in Africa, South of Sahara.

In smallpox eradication, the continued surveillance discovered human monkeypox resembling smallpox infection. Extensive research revealed that the disease does not frustrate the eradication. In polio eradication, studies are underway to elucidate the behaviour of vaccine virus after the anticipated stoppage of polio vaccination. Thus, global eradication of any disease will need substantial research.

In conclusion, eradication effort will be one of most

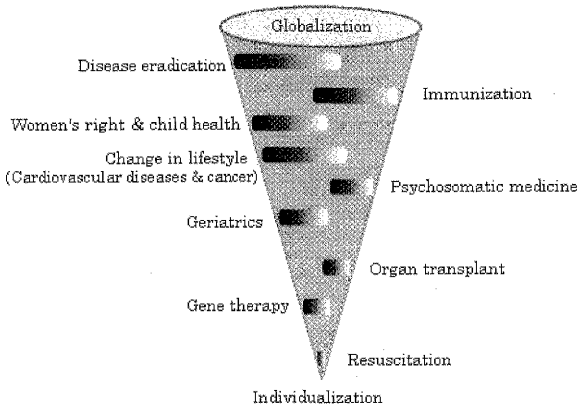

Figure 1 Global eradication is a logical consequence of the evolution of medical technology in the mainstream of the 21st century. Note this is contrasted by the revival in the trend of individualization on the other extreme end.

valuable human efforts to eradicate permanently human misery, but its initiation must be well prepared and conducted under a strong leadership. The failure of malaria eradication in 1970's was a good lesson as it lacked adequate scientific analysis of feasibility world-wide at the inception (Figure 1).

Toward the 21st century, it is hoped Japan would contribute significantly to this development.

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WORK SHOP: FOR MEANINGFUL INTRODUCTION OF ADVANCED TECHNOLOGY TO STUDIES OF MALARIA EPIDEMIOLOGY AND CONTROL

1 EVALUATION OF THE SINGLE-STEP SCREENING METHOD FOR QUICK DETECTION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G-6-PD) DEFICIENCY IN THE FIELD SURVEY

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Primaquine is known as the only effective gametocidal drug against *Plasmodium falciparum*. Glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is the most frequent hereditary enzyme abnormality of erythrocytes. The prevalence of the G-6-PD deficiency is higher in malaria endemic area. If the G-6-PD deficient individuals receive primaquine treatment, acute hemolytic attack may occur. Single step screening method (Hirono *et al.*, 1998) is a newly improved qualitative G-6-PD screening test which utilizes coloring of formazan formation on anion-exchange resin associated with enzyme reaction. G-6-PD deficient individuals are identified if their G-6-PD activity is less than 10% of normal enzyme activity. The color of the resin reached to plateau within 40 min at ambient temperature of 25°C. On the other hand, the resin with G-6-PD deficient samples colored slowly. Both of reagents and test tubes kept their reactivity in long- term storage (33°C, 14 days), high temperature (70°C, 6 hr), and continuous shaking (24 hr). In case of anemia, the G-6-PD deficient sample was verified from others within 20 min when the samples were diluted to 4 times (calculated hemoglobin concentration was 3 g/d*l*). High sensitivity against sunlight is the only problem for field survey. The reaction tubes turned to purple within 1.5 min under the direct sunlight in tropical area (Indonesia). We could keep its reactivity by keeping test tubes in a dark place or by covering with black plastic-bag until use. When test tubes are stored without MTT-PMS mixture, the test tubes could be kept without shielding. We made a test kit and succeeded to complete all procedure without any electric equipments in field survey. The result of Single-step screening method was well correlated with formazan-ring method in the field condition.

2 PROGUANIL POLYMORPHISM AND EFFICACY AGAINST MALARIA

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Variations in the human cytochrome P450 isoenzyme *CYP2C19* and the parasite *dihydrofolate reductase* (*dhfr*) genes, related to poor metabolism of proguanil to cycloguanil (a strong DHFR inhibitor) and resistance to cycloguanil respectively, have both been assumed to be associated with poor antimalarial effect by proguanil. To study this relevance, 95 subjects (median age, 7 years) with uncomplicated *Plasmodium falciparum* or *P. vivax* infections in Vanuatu, where we have recently found extremely high frequencies of *CYP2C19* mutations, received proguanil treatment for 3 days (adult relative dose of 300-500 mg/day) and were followed up for 28 days. Blood samples were collected on filter paper for determining human *CYP2C19* mutations, blood concentrations of proguanil/cycloguanil and parasite *dhfr* mutations. We observed a similarly high antimalarial efficacy of proguanil against both infections in 62 patients with *CYP2C19-related poor metabolizer (PM) genotype as in 33 with extensive metabolizer genotype, even though significant cycloguanil blood concentrations were not detecable in the PMs. Parasite genotyping revealed two dhfr* mutations (residues 59 and 108) in all 28 *P. falciparum* isolates tested, suggesting moderate resistance to cycloguanil. The results suggest that the parent compound proguanil has significant intrinsic efficacy against falciparum and vivax malaria independent of the metabolite cycloguanil. Proguanil represents an important antimalarial even in a population with a high frequency of the *CYP2C19-related PM genotype status.*

3 A NOVEL GENE CLONING OF OOKINETE SURFACE PROTEIN FROM PLASMODIUM VIVAX, AND POLYMORPHISM IN NATURAL PARASITE ISOLATES

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In many malarious regions outside of Africa, development of transmission-blocking vaccine will require activity against both *Plasmodium falciparum* and *P. vivax*. Work on *P. vivax* transmission-blocking vaccines has been hampered by the inability to clone the vaccine candidate genes from this parasite. To search for genes encoding the ookinete surface proteins from *P. vivax*, we aligned the gene sequences of the eight known proteins in P25 subfamily (Pfs25, Pgs25, Pys25, and Pbs25) and in P21/28 subfamily (Pfs28, Pgs28, Pys21, and Pbs21) and synthesized degenerate PCR oligonucleotides. We used genomic DNA, genomic library or splinkerette genomic library of *P. vivax* SalI as templates of PCR. To determine the polymorphism of Pvs25 and Pvs28, we used genomic DNA extracted from *P. vivax* isolates. Analysis of the amino acid sequence of Pvs28 revealed a secretory signal sequence, four EGF-like domains, a $(GSGGE/D)_6$ repeat and then a short hydrophobic region. Pvs28 is the presumed homologue of P21/28 subfamily member because the fourth EGF-like domain has four rather than six cysteines. Analysis of the deduced amino acid sequence of Pvs25 revealed a similar structure to Pvs28. The presence of six rather than four cysteines in the fourth EGF-like domain suggested that Pvs25 is the homologue of P25 subfamily member. Comparison of the primary structures of Pvs25, Pvs28, Pfs25 and Pfs28 obtained from field isolates suggested that there were some point mutation sites. In conclusion, we found two novel ookinete surface protein genes, Pvs28 and Pvs25 from *P. vivax* that are candidates of transmission-blocking vaccine. We also found some point mutation sites in Pvs25 and Pvs28 that result in antigenic polymorphisms.

4 IMMUNOGENETIC ANALYSIS OF THE PATIENTS WITH FALCIPARUM MALARIA IN THAILAND

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To investigate the host genetic factors affecting the clinical course of falciparum malaria, polymorphism of HLA-B, DRB1, TNF-α, promoter region, PECAM (CD31) gene were analyzed in the outpatients and hospital patients with different clinical severity of malaria.

Two hundred and 20 outpatients with positive blood smear of *P. falciparum* at Mae Sod malaria clinic, located at the border between Thailand and Myanmar, were examined for their CBC, Hb, Ht, and parasitemia. At the same time, physical examination including palpebral anemic change, bulbar icterus, and hepatosplenic palpitation was done by the same physician. After the diagnosis, all the subjects were immediately treated by anti-malarial drugs. We have also collected blood samples from 14 severe malaria patients at Mae Sod National Hospital. The nationality of the patients was Myanmars (115 from Karen, 105 from other Burmese). Thirteen cerebral malaria patients were also examined for their HLA-B alleles. The frequency of HLA-B*4601 significantly increased in the patients compared with the outpatients at Malaria clinic with mild symptoms ($Pc < 0.02$). TNF-α 5'-flanking region which is located within classIII region of HLA, was revealed to be polymorphic in -238, -308, -857, -863, -1031 positions from the initiation codon of TNF-α. In the patients there were at least 5 linkage groups and one of the type-857T, -863C, -1031T that had been detected to be a high producer of TNF-α increased in the cerebral malaria patients. The same kind of study was the Faculty of Tropical Medicine, Mahidol University. Although Thais are different ethnic groups from Myanmars, the same genotype of the TNF-promoter increased in the cerebral malaria group.

5 ANTIMALARIAL NATURAL PRODUCTS FROM *DICHROA FEBRIFUGA* (JOH-ZAN)

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A number of medicines such as chloroquine and quinine are available for treatment of malaria, but the rapid developrment of drug resistance is a serious problem. Medicinal agents based on novel mechanisms of action are, therefore, required to overcome emergence of resistance and to control an ever-increasing number of epidemics caused by the malaria parasite. As part of a multidisciplinary research program on antimalarial natural products, we are screening plant extracts that are alleged to have antimalarial activity. The methanol extract of the roots of *Dichroa febrifuga* (Japanese name: Joh-zan) shows high potency against *Plasmodium falciparum*. In China, the roots of *D. febrifuga* have been employed against malaria fevers, and no parasite which is resistant to *D. febrifuga* has been reported. Febrifugine and isofebrifugine were isolated as active principles against malaria, and febrifugine was once submitted to clinical test. However, it is told that this compound was never launched because of their serious adverse reactions, such as vomiting and diarrhea. To reexamine their activity and utility, the constituents of the methanol extract of *D. febrifuga* have been investigated in detail. As the results, powerful antimalarial compound with higher therapeutical selectivity than febrifugine was isolated. C-252, the composition of the roots of *D. febrifuga* shows high antimalarial activity (selective toxicity: 830) between chloroquine-sensitive and resistant strain of *P. falciparum in vitro*. This result suggests that C-252 could form the basis of a new antimalarial agent for the treatment of drug-resistant human malaria.

6 EXPRESSION OF CATALYTIC SUBUNITS OF MITOCHONDRIAL COMPLEX II FROM *PLASMODIUM FALCIPARUM* IN *ESCHERICHIA COLI*

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The progress in malaria vaccine development is rapid in recent years, but the schedule for the practical use of it is still rather vague. To make matters worse, chemotherapy of malaria using chloroquine and alternative compounds has been followed by the appearance of resistant parasites as shown in many epidemiological studies. The major reason for this vicious cycle is that the modes of action of many antimalarials are poorly understood. This lack of knowledge would be efficiently overcome by exploring the biology of *Plasmodium* in the asexual erythrocytic stage and focusing on preferential targets.

The morphological difference and the favorable effect of low O2 level on the *in vitro* cultivation of *Plasmodium falciparum* suggests that the physiological role of malarial mitochondria, including that in energy metabolism, is different from human host. The studies so far did not support the existence of complete TCA "cycle" in *Plasmodium*, but suggested the functional parasite electron transport system. For elucidating the role of this organelle, we have focused on complex II (succinate dehydrogenase, SDH) because it is exclusively mitochondrial enzyme and plays a unique role as a direct link between these two major mitochondrial systems. And furthermore, this enzyme functions as fumarate reductase (FRD), opposite to SDH, under anaerobic condition in some parasites such as adult *Ascaris suum*. Complex II is generally composed of four subunits, and we have already cloned the genes for two catalytic subunits (flavoprotein subunit: Fp and iron-sulfur subunit: Ip) from *P. falciparum*. Northern analysis and RT-PCR also demonstrated the expression of two genes at intraerythrocytic stage parasite.

The physiological function of the enzyme encoded by these senes should be clarified, but the protocol for preparing workable quantities of functional mitochondria has not been established yet. So, we planned to reconstruct parasite complex II in *E. coli*. DNA Fragments that encode putative mature region of each subunit of Fp/Ip were independently ligated into expression vector, and recombinant protein were successfully expressed. Antisera were raised against each recombinant protein and immunofluorescence assay on acetone-fixed *P. falciparum* and *P. yoelii* parasitized-erythrocytres demonstrated the expression of Ip, confirming the result of Northern analysis. Furthermore, the binding of prosthetic group flavin adenine dinucleotide (FAD), which is essential for enzymatical activity of complex II, to recombinant Fp was demonstrated using antibody against FAD. The results encouraged further effort toward the overall goal of our study, to clarify the role of parasite mitochondria and show targeting point for organized chemotherapy.

Panel presentation

1 COMPARATIVE ANALYSES OF NONSTRUCTURAL PROTEIN NS5 OF DENGUE VIRUS TYPE 1

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Dengue viruses (DEN) cause serious clinical manifestation. However, understanding of virus replication is still not enough. To clarify the biological activity of DEN nonstructural protein, we examined the difference in the expression and antigenicity between two DEN-1 strains, i.e. Mochizuki and A88 (Fujita *et al.*, 1997). The Mochizuki strain has been passed through mouse brains and subsequently through mosquito cell cultures for a number of generations, while the A88 strain isolated from the blood of DHF patient in Indonesia, has been cultivated in mosquito cell cultures for a number of generations.

Mochizuki and A88 viruses were cultivated in Vero and C6/36 cells. Virus reproduction was confirmed by RT-PCR, western blot and virus titration. The growth rate and yields of Mochizuki strain were higher than those of A88. Expression of DEN-1 nonstructural protein NS5 was compared between Mochizuki and A88 strains. By western blot analyses, Mochizuki strain NS5 was clearly detected at 3 days post infection, especially in Vero cells. On the other hand, A88 NS5 protein was not detected, although viral RNAs were detected by RT-PCR using DEN-1 specific primers. The nucleotide and amino acid sequence homology are 95% at the NS5 region. The results of western blot indicate that the antigenicity of Mochizuki NS5 is different from that of A88 NS5. The properties of virus replication seem to be related with biological activity of NS5. Further analyses are necessary.

2 A STUDY ON THE APPROACH OF THE USE OF INSECTICIDE AEROSOL CANS COMBINED WITH ACTIVE SURVEILIANCE FOR DENGUE CONTROL

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In dengue control, early case detection in sentinel health facilities, early warning system and prompt countermeasures of vector control have been considered essential. Concerning of early detection, a number of commercial kits for anti-dengue IgM and IgG antibody detection have become available in the past few years. On the contrary, there are few progresses in countermeasures for dengue control. WHO recommended the use of household aerosol insecticides as the personal protective measures. To evaluate the feasibility and the efficacy of new approach combining dengue active serological surveillance among febrile people with the provision of insecticide aerosol cans for neighborhoods of dengue patients, we had conducted the implementing study from February to December, 1997 in two communes (Binh Minh and Trang Bom) of southern Vietnam.

The insecticide aerosol cans were provided for the neighborhood of dengue patients in June, July, August, September and October in Binh Minh. The ultra low volume fogging of insecticide (usual approach in this district) were done around the patient's in March, May, July, August and September in Trang Bom as a control area. The serum samples of febrile cases collected in

local health facilities were 396 in Binh Minh and 758 in Trang Bom. The overall positive rate for dengue IgM antibody is 5.6% (22/396) in Binh Minh and 5.7% (43/758) in Trang Bom. The peak of positive rate and the number of IgM positive patients was August in Binh Minh (17 positive cases among 110 febrile patients and positive rate: 15.5%) and one month early in Trang Bom (21 positive cases among 137 febrile patients and positive rate: 15.3%) though zero case was positive in July in Binh Minh.

When we compared the number of dengue haemorrhagic fever patients who were graded as third and forth in WHO criteria, the total sixteen patients were reported in Binh Minh and forty three patients in Trang Bom in 1997. To compare the patients number of previous year, the number of cases in 1997 was 28.6% of previous year (56 cases) in Binh Minh, the number of case in 1997 was 48.3% of previous year (89 cases) in Trang Bom. The morbidity rate in Binh Minh was reduced dramatically from $413/10^5$ to $118/10^5$, and the morbidity rate in Trang Bom was also reduced from $789/10^5$ to $381/10^5$. The provision of insecticide aerosol cans cost five million and one hundred thousand Vietnamese Don (which was equivalent to 393 US dollars). That was lower than the cost of ultra low volume insecticide spraying which cost seven million and one hundred eighty thousand Vietnamese Don (which was equivalent to 553 US dollars).

Our study suggested that this new combined approach had an advantage in the cost effectiveness in dengue control (Acknowledgements: Dainihon Jochugiku Co., Ltd. for providing aerosol cans).

3 JAPANESE ENCEPHALITIS VIRUS RECOMBINANT NS5 PROTEIN EXPRESSED IN *ESCHERICHIA COLI* EXHIBITS RNA-DEPENDENT RNA POLYMERASE ACTIVITY

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Flavivirus genomic RNA is translated into a large polyprotein that is processed into structural and nonstructural proteins. Sequence analyses of flavivirus nonstructural protein NS5 suggests that NS5 protein is an RNA-dependent RNA polymerase (RdRp) with sequence motifs in the C-terminal region. Recently RdRp activity in recombinant NS5 protein of dengue type 1 virus and NS5B of hepatitis C virus (HCV) have been reported, confirming the predicted enzymatic activities. However RdRp activity of Japanese encephalitis virus (JEV) is still unknown.

To assess the RdRp activity of JEV, the complete and 2/3 of C-terminal NS5 gene both containing the putative RdRp motifs, were expressed in *Escherichia coli* as histidine-tagged protein and purified using metal affinity resin column. The purified recombinant proteins had a predicted molecular weight of 103 kDa (JEV-NS5F) and 65 kDa (JEV-NS5S) respectively. The complete form and not the N-terminal deleted mutant of NS5 protein possessed RdRp activity, indicating that protein integrity is required for RdRp activity of JEV NS5 protein.

4 CHARACTERIZATION OF POLIOVIRUSES ISOLATED FROM PAKISTANI CHILDREN II

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The World Health Organization (WHO) made the resolution to eradicate poliomyelitis from the earth by

the year 2000 in 1988. Although the number of reported polio cases has markedly decreased after the introduction of National Immunization Days (NIDs) in 1994, polio has been one of the indigenous infectious diseases in children in Pakistan. We tried to characterise the polioviruses isolated from poliomyelitis and diarrheal patients, 1995-1996.

Eighty-one stool specimens collected from patients were inoculated into Lα cells (devided from Prof. Nomoto, Institute of Medical Science, Univ. of Tokyo). Isolated polioviruses were identified using antisera to polioviruses and intratypically differentiated by the PCR-RFLP method; amplified PCR products were digested with restriction enzymes, DdeI, HpaII or HaeIII. The phylogenetic similarity of these sequences was compared using the UPGMA software packages.

Thirty-three poliovirus strains were isolated from 21 poliomyelitis and 11 diarrheal patients; 18 for type 1, 1 for type 2, and 14 for type 3. A mixture of type 1 and 2 was isolated from a diarrheal patient. Fifteen strains of type 1 and 13 strains of type 3 showed different RFLP patterns from that of Sabin 1 and 3, respectively. A type 2 and a type 3 strains were identical to Sabin strains. Three strains of type 1 did not identified by this method. We conducted to determine the nucleotide sequences of amplified genomic fragments of the wild poliovirus isolates. Twelve isolates of type 1 were classified into 3 distinct genotypes, and 12 type 3 isolates were divided into 2 genotypes.

In Pakistan, hundreds of polio cases were reported in 1995 and 1996. Our results show that almost of polioviruses isolated from polio cases were wild types and one polio case was associated with vaccine strain. Three or more genotypes of type 1 and 3 wild polioviruses were circulating in this area. For the eradication of wild poliovirus infection it is necessary to increase vaccine coverage in all areas of Pakistan and to identify infectious agents of polio-like and acute flaccid paralysis patients.

5 THE RISK OF HEPATITIS VIRUS INFECTION AMONG JAPANESE STAYING IN DEVELOPING COUNTRIES

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Hepatitis virus infection is common in Japanese staying in developing countries. We investigated the risk of the infection comparing antibodies to the viruses in Japanese serum before and after the stay.

Sera of 109 Japanese (Median age: 38.8 y.o., Average length of stay: 17.2 months) were obtained before and after the stay in developing countries (Asia: 79, Middle East: 5, Africa: 8, Latin America: 17). Antibodies to hepatitis A virus (total anti-HAV), to B core antigen (total anti-HBc), to C virus (total anti-HCV) and to E virus (IgG anti-HEV) were examined by EIA. Japanese who had the antibody before the stay or had vaccinations were excluded from the subjects of each virus (No. of subtracted subjects; HAV: 74, HBc: 79, HCV: 109, HEV: 109).

Five cases (6.8%) seroconverted to anti-HAV and one cases (0.9%) seroconverted to anti-HEV. The places of their stay were Asia (4) and Africa (1) in anti-HAV cases, and Asia in anti-HEV case. These cases did not have a history of hepatitis during the stay in the countries, and the liver functions were normal after the stay. Seroconversion to anti-HBc or anti-HCV was not detected.

Among hepatitis viruses, the risk of HAV and HEV infections, food and water-borne infections, are relatively high compared to HBV and HCV infections which are transmitted by parenteral exposure. Therefore, preventive measures such as the health education and vaccination should be provided for Japanese staying in developing countries with priority in HAV and HEV infections.

6 PREVALENCE OF HUMAN HERPESVIRUS 8 (HHV8) IN A YOUNG UGANDAN POPULATION

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We studied the seroprevalence and transmission of Kaposi's sarcoma associated herpesvirus (KSHV/HHV8), among 215 Ugandan children, adolescents and young adults. We measured antibodies to a latent nuclear antigen (LANA) and a lytic cycle protein encoded by orf 65. Infection with KSHV/HHV8 occured during early childhood and reached adult levels (40-50%) before the age of puberty. KSHV/HHV8 was not associated with the quality of the water supply, household size, previous blood transfusions, number of boy/girl friends or marital stasus. Transmission of KSHV among Ugandan children follows a horizontal pattern.

7 COMPARISON OF THE CYTOKINE INDUCTION LEVELS OF VEROTOXIN-PRODUCING *ESCHERICHIA COLI* ISOLATED FROM HUMAN, CATTLE AND SWINE

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Vero (Shiga-like) toxin-producing *Escherichia coli* (VTEC) strains are the predominant cause of hemorrhagic colitis and hemolytic uremic syndrome in humans. Verotoxins are thought to be associated with both the development of hemorrhagic colitis and the occurrence of systemic complications. However, in addition to Verotoxins, most VTEC strains have the ability to lyse erythrocytes (hemolysis). In this study, we focused on the actions of enterohemolysin (EntHly), a pore-forming cytotoxin, investigated the frequencies of EntHly in VTEC strains isolated from human, swine and cattle, and compared the *in vitro* cytokine responses induced by both EntHly$^+$ and EntHly$^-$ strains. We found that a marked increase in IL-1β production induced by EntHly-producing strains, and suggested that EntHly may play an important role in pathogenic mechanisms of VTEC infections.

A total of 160 human VTEC strains isolated from Japan in 1996 were investigated for production of EntHly. Twenty-three cattle and 11 swine VTEC strains were obtained from our culture collection. Hemolysin assays were performed as described previously (Bettelheim, 1995). Bacteria were grown on Tryptic Soy Agar base supplement with 10 mM $CaCl_2$ and 5% defibrinated sheep blood. PCR for detection of EntHly was performed for some VTEC strains. Primers 5′-GGTGCAGCAGAAAAAGTTGTAG-3′ and 5′-TCTCGCCTGATAGTGTTTGGTA-3′ were designed to amplify the EntHly gene. EntHly$^+$HB101 was derived by transformation with plasmid pF60:Tn1. This plasmid contains an EntHly determinant from F60 (O128, EntHly$^+$), a clinical isolate from a human VTEC infection. *E. coli* HB101 is a nonpathogenic human isolate (EntHly$^-$). Human monocytes were isolated from peripheral blood. Bacteria (VTEC, EntHly$^+$HB101, HB101 strains) from overnight cultures were inoculated into RPMI1640/10% FBS medium and brought into log-phase growth by culture for 5 hr, supernatants were harvested and sterile filtered. Approximately 10^5 monocytes were incubated with the supernatants for varied times. IL-1β and TNF α were assayed in cell culture supernatants with the use of ELISA kit.

(1) Most VTEC strains isolated from human (98%) and cattle (65%) were EntHly positive, the EntHly was plasmid-encoded. In contrast, all of the VTEC strains isolated from swine were EntHly negative. (2) An

increased IL-1β and TNFα levels were determined from monocytes stimulated by EntHly$^+$ VTEC culture supernatants. (3) EntHly$^+$ recombinant strain (EntHly$^+$ HB101) remarkably induced the IL-1β productions. In contrast, HB101 strain did not.

In conclusion, IL-1β, the cytokine which enhances the cytotoxic potency of Verotoxin toward human endothelial cells, was induced by EntHly-producing strains. EntHly may contribute to the development of VTEC infections.

8 THE ISOLATION FREQUENCY OF DIARRHEAGENIC BACTERIA IN VIENTIANE, LAOS

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The etiological agents of diarrhea in Vientiane, Laos, were studied in 1996 and 1997. A total of 880 diarrheal cases visiting medical facilities were examined for *Shigella, Salmonella, Escherichia coli, Vibrio, Aeromonas, Campylobacter* and rotavirus. *Shigella,* enterotoxigenic *E. coli* and enteropathogenic *E. coli* were found to be main causative organisms. The isolation frequency of *Shigella,* enterotoxigenic *E. coli* and enteropathogenic *E. coli* was 16.8% (148/880), 17.2% (111/645), 11% (97/880), respectively. *Salmonella, Campylobacter* and rotavirus were of relatively low frequency with 0.6% (5/880), 4.4% (39/880), and 6.1% (9/148), respectively. *Aeromonas* and *V. cholerae* were not found. *Shigella* strains isolated comprized 82 *S. flexneri,* 60 *S. sonnei,* 4 *S. boydii,* and 1 *S. dysenteriae* (not type 1). Almost all *Shigella* isolates were highly resistant to tetracycline, about 50% to ampicillin.

9 THROMBOMODULIN LEVELS IN PATIENTS WITH TYPHOID FEVER

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Serum thrombomodulin (TM) and creatinine levels were determined in 7 male Japanese febrile patients with typhoid fever and in 6 male Japanese healthy controls. The serum TM values of the patients and controls were 5.04±1.69 FU/m*l* and 2.93±0.74 FU/m*l*, respectively, and a significant difference was identified between them ($P<0.025$). The serum creatinine levels of the patients and controls were 0.93±0.16 mg/d*l* and 0.77±0.10 mg/d*l*, respectively, and the fact that no significant difference was identified between them may indicate that delayed clearance from kidney was not predominant as explanations for the elevated serum levels of TM. Although the numbers of patients and controls were small and more studies are waited, we found elevated serum TM levels during the course of a febrile S. serovar Typhi infection and serum TM levels may reflect the disease acitivity of typhoid fever.

10 IDENTIFICATION OF THE RECEPTOR FOR *AEROMONAS SOBRIA* HEMOLYSIN

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The motile *Aeromonas* species, particularly *A. hydrophila* and *A. sobria,* have been recognized as pathogens associated with acute gastroenteritis in both adults and children. Aerolysin secreted by the human pathogen *A. hydrophila* is one of the important virulence factor and is well characterized. Aerolysin is secreted as a dimeric inactive precursor which can be activated by proteolytic clevage of a C-terminal peptide. Both aerolysin and its protoxin interact with the target cells by binding to specific receptors. To date, all identified receptors for aerolysin were found to be GPI anchored. However, different receptors were found in different cell types and a given cell type was found to have more than one receptor. For example, aerolysin was shown to bind to Thy-1 on T-lymphocytes, to 47 kDa receptor on rat erythrocytes, and to 80 kDa receptor on baby hamster kidney cells.

There have been few studies on the toxins produced by *A. sobria,* and the biological properties of these toxins still remain unclear. *A. sobria* hemolysin is highly similar (68% at the amino acid level) to aerolysin. These toxins may have the same functional activities. Recently, Fujii *et al.* demonstrated that hemolysin is responsible for the diarrhea of *A. sobria,* because isogenic mutant strain lacking the hemolysin gene did not cause diarrhea. Accordingly, it is very important to clarify the receptor molecule for *A. sobria* hemolysin on the intestinal cells to understand the signal pathways that leads to diarrhea at the molecular level. Using immunoprecipitation method with hemolysin and its antibody, here we report that a cell surface glycoprotein (p66) with a molecular mass of 66 kDa can bind to hemolysin in intestine 407 cells, the human intestinal cell line.

11 THE TREATMENT OF LEPROSY; CURRENT SITUATION AND PROBLEMS

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Leprosy had been proved to be a really curable disease after WHO Study Group on Chemotherapy of Leprosy recommended MDT (multidrug therapy) as the most effective treatment in 1982. In 1992, the Member States of WHO declared their intention to eliminate leprosy as a public health problem by the year 2000. Elimination was defined as the reduction of prevalence to less than one case per 10,000 population. At the beginning of 1998, there were still 32 countries where the prevalence was beyond the global target. In 1997, WHO Expert Committee on Leprosy proposed that the duration of MDT for MB cases should be shortened to 12 months (from 24 months) and single dose of ROM (rifampicin+ofloxacin+minocycline) can be indicated for the cases of single patch. Although these proposals were approved at the 15th International Leprosy Congress in September 1998, the duration of treatment of leprosy has been controversial subject.

Through the comparison of the situation of leprosy in 2 countries that we studied, one is Morocco as the representative of low endemic country and the other is Myanmar as a well known endemic country, some of the

current global problems for leprosy control became clear. We also studied some of our cases retrospectively to consider about the appropriate duration of chemotherapy.

In Morocco, great reduction of prevalence of leprosy has been done during these 15 years, having appropriate regimen and enough support of NGO. In Myanmar, although the prevalence decreased rapidly after the implementation of MDT, there was no decrease of new cases. From our experiences, LL case with high Bacterial Index showed late reversal reactions even after 10 months from the completion of MDT.

For the success of Leprosy Control, enough duration of treatment and follow-up is necessary along with effective chemotherapy. In this aspect, Moroccan system is one of the ideals but this regimen might have not been afforded without great support of NGO. In Myanmar, there seems to be considerable amount of backlog (hidden) cases that are the suspicious sources of infection.

Since the MDT of 24 doses cannot be considered to be enough for LL cases from our experiences, we are concerning about possible relapse cases that may emerge more often after the shortening of MB regimen is introduced.

Leprosy, as a super chronic disease, needs long follow up with careful observation. Only after enough duration of follow-up, we can judge the efficiency of current regimen.

12 ROLE OF HUMAN POLYMORPHONUCLEAR NEUTROPHILS IN HOST DEFENCE AGAINST INFECTION WITH *PENICILLIUM MARNEFFEI*

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Penicillium marneffei is an important opportunistic fungal pathogen. Host defense mechanisms against *P. marneffei* are not fully understood. We investigated the fungicidal activity of human polymorphonuclear leukocytes (PMN) against *P. marneffei*. The yeast cells were cocultured *in vitro* with peripheral blood-derived PMN for 24 hr. Microscopic examination was also performed to examine the germination of yeast cells and their transformation to hyphal from during culture. Unstimulated PMN inhibited the growth of fungus only at a higher effector/target (E/T) ratio and the morphological change even at a lower E/T ratio. In further experiments, we examined the effects of various PMN-activating cytokines including granulocyte-macrophage colony stimulating factor (GM-CSF), G-CSF, interleukin (IL)-8, interferon (IFN)-γ and tumor necrosis factor (TNF)-α on their activity. Among these cytokines, GM-CSF, G-CSF and IFN-γ induced PMN fungicidal activity, but other ones did not or showed a marginal effect. In contrast, all these cytokines enhanced the inhibitory activity of PMN against the morphological changes of fungus. These antifungal activities were most strongly induced by treatment with GM-CSF. The combined use of any of the above cytokines failed to synergistically enhance antifungal PMN activity. Our results demonstrated that cytokine-activated PMN exert a significant antifungal activity by suppressing the growth and germination of *P. marneffei*. Our results suggest that PMN may contribute to host resistance to infection against this fungal pathogen.

13 THREE CASE REPORT OF INFECTION DUE TO *PENICILLIUM MARNEFFEI* WITH AIDS PATIENTS IN NORTHERN THAILAND

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We had a co-operative study about "Treatment of Acute Respiratory Infection in Thailand" from April, 1994 to March, 1997. And we are studying about "Treatment of Respiratory Infections with AIDS in Thailand". Disseminated infection with the fungus *Penicillium marneffei* is one of the most common opportunistic infections in AIDS patients in northern Thailand. We report 3 cases of infection due to *P. marneffei* with AIDS patients who were admitted to Nakorn Ping Hospital in Chaing Mai in order to treat community acquired pneumonia and review the literature. First case was 33 year-old male who complained of fever, cough and sputum. He was treated with penicillin G and gentamicin, but he died 2 days after admission. *P. marneffei* was detected from sputum in the day of his death. Second case was 24 year-old male with similar symptom. He was treated with antibiotics and amphotericin B, and his clinical course was good. *P. marneffei* was detected from blood and skin on admission. Third case was 31 year-old female with similar symptom. She was treated with antibiotics and antifungal therapy, and her clinical course was good. *P. marneffei* was detected from blood on admission.

14 STUDY ON THE SALIVARY GLAND PROTEINS OF MALARIA VECTOR MOSQUITO, *ANOPHELES STEPHENSI*

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During blood feeding, *Anopheles stephensi* extremely reduces the volume of the salivary glands. To observe the saliva consumption, we collected and dissected the salivary glands of *An. stephensi* before feeding, during probing, and after fully blood feeding. By the SDS-PAGE and Western blotting, several proteins, whose molecular size were 33, 35, 45 and 70 kDa, depleted after the blood feeding. Proteins in the salivary glands were consumed when mosquitoes were probing on mouse, and even when mosquitoes were taking blood into the midgut. Moreover, on feeding of *An. stephensi* infected with *Plasmodium berghei,* in prediuresis we found sporozoites which might come from the salivary gland of the mosquito and furthermore we detected the salivary protein, which molecular size were 90 kDa, from the midgut content of the mosquitoes after full blood meal. These results indicate that mosquitoes secret their saliva into the host and then take their own saliva into the midgut together with blood.

15 A FUNCTIONAL SINGLE-CHAIN FV DIRECTED TO A RODENT MALARIA PARASITE SPECIFICALLY INHIBITS THE DEVELOPMENT OF OOCYSTS IN MOSQUITOES

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Pbs21 is a membrane protein identified on the macrogamete, zygote, ookinete and oocyst stages of the rodent malaria parasite *Plasmodium berghei*. A monoclonal antibody (mAb), called 13.1, directed to Pbs21 has been reported to inhibit the development of oocysts in *Anopheles stephensi* mosquitoes.

1) To clone and express a single-chain antibody fragment (scFv) from 13.1 hybridoma cells and analyze its functional activities *in vitro*. 2) To determine whether the molecularly engineered scFv can block the malaria transmission *in vivo* (mouse model).

The V regions of heavy and light chains were cloned and linked by a sequence encoding for (Gly_4Ser_3) and expressed by a recombinant baculovirus. Following purification, the scFv was analyzed the binding activity by immunoblotting, inhibition ELISA and IFAT. Furthermore, the scFv was injected into mice intravenously and mosquitoes were allowed to feed blood from the mice. After 14 days, the mosquito midguts were dissected and the number of oocysts were counted.

Large amounts of the 13.1 scFv were produced in insect cells infected with the recombinant baculovirus. Following purification on a Ni affinity column, a single protein was eluted from the column, as determined by SDS-PAGE. Immunoblotting and inhibition ELISA revealed the 13.1 scFv exhibit similar binding properties as the parent 13.1 mAb. Finally, significant inhibition of oocyst development in the mosquito midguts was achieved with inoculation of 13.1 scFv.

Our data show that the 13.1 scFv produced by the *in vitro* baculovirus expression system fully retains the antigen binding properties and the functional properties of the parent 13.1 mAb. In addition, this functionally active scFv may be used as a model for generating a refractory mosquito against malaria parasites.

16 APPLICATION TO RESEARCHES AND DIAGNOSES OF A NEWLY DEVELOPED MALARIA PARASITE DETECTION METHOD BY FLOW CYTOMETRY

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Lack of effective vaccine and wide spread of drug resistance has still made malaria a serious public health concern. Basic studies aiming at development of vaccines and drugs will be more energetically pursued and in these studies *in vitro* and *in vivo* it will be required to analyse a lot of sample at the same time, detecting and counting parasites. It is also necessary to examine a plenty of blood samples for diagnosis purposing malaria control in the field. The conventional microscopic examination as golden standard to detect and count malaria parasites is a time consuming and rather subjective method depending on the technical ability of examiners. In this situation, we have developed a simple, sensitive and objective flow cytometric method to detect

and count parasites freed by a newly designed lysing solution and stained with acridine orange in cultured or patient blood samples. The parasite number analysed by this method correlated closely with parasitemia over 0.005% in cultured samples. Stage-specific parasites could be analysed differentially from one another. The effects of antimalarial drugs on parasite growth were examined by three methods (microscopic examination of thin smears stained by Giemsa, [H3] uptake study, and this flow cytometric method) and very similar dose-response curves were obtained. Seven malaria (*P. falciparum* or *P. vivax* infected) patient blood samples were also analysed by this method. Although parasites could be detected in all of the patient samples examined, the sensitivity in clinical blood samples was lower than that in cultured samples. We have attempted the device of the preparation of samples and the improvement of lysing solution to differentiate parasites from platelets to get satisfactory sensitivity for clinical application of this method. We have also tried to apply flow cytometry with imaging divice, which may be enable to differentiate four species of *Plasmodium* in this method.

17 INFLUENCE OF *SCHISTOSOMA MANNONI* INFECTION ON SUSCEPTIBILITY TO *PLASMODIUM CHABAUDI* IN MICE

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In mice and humans infected with *Schistosoma mansoni,* the polarization of helper T cell responses are induced to a parasite specific antigen. Several studies have demonstrated that *S. mansoni* infection changed not only immune responses but also the course of infection for other pathogens. Considering the fact that people in *S. mansoni* endemic areas often have multiple parasitic infections simultaneously, *S. mansoni* infection might affect the onset and development of other parasite diseases. In the present study, we infected mice with *S. mansoni,* and challenged it with *Plasmodium chabaudi* thereafter.

Mice were infected intraperitoneally (i.p.) with 10^6 *P. chabaudi* AS-parasitized erythrocytes (PRBC). Eight weeks prior to the *P. chabaudi* infection, some mice were infected with 25 cercariae of *S. mansoni.* In susceptible A/J mice, *S. mansoni* infection resulted in significantly decreased mortality, and parasitemias were reduced to subpatient levels 22 days after *P. chabaudi* infection. *In vitro* IL-10 and IFN-γ production for *P. chabaudi* antigen in spleen cells were significantly increased by *S. mansoni* infection, however, *S. mansoni* infection did not affect the level of NO metabolite, NO_3^-. In addition, serum and spleen cells from *S. mansoni* infected mice did not transfer the resistance against *P. chabaudi.*

Our findings suggest that *S. mansoni* infection provided susceptible mice with the resistance against *P. chabaudi* and influenced the cytokine production induced by *P. chabaudi.* Further analysis about the mechanism and effector of this resistance is in progress.

18 DETECTION OF SUBPATENT LEVEL OF MALARIA PARASITES BY NESTED POLYMERASE CHAIN REACTION (PCR) AND ITS CORRELATION WITH MICROSCOPY IN MYANMAR PATIENTS

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Microscopy has been an essential method for the diagnosis of malaria. Polymerase chain reaction (PCR) is an alternative approach used as a sensitive methodology for detection of malaria infection. In the present study, we applied a nested PCR method based on the amplification of small subunit ribosomal RNA (SSUrRNA) gene, to compare its sensitivity to that of routine microscopy among Myanmar malaria patients and to detect the subpatent level of parasitaemia. A total of 96 Myanmar male patients aged between 18 and 48 years admitted to Clinical Malaria Research Unit, Defence Services General Hospital in Yangon, Myanmar were randomly selected for collection of blood samples in January 1997 and May 1998. Patients came from different parts of malaria endemic areas in Myanmar where they started to have the initial symptoms related to malaria.

Microscopy and PCR methods revealed 74 and 82 cases of *Plasmodium falciparum,* 1 and 2 cases of *P. vivax,* 3 and 8 cases of *P. falciparum* and *P. vivax* mixed infection and 18 and 4 negative cases, respectively. Among negative cases, we could detect the parasite DNA after the parasitaemia in blood smear was negative for more than 5 days. This showed that nested PCR is useful and sensitive for the detection of the presence of subpatent level of parasitaemia in blood smear negative cases. In some cases, however, we did not detect the parasite DNA even within 4 days of treatment. Individual cases will be discussed.

19 CHEMOTHERAPEUTIC MALARIA CONTROL TRIAL WITH DIAGNOSIS KIT (ICF-PF) AND SINGLE STEP G6PD DEFICIENCY TEST IN THE SOLOMON ISLANDS

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Chloroquine has been commonly used for malaria mass treatment in the field study. However the analyses of epidemiological data and further mathematical model analyses that primaquine, which can effectively kill malaria gametocytes, should be involved to control malaria prevalence. However it is well known that hemolysis might occur when primaquine administered to patients who lack glucose 6-phosphate dehydrogenase (G6PD). Therefore G6PD deficiency test should be incorporated before primaquine treatment.

In this study we tried to diagnose falciparum malaria with commercial kit (ICT-Pf) and newly developed single step screening G6PD deficiency test before mass treatment with primaquine as well as chloroquine and Fansidar. We conducted the survey in malaria endemic area in Solomon Islands in January 1998. The study subjects were 392 local inhabitants and their mean age was 15.2 years old. Among them 293 were examined

by both ICT-Pf kit and microscopic examinations with Acridine Orange (AO) and Giemsa staining. The remaining 99 subjects were diagnosed only by microscopic examinations. Primaquine was not given in the case of G6PD deficiency.

ICT-Pf showed that 83 (28.3%) of 293 subjects were positive for *Plasmodium falciparum* (Pf). G6PD deficiency test showed that 29 (7.4%) of 392 subjects were G6PD deficient. The sensitivity and specificity of ICT-Pf kit were 0.85 and 0.82, respectively compared with Giemsa staining. Compared with AO test, the sensitivity and specificity of ICT-Pf kit were 0.85 and the 0.84, respectively. Anyone can easily diagnose Pf in a few minutes by ICT-Pf kit without any training. ICT-Pf kit is suitable for Pf diagnosis especially in the field. Furthermore we could detect the deficiency within 40 min by the newly developed G6PD deficiency test. Therefore we could rapidly treat malaria patients on the examination site with chloroquine and primaquine after the combination of these two rapid diagnosis methods.

20 CHANGE IN MALARIA VECTORS AND MALARIA SITUATION IN NORTHERN THAILAND DURING 30 YEARS FROM 1968

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To evaluate a long-term change in malaria and its vector situation, and to avoid missing and scattering valuable information in malaria epidemiological surveys and studies carried out by Office of Vector Borne Diseases Control 2 (previous Malaria Center 2) with collaborators in the past time, reformatting, computing and analysis of all available data of malaria concerned in northern Thailand have started. Firstly all readable stocked data at provincial, district and county malaria offices were photocopied, examined qualities, and decided the format for the original electric database, which was composed of 5 items (general back ground, vector control measurement, environmental conditions, malaria incidence and vector abundance) of 34 parameters. It was found that a total of 4,061 surveys had been completed for 30 years since 1968 in 13 provinces with more than 400 surveys in 7 provinces. Problems for analysis are: that about 20% of the data lacks key parameters such as the number of human baits and so on, that data before 1968 are too weak to analyze, and that the surveys had mainly been focused to problem areas, period, and recent 10 years. Nevertheless, a rough analysis based on 436 data sets of Maehongson Province, where malaria has been being serious, revealed clear temporal changes in vector and malaria situation suggesting recent natural and social environmental changes in the province. We believe the completion of this database and analysis is helpful to yield more reasonable and harmonious malaria control strategy under unstable environment with radical developing.

21 CLINICAL STUDY ON FIFTEEN CASE OF IMPORTED MALARIA

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Ten imported malaria patients admitted to our hospital and 5 to other hospital in Okinawa within last ten years were studied. These cases comprised 8 (53.3%) *Plasmodium vivax* (Pv), 6 (40.0%) *P. falciparum* (Pf) and one unidentified case. Eighty percent of these were in their 20s and 30s. Ten (66.7%) were Japanese and 5 (33.3%) were non-Japanese. Four of 8 Pv cases were contracted in Southeast Asia, 2 were in India and 2 were in Africa. Chroroquine or fansidar was used in the acute phase treatment of most Pv cases, and

then primaquine was used for radical cure. One Pv male case contracted in Indonesia was relatively resistant to conventional radical therapy with primaquine (15 mg daily for 14 days). He received mefloquine and radically cured by 2 cycles of primaquine 15 mg daily for 14 days. Three of 5 Pf cases were contracted in Africa and 2 were in Southeast Asia. Mefroquine or fansidar was used in most cases of Pf. In one case of chloroquine resistant Pf, fansidar was effective. One Pf male case contracted in Philippine developed severe cerebral malaria with renal failure and DIC. He was treated with intravenous qunine, mefroquine and fansidar, and needed hemodialysis and plasma exchange. In these management, he had a remarkable improvement within a month.

All 15 patients had a good improvement finally in this study, but drug resistance is becoming a problem also in imported case of malaria.

22 NINE CASES OF IMPORTED MALARIA IN KYUSHU; CLINICAL ANALYSIS OF DURING LAST ONE YEAR

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We experienced nine cases of imported malaria during last one year in the Kyushu district and contributed to treatment of these cases. Of these, seven were consulted for drug supply, and two were our cases.

These cases involved six tertian, two falciparum and one ovale malaria. Among consulted cases, sulfadoxine pyrimethamine was used for four cases, doxycycline for one, doxycycline and sulfadoxine pyrimethamine for one case, doxycycline and quinine for one case. Both of our cases were treated with mefloquine. Because sulfadoxine pyrimethamine was not effective enough for one tertian case and one falciparum case, the treatment was altered to quinine and minocycline, mefloquine, respectively. We supplied primaquine to six cases of tertian and ovale as well as mefloquine to one falciparum case. Orphan antimalarial drugs were not chosen for initial treatment for all consulted cases. There seemes to be an urgent need for immediate supply of these antimalarial drugs.

23 CYSTEIN PROTEINASE GENE AS A BASIS FOR DIFFERENTIATING *ENTAMOEBA HISTLYTICA* AND *ENTAMOEBA DISPAR* BY PCR

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The enteric protozoan parasite, *Entamoeba histolytica* is the causative agent of human amebiasis. It has long been known that although about 500 million people each year have amebiasis, only about 10% experience symptomatic disease. After much research and argument, it is now generally accepted that the previous classification *E. histolytica,* is now separated into two species: noninvasive *E. dispar* and potentially invasive *E. histolytica.*

A WHO-Pan American Health Organization-United Nations Educational, Scientific, and Cultural Organization Expert Panel recently recommended the development of improved methods, for the specific diagnosis of *E. histolytica* infection. To this end, we are able to design new sets of primers that uniquely identify *E. histolytica* from *E. dispar* based on the gene coding for

E. histolytica cysteine proteinase, an implicated virulence factor in the pathogenesis of amebiasis and plays a key role in tissue invasion and disruption of host defenses. Using WRG-1 (5'-CAGTTGATTGGAGAAGTATTATGAA-3') and WRG-2 (5'-CATGTTGTTGGAGTCTCTAGC-3') primer pair, a 570 bp nucleotide was amplified in both *E. histolytica* and *E. dispar*. On the other hand, a 755 bp nucleotide was amplified using WRG-3 (5'-TTCTAAAAACAATAAACACTTCA-3') and WRG-4 (5'-TTTCTAACTATCCAACATTCTT-3') primer pair for all *E. histolytica* reference strains but not in *E. dispar*. All DNA extracts from *E. histolytica* and *E. dispar* cysts collected from the Philippines are presently being tested against the new sets of primers. At the same time, the sequence of the amplified PCR product is being determined for the construction of a possible probe in the identification of pathogenic *Entamoeba*.

24 BACTERIAL EXPRESSION OF A HUMAN MONOCLONAL ANTIBODY FAB FRAGMENT RECOGNIZING AN *ENTAMOEBA HISTLYTICA* SURFACE ANTIGEN

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Despite the medical importance of *Entamoeba histolytica,* an effective vaccine to prevent amebiasis has not yet been developed. However, it has been reported that passive immunization with human anti-amebic antibodies obtained from patients with amebic liver abscess exerted a protective effect against amebic liver abscess formation in a mouse model. Therefore, such antibodies may be useful in attempts to reduce mortality from amebiasis by passive immunization. We have prepared recombinant human monoclonal antibody Fab fragments in *Escherichia coli.*

Lymphocytes were separated from the peripheral blood of a patient with an amebic liver abscess. Poly (A) RNA was isolated from the lymphocytes and then genes coding for the light chain and Fd region of the heavy chain were amplified by a reverse transcriptase-polymerase chain reaction. Amplified DNA fragments were ligated with a plasmid vector pFab1-His2 and introduced into *E. coli.* Bacterial colonies were transferred to nitrocellulose membranes and then screened for the production of antibodies by incubation with *E. histolytica* antigens followed by horseradish peroxidase-conjugated antibodies of the patient. One positive clone was selected for further analysis. The monoclonal Fab fragment reacted with 10 reference strains of *E. histolytica,* as shown by an indirect fluorescence antibody test using fixed trophozoites. In contrast, the antibody failed to react with other enteric protozoan parasites. Incubation of intact trophozoites with the antibody fragment localized the epitope on the cell surface. Western immunoblot analysis showed that the molecular mass of the *E. histolytica* antigen recognized by the monoclonal antibody Fab fragment was 260 kDa under nonreducing conditions. The Fab fragment significantly inhibited the adherence of *E. histolytica* to human erythrocytes.

25 DETECTION OF *ENTAMOEBA HISTLYTICA* -SPECIFIC ANTIGEN BY SANDWICH ELISA

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Entamoeba histolytica and *Entamoeba dispar* are morphologically inseparable, but only *E. histolytica* is responsible for invasive amebiasis. Therefore, distinguishing between *E. histolytica* and *E. dispar* is important for clinical and epidemiological reasons. We have prepared several antibodies specific for *E. histolytica*

and/or *E. dispar*. We tried to detect *E. histolytica* antigen(s) by sandwich enzyme-linked immunosorbent assay (ELISA) using an *E. histolytica*-specific monoclonal antibody. The wells of ELISA plates were coated with rabbit polyclonal antibody to *E. histolytica*. Various concentrations of *E. histolytica* antigen were added, incubated at 23°C for 1 hr, then washed with phosphate buffered saline (PBS) containing Tween 20. The plates were then treated with horseradish peroxidase-labelled monoclonal antibody and substrate. When the antigen was suspended in PBS, 25 trophozoites per well were detected by the system. Even if the antigen was mixed with feces, 50 trophozoites per well could be detected. On the other hand, when *E. dispar, Giardia intestinalis,* and *Blastocystis hominis* were used as antigen at a concentration of 1,000 per well, positive reactions did not occur. The sandwich ELISA system using the monoclonal antibody seems to be useful for the detection of *E. histolytica*.

26 DNA POLYMERASE ACTIVITY IN ENCYSTING *ENTAMOEBA INVADENS*

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There is no axenic encystation system for *Entamoeba histolytica*. In this respect *E. invadens,* a parasitic protozoan of reptiles, is a useful model for *E. histolytica,* because it resembles the human pathogen in morphology and life cycle and encysts *in vitro*. Using this model system, we examined the level of DNA polymerase activity of *E. invadens* during encystation induced *in vitro*. We first characterized DNA polymerase activity of trophozoites of *E. invadens* comparing it with that of *E. histolytica* and found that the activity of *E. invadens* was lower than that of *E. histolytica* at pH 2, 4 and 6, and was higher at pH 8 and 10. The activity of *E. invadens* was completely inhibited by high concentrations of K^+. Among inhibitors of mammalian DNA polymerases, aphidicolin and *N*-ethylmaleimide inhibited the activity, but 2′, 3′-dideoxythymidine-5′-triphosphate did not. Thus sensitivity of the *E. invadens* activity to salt and inhibitors of mammalian DNA polymerases was basically the same as that of *E. histolytica* in our previous results. The level of DNA polymerase activity of cysts decreased as encystation proceeded when compared with that of trophozoites. Thus the results indicate that encystation is accompanied by a reduced level of DNA polymerase activity so that this correlates to the previous report that two nuclear divisions during cyst maturation occur without DNA synthesis.

27 INHIBITORY EFFECT OF APHIDICOLIN ON GROWTH AND ENCYSTATION OF *ENTAMOEBA INVADENS*

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Aphidicolin, a mycotoxin, is a specific inhibitor of eukaryotic nuclear replicative DNA polymerases and treatment with this drug accumulates the cells at the G1/S border. We previously reported that aphidicolin inhibited DNA polymerase activity and growth of *Entamoeba histolytica* trophozoites. Because no axenic culture medium which induces encystation is available for *E. histolytica, E. invadens,* a parasitic amoeba of

reptiles, which can encyst in axenic condition, is a useful model for encystation of *E. histolytica*. The present study was conducted to determine whether encystation as well as growth of *E. invadens* is affected by aphidicolin. Trophozoites of IP-1 strain of *E. invadens* were axenically cultured in BI-S-33 medium and encystation was induced by glucose depletion and reduction of osmotic pressure using 47% LG medium. The growth of *E. invadens* was inhibited by aphidicolin in a dose-dependent manner. Encystation was also inhibited by aphidicolin in a dose-dependent manner. Encystation was intensely inhibited when trophozoites were cultured with aphidicolin before being transferred to encystation medium containing the drug compared with that without the pretreatment. The inhibitory effect of aphidicolin on growth and encystation was reversible because these were restored when the drug was removed by replacing with drug-free medium. These results indicate the reversible inhibitory effect of aphidicolin on growth and encystation of *E. invadens*, and suggest the importance of DNA synthesis before the onset of encystation.

28 SEROEPIDEMIOLOGICAL STUDY OF ENTOAMOEBIASIS USING MICRO-CHEMILUMINESCENCE ELISA IN NEPAL

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Entamoebiasis histolytica is a worldwide distributed disease, especially in tropical and subtropical regions. However, few sero-epidemiologic researches of this disease has been reported in Nepal, so far. In this study, we measured the specific antibody of *Entamoeba histolytica* in the sera of healthy Nepalese by micro-chemiluminescence enzyme-linked immunosorbent assay (micro-chemiluminescence ELISA), and analyzed the sero-positive rate.

Test sera were collected from two hundred and twenty Nepalese, aged sixteen through eighty-four in the capital, Kathmandu, and in a village of central highland, Dzong in March, 1997. The method of micro-chemiluminescence ELISA was conducted as follows: 3 $\mu g/ml$ of the antigen of *Entamoeba histolytica* was placed in a microtitre plate. After washing, 1:1,000 diluted test serum was added and the plate was incubated. The plate was then washed, alkaline phosphate labelled anti-human IgG, diluted 1:50,000, was added, and the plate was incubated. After further washing, Lumi-phos 530™ was added. The luminescence was determined when the enzymic reaction reached a plateau. The examination of Nepalese sera results as follows: The average of chemiluminescence value of the test sera were $4,121 \pm 1,848$ counts per second (cps). Data over the converged average ± 2 S.D. ($3,578 \pm 2,317$ cps) were regarded as positive. As a result, 25/220 test sera (11.4%) were positive, and 195/220 (88.6%) were negative. Classified with regions, 21/116 test sera (18.1%) were positive in Kathmandu, 4/104 (3.8%) were positive in Dzong. In sex, 14/103 test sera (13.6%) were positive in male, 11/115 (9.6%) were positive in female. Comparing to colorimetric ELISA, which is usually used for clinical test for amoebiasis, micro-chemiluminescence ELISA needs less amount of test serum. Therefore this micro-chemiluminescence ELISA is suitable method for overseas seroepidemiological research, in which large number of test sera should be collected. Since the people whose antibody indicated positive, were asymptomatic, their physical condition of entamoebiasis seems to have had focuses on the gut without apparent symptom, or have already cured althought they had had focuses before.

The positive rate in an urban area was much higher

than that in a rural area. Moreover, the positive rate of male was higher than that of female. The causes of such difference in the classifications are to be studied.

29 SEROEPIDEMIOLOGY OF CRYPTOSPORIDIOSIS

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Cryptosporidium parvum is one of the causative agents for diarrhea and distribute worldwide. In Japan little attention had been paid to the protozoan infection until an outbreak in 1994. Although cases of the infection in abroad are increased, it is suspected that some of the cases have not been properly diagnosed, since its symptom, diarrhea, usually last within 1-2 weeks naturally. In this study, we used a quantitative ELISA method to measure antibodies to the parasite antigens and compared the results with those obtained by a stool examination.

Serum samples from Japanese students, Japanese criptosporidiosis patients, and Indonesian patients with diarrhea admitted to a hospital in Surabaya. IgG antibodies to *C. parvum* were quantitatively measured by ELISA.

The cut off point of this ELISA system was calculated as mean +3SD of 139 Japanese students' anti-*C. parvum* IgG. Only one student's sample was judged as positive. Out of the 516 Indonesian samples, 45 (8.7%) were ELISA positive and the rate was double of the oocyst positive rate (4.2%). Oocysts were detected only in younger age groups, under 10 years old. On the other hands, ELISA positive cases were lower in a age group under 1 year old (5%) compared with older age groups (11-15%). In the age group under 1 year old, the oocyst positive rate (5.8%) exceeded its ELISA positive rate (5.0%). Anti-*C. parvum* IgG levels were higher in younger age groups. It was suggested that older age groups had acquired not complete but partial resistance to *C. parvum* infection and this may account for their oocyst negative and low anti-*C. parvum* IgG levels of the ELISA positives.

Anti-*C. parvum* IgG levels measured in Japanese patients after the onset of diarrhea shows that the levels were positive in a limited period, during 2 to 6 weeks post the onset of diarrhea. This may explain that ELISA and oocyst double positives are rare and ELISA positive rate was double of the oocyst positive rate.

30 DETECTION OF *CRYPTOSPORORIDIUM PARVUM* DNA IN *APODEMUS ARGENTEUS* IN RESIDENTIAL QUARTERS BY PCR

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Waterborne outbreaks of cryptosporidiosis have been relevant to contamination by animal and human excretions containing *Cryptosporidium* oocysts. It has been already reported that just neonatal calves excrete the oocysts, but not adult cattle because they acquire immunity to the parasites. However the transmission route of the parasites to calves is still unknown.

We surmised that small animals living near cattle

sheds, such as wild mice, were the carriers/reservoirs of *C. parvum* which supplied the oocysts to big animals, e.g., calves, whose excreta contained a lot of oocysts and they might contaminate environmental water source.

Forty three wild mice (32 mice of *Apodemus specious,* 10 mice of *A. argenteus* and a mouse of *Eothenomys smithii*) were captured with traps in the suburbs of Nagasaki City, Jan. 1998. The intestinal contents of five mice (12%) out of them were positive to a specific FITC labelled monoclonal antibody (Wako 297-54301) to the oocyst wall and also *C. parvum* DNA was detected in the intestinal tissue by a nested PCR (Ochiai *et al.,* 1998).

We found that the reproductive stages of *Cryptosporidium* in intestinal organs was more useful to prepare DNA templates for a PCR-based test than the oocysts in feces because it was tricky to break oocyst wall to extract DNA from them, and the infection source of *Cryptosporidium* unexpectedly exsists in a daily milieu near a city.

31 INTESTINAL BLOCKAGE BY CARCINOMA AND *BLASTOCYSTIS HOMINIS* INFECTION

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Blastocystis hominis is frequently identified in stool specimens submitted to clinical microbiology laboratories. However, its pathogenic potential remains poorly understood and controversial. Recently, we detected heavy infections of *B. hominis* in 4 individuals with intestinal obstruction due to cancerous growth. After resection of the occluding masses, the infections spontaneously resolved, without specific chemotherapy. It appears the *B. hominis* infection was coincidental and not related to the neoplastic growth. We suggest that the intestinal obstruction and concomitant stool retention, plus hemorrhage from the cancerous lesions, may have permitted the more abundant growth of *B. hominis.* This is the first report of a possible relationship between intestinal obstruction and a concomitant *B. hominis* infection.

32 STUDIES ON *LEISHMANIA* SPECIES ISOLATED FROM THE GREAT GERBIL (*RHOMBOMYS OPIMUS*) IN XINJIANG UYGUR AUTONOMUS REGION IN THE PEOPLE'S REPUBLIC OF CHINA

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The great gerbil, *Rhombomys opimus,* is a well-documented reservoir of *Leishmania major* in the central Eurasia, the causative agent of Old World zoonotic cutaneous leishmaniasis, and is also known to be the

natural host of *L. gerbilli* and *L. turanica*. To understand the transmission cycles of *Leishmania* infection among the great gerbils, an epidemiological research was done in 1997 at Karamay, Xinjiang Uygur Autonomus Region in the People's Republic of China where cutaneous leishmaniasis has recently been endemic. As a part of the research, 3 isolates of *Leishmania* parasites (KMA2, 4, and 7) were established in cultures from the auricula of gerbils and five isolates were established from 3 species [*P. mongolensis* (KMP3 and 4), *P. andrejevi* (KMP2), *Seregentomyia arpaklesis* (KMP1 and 6)] of sandflies. To identify the species of *Leishmania* isolates, the sequences of a region of the mini-exon gene which is unique in trypanosomatid protozoa were determind. Based on the sequence comparisons with *L. gerbilli* (MRHO/CN/60/GERBILLI), *L. turanica* (MRHO/CN/92/Qitai) and *L. major* (MHOM/Israel/83/LT252), KMP3 and KMA4 were identified as *L. gerbilli,* and KMP2, KMA2 and KMA7 were identified as *L. turanica*. *L. major* has not been found in this study. To demostrate the pathogenicity of these species, histopathological examinations were performed on HE stained tissue sections of great gerbils from which the isolates were established. The large number of amastigotes were observed in the auricula of the infected gerbils, but no significant pathological changes were seen. No amastigotes were observed in spleen and liver. These results indicate that the coexistence of *L. gerbilli* and *L. turanica* in a population of the great gerbils in Karamay and *L. gerbilli* and *L. turanica* are transmitted by *P. mongolensis* and *P. andrejevi* respectively to the great gerbils.

33 LEISHMANIASIS IN TURKEY

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Leishmaniasis, both visceral and cutaneous is still a health problem in Turkey. Visceral leishmaniasis (VL) is consistent with Mediterranean type of VL with a fatal debilitating disease which is most encountered in infants between 0-15 years old. Human VL is mainly observed in western and southern regions, rarely other regions, of Turkey along Ege and Mediterranean coasts as endemic or sporadic. Strains isolated from human cases were identified *Leishmania infantum* using different technics.

Canine VL (CVL) is also endemic and showing a higher prevalance than human VL. Reservoir studies in endemic and sporadic regions has not been carried out yet. The parasites isolated from dogs were also identified as *L. infantum*.

Cutaneous leishmaniasis is seen sporadically in most regions except southeastern part of Turkey where the disease is still higly endemic. Up to date, a total of 17 different *Phlebotomus* species and subspecies were reported. Since there is no report on *Leishmania* promastigotes detected in sandflies, some species can only be considered as suspect vector of leishmaniasis in Turkey.

34 STRUCTURE ANALYSIS OF *TRYPANOSOMA CRUZI* TRANS-SIALIDASE GENE FAMILY: RELATION TO PROTEIN LOCALIZATION AND ENZYME ACTIVITY

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Trans-sialidase of *Trypanosoma cruzi* is a unique enzyme which catalize the transfer reaction of terminal sialic acid from host derived glycoconjugates to the mucin-like acceptor molecules on the parasite surface. The sialylated molecules and the enzyme itself are involved in host-parasite interactions and initial stage

of trypomastigote invasion into host cells. Trans-sialidase is highly expressed in trypomastigote stage and the genes for these proteins are arranged in clusters of tandem array. The other type of trans-sialidase is detectable at the epimastigote stage of the parasite. The trans-sialidase genes of these two stages are localized at the different chromosomes and may be regulated independently. Comparative analysis of the obtained genes revealed that catalytic domain of these two trans-sialidase molecules shear more than 80% of similarities, however amino- and carboxyl-terminal regions have no similarities. Most remarkable differences are at their C-terminals. The C-terminal region of trypomastigote type is tandem repeats of 12 amino acid unit and these are followed by hydrophobic amino acid sequence which is replaced by GPI anchor structure. No these repeats and no GPI exist in trans-sialidase of epimastigote. These are consistent with the analysis of the epimastigote type protein. One of the important information remained to be obtained is the localization of trans-sialidase in epimastigote and the genes which give enzyme activity. Here we show that there are at least four types of C-terminal amino acid sequences in trans-sialidase genes expressed in epimastigote and we have obtained enzyme activity from only one of these types, by expressing in *E. coli*.

35 COMPARATIVE ANALYSIS OF cDNA EXPRESSION PROFILES BETWEEN LONG SLENDER AND SHORT STUMPY BLOODSTREAM FORMS OF *TRYPANOSOMA B. BRUCEI* USING FLUORESCENT DIFFERENTIAL DISPLAY METHOD

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African trypanosomes are member of the order Kinetoplastida, causing human sleeping sickness and nagana disease in cattle. During the ascending parasitemia, long slender, actively dividing forms predominate in the host blood and tissue fluids. At the peak of parasitemic wave, long slender forms differentiate into non-dividing, short stumpy forms, which have a limited life span (24-36 hr) in the host blood. This mechanism of slender to stumpy differentiation is still unclear. Therefore in order to clarify the mechanism, we compared the cDNA expression profiles between long slender and short stumpy forms of pleomorpic clone of *Trypanosoma b. brucei* (GUT at 3.1) using Differential Display (DD) techniques.

Long slender forms were harvested from *in vitro* cultured bloodstream forms. Short stumpy forms of the same clone were harvested from 5-6 days after infection of Balb/c mice and isolated from blood by DEAE-cellulose chromatography. Total RNAs of both forms were isolated and subjected to the DD analysis. Totally, about 8,000 cDNA bands were compared. As a result, 16 cDNAs showed increased expression in short stumpy forms compared to long slender forms, while 33 cDNAs showed decreased expression. One cDNA which was expressed in long slender forms, not in short stumpy forms, was randomly selected, a full-length of which was cloned with 5' RACE and subjected to sequence analysis. BLAST homology search revealed that the cDNA (gene) is a novel variant surface glycoprotein (VSG) gene. However, the relation of the VSG-expression and the difference of the nature of those forms is still unclear.

36 IMMUNOGENETIC ANALYSIS OF CHAGAS' DISEASE

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Chagas' disease is caused by a protozoan parasite *Trypanosoma cruzi*. Whether the infected individuals are able to eliminate the organism or develop chronic Chagas' disease with cardiopathy is unpredictable. In the present study, in order to reveal the host genetic factors that influence susceptibility to Chagas' disease, we typed 44 seropositive Chagas' patients and 138 seronegative healthy controls in Guatemala for their *HLA-B, DRB1, MICA* (MHC class I chain-related gene A) and *TNFα* promotor alleles. The frequency of *HLA-B35* was significantly increased in the seropositives ($x^2=17.95$, $Pc<0.0004$, OR=4.69, 95%Cl 2.22-9.92). *MICA-A5* was increased in the seropositives but the difference was not significant after correction ($x^2=7.62$, $Pc=0.223$, OR=2.04, 95%Cl 1.01-4.13).

The increased risk for the individuals who had both *HLA-B35* and *MICA-A5* ($OR_{B35/A5}-1=16.55$) was much higher than the sum of the increased risk for HLA-B35 ($OR_{B35}-1=5.41$) and *MICA-A5* positive individuals ($OR_{A5}-1=2.15$), indicating that the effects of *HLA-B35* and *MICA-A5* on susceptiblity were synergistic. Therefore, we suggest that the susceptibility to infection with *T. cruzi* is determined by the interaction of two genes, *HLA-B35* and *MICA-A5*.

37 COMPARISON OF THE POLYMERASE CHAIN REACTION WITH THREE SEROLOGICAL METHODS FOR THE DIAGNOSIS OF CHAGAS' DISEASE

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PCR-based diagnostic test for Chagas' disease was compared with three serological methods; rapid agglutination test (RLAT), immuno-double diffusion test (IDDT) and ELISA. We tested 143 blood samples collected more than two years ago at a hospital in an active transmission area, Guatemala. Forty eight out of 143 were seropositive by the three methods. Twenty four samples out of 48 seropositive cases showed the PCR products of *Trypanosoma cruzi*. Five hemocultures (21%) out of 24 cases with positiveness by PCR became positive. The results by PCR did not highly correspond with those of serological tests. One of the reasons is surmised that the blood samples were too old to get successful PCR products because the samples were kept after the collection with just EDTA not with guanidine-EDTA (Avila *et al.*, 1991).

To establish the PCR protocol for Chagasic diagnosis we compared two different DNA extraction methods: phenol-chloroform or IsoQuick kit (ORCA Research, Inc.), and two different sets of primers: BP1/BP2 (Silber *et al.*, 1997) specific primers to nDNA or 121/122 (Gomes *et al.*, 1998), specific primers to kDNA.

The PCR product obtained from phenol-chloroform extraction revealed smears instead of a target product on an electrophoresis, on the other hand IsoQuick showed apparently a distinctly positive band in a clear background.

The primers BP1/BP2 cannot distinguish *T. cruzi*

(692 bp) from *T. rangeli* (615 bp) each other since the respective PCR products is almost similar size. In the case of the primers 121/122, the target size of *T. cruzi* (330 bp) is closed to that of *T. rangeli* (approx. 350 bp) so that the both parasites can be neither identified from the main targets. However the size of artifact products by hairpinning is respectively different between them; that of *T. cruzi* (approx. 650 bp) is smaller than that of *T. rangeli* (>700 bp). *T. cruzi* and *T. rangeli* parasites are distinguishable by just one set of primers (121/122), but only in the case of single infection, not multi-infection.

We can recommend a combination of DNA extraction with IsoQuick kit and the specific primers to kDNA (121/122) for Chagasic PCR diagnosis. These primers are useful for PCR diagnosis in the cases of a single infection with *T. cruzi* or *T. rangeli,* but in the cases of the multiple infection probably the PCR by the primers makes a wrong diagnosis. The PCR-based test is a kind of complemental diagnostic examination, that is, the negativeness by PCR diagnosis means always non-chagasic. The results of not only PCR-based tests but also serological tests and others should be considered for the decision of Chagas' disease and for epidemiological studies.

38 COMPARATIVE STUDIES ON EPIDEMIOLOGY OF CHAGAS' DISEASE BETWEEN BOLIVIA AND NORTHEAST REGION OF BRAZIL

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In the present studies we compared several aspects of triatomine bugs and seroepidemiologic features on *Trypanosoma cruzi* (*T. cruzi*) infection in Santa Cruz-Bolivia and the northeast region of Brazil. The seroprevalence on *T. cruzi* infection were evaluated by HA, IFA and ELISA. The anti-*T. cruzi* antibodies in Abapo and El-Torno were positive rate at 84.3% and 61.1% respectively. In addition, we found 31 cases of suspected congenital Chagas' disease among 1,200 newborn babies, based on the parasitemia positive of *T. cruzi* by the Strout method. It has been confirmed that congenital infection of *T. cruzi* by the xenodiagnosis. In contrast to Santa Cruz, acute or congenital Chagas' disease have not been recently reported in the northeast region of Brazil. We could captured many *Triatoma infestans,* the primary vector of *T. cruzi* have greatly reduced houses infestations in Abapo and El-Torno, *T. cruzi* positive rate of them were 78% and 83% respectively, but in northeast region of Brazil, scarcely captured *T. brasiliensis* and *T. pseudomaculata* and 8.6% of them showed positive of *T. cruzi.* Analysis of the relationship between the reactors and triatomine bugs, interviewed for inhabitants when collected blood showed that most of them were free of such knowledge. These observations suggest there is a distinct difference in the prevalence of *T. cruzi* infection between Santa Cruz-Bolivia and Northeast region of Brazil. The vector control will constitute the main factor in the control of Human Chagas' disease in Santa Cruz of Bolivia. Other factors have poor condition of economical, sociological and educational for health care to inhabitants in endemic area.

39 STUDY OF CROSSREACTING ANTIGENICITY BETWEEN *ASCARIS LUMBRICOIDES* ANTIGENS AND HUMAN COLONIC MUCOSA

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The present study was conducted to determine the crossreacting antigenicity between *Ascaris lumbricoides* antigen (As Ag.) and human colonic mucosa (Cm Ag.). *A. lumbricoides* were homogenized in phosphate-buffered saline (PBS) to prepare As Ag. and rabbits were immunized with As Ag. in order to produce anti-*A. lumbricoides* serum (anti-As abs), anti-As abs was absorbed in *E. coli* to eliminate anti-*E. coli* serum.

Seven normal human colonic mucosa were obtained from biopsy specimens at colonoscopy and those were respectively homogenized in PBS and Cm Ag. were prepared.

Immunohistological staining of embedded colonic mucosal section was performed by immunofluorescence technique using anti-As abs.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional gel electrophoresis were performed against Cm Ag. and chemiluminescence Western blotting analysis of Cm Ag. using anti-As abs were performed followed by treatment with horseradish peroxidase-labeled goat anti-rabbit IgG.

As a result, crypts of colonic mucosa, especially basal membrane part, were stained using immunofluorescence technique. Moreover, we detected that mainly molecular weights 41 kDa and 38 kDa proteins were cross-reacting antigens between As Ag. and Cm Ag. by chemiluminescence Western blotting analysis, and isoelectric point of both proteins were pI 5.8.

We demonstrated that the presence of common antigenicity between As Ag. and Cm Ag. and molecular weights of these cross-reacting antigens were almost equal to antigen detected by colitis colon associated IgG (CCA-IgG) which was found in the colonic mucosa of ulcerative colitis by DAS.

There was a resemblance between these cross-reacting antigens and antigen detected by CCA-IgG. In conclusion, it is thought that these cross-reacting antigens might be closely related with the pathogenesis of ulcerative colitis.

40 IMMUNODIAGNOSTIC APPLICATION OF THE RECOMBINANT *TOXOCARA CANIS* ANTIGEN FOR HUMAN TOXOCARIASIS CANIS AND CATI

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The diagnosis of human toxocariasis canis caused by infection with the second stage larvae (L_2) of *Toxocara canis* depends mainly on the detection of anti-*T. canis* antibodies by ELISA using *T. canis* L_2 excretory-secretory antigens (TES). However, the TES commonly used cross-reacts with sera from patients infected with various helminths, and is laborious to obtain in large amounts from cultured *T. canis* L_2. So, we developed a recombinant antigen to substitute for TES in the immunodiagnosis of human toxocariasis. A clone (clone 2) derived from a *T. canis* L_2 cDNA library was selected for protein expression. IPTG-inducible pET-32b(+) plasmid was used as an expression vector. Recombinant *T. canis* protein (38 kDa) produced in

Escherichia coli was purified using TALON™ Metal Affinity Resin, and its specificity as an antigen was evaluated by IgG-ELISA using 118 serum samples from patients infected with 15 different helminths. The recombinant antigen reacted intensely with all 4 serum samples from suspected toxocariasis patients at an antigen concentration of 125 ng/m*l* and serum dilution of 1:200, yielding no false negative reactions. Cross-reactivities were observed in only one case each of gnathostomiasis, paragonimiasis, and spirometriasis out of 114 samples from patients infected with 13 different helminths. In the case of TES used for comparison, cross-reactions were seen in 49 cases out of the 114 serum samples and involved 10 different helminth infections, indicating that the specificity of the recombinant antigen is much higher than the TES widely used. The recombinant *T. canis* antigen developed can be recommended for the immunodiagnosis of human toxocariasis and may provide more reliable diagnostic results than the currently available TES.

41 *STRONGYLOIDES RATTI* ADDITIVE EFFECT OF TESTOSTERONE IMPLANTATION AND CARBON INJECTION ON THE SUSCEPTIBILITY OF FEMALE MICE

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There is sex difference in susceptibility to parasites. In *Strongyloides ratti*-mice model, it was known that male mice were highly susceptible to *S. ratti* than female mice and testosterone implantation could render female mice susceptible similar to native male mice. This effect was observed during migrating phase of *S. ratti*. Recently it was reported that testosterone could affect various cell's function, including macrophages. We tried to examine whether the testosterone could affect susceptibility through macrophages. Testosterone was implanted in female mice in advance and macrophages were blocked by carbon injection. After carbon injection, 2,000 *S. ratti* L3 were infected subcutaneously. The effect of each treatment was assayed by counting worm number arrived to the head of infected mice. Testosterone treatment alone or carbon treatment alone effectively increased the worm number as previously reported by others. The worm number observed in mice treated with testosterone and carbon was significantly higher than that treated with testosterone alone or carbon alone. Furthermore, it seemed to be the sum of worm number in each treatment group. Serum testosterone level was elevated in testosterone and carbon treated group as compared with that of testosterone treated group. To adjust serum testosterone level equally between these groups, the amount of implanted testosterone was adjusted before *S. ratti* L3 infection. Testosterone (adjusted dose) and carbon treatment also increased the worm number equally to the sum of each treatment alone. This experiment revealed that testosterone and carbon treatment had an additive effect on susceptibility in female mice. It might be suggested that testosterone could increase the susceptibility of female mice to *S. ratti* independently of macrophages because testosterone could affect the worm recovery after macrophage blockade.

42 PREVALENCE OF *STRONGYLOIDES STERCORALIS* INFECTION AMONG INHABITANTS OF YORON ISLAND IN KAGOSHIMA PREFECTURE AND CLINICAL STUDY ON SYMPTOM IN PATIENTS WITH STRONGYLOIDIASIS

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Strongyloides stercoralis which is soil-dwelling nematode is prevalent in the tropics and subtropics soil. In humans, infestation with this nematode most commonly involves the upper small intestine. Clinical signs and symptoms are minimal digestive syndrome. However, in the presence of immunosuppression, invasive strongyloidiasis may develop into a life-threatening disease.

We examined 611 cases inhabitants of Yoron Island in Kagosgima Prefecture. The patients with *S. stercoralis* was 64 cases. The positive rate was higher in sixties.

The most common complaints of 64 patients with *S. stercoralis* were borborygmus (45.6%), abdominal pain (26.3%) and diarrhea (26.3%).

We treated 32 patients with ivermectine and symptoms improved after treatment described below: The cure rate was 100% after 4 weeks after. Sixteen of the patients (88.9%) with digestive syndrome improved. Seven of the 9 patients (77.8%) with itching of anus, four of the 5 patients (80.0%) with discomfort of pharynx and seven of the 12 patients (58.3%) improved.

43 SEROEPIDEMIOLOGY OF AN EMERGING PARASITIC DISEASE, NEUROCYSTICERCOSIS IN IRIAN JAYA, INDONESIA

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Based on our previous report on taeniasis/cysticercosis in Indonesia as an emerging disease (Simanjuntak *et al.* 1997, Parasitology Today, 13, 321-323), we have done seroepidemiological study of cysticercosis in Irian Jaya, Indonesia. For this study, we have examined a total of 138 serum samples including 18 from suspected neurocysticercosis (due to anamnesis of epileptic seizures), 31 from suspected cysticercosis (due to subcutaneous or muscle nodule(s)), 12 from taeniosis (due to fecal examination or anamnesis of expulsion of proglottids) and 77 from people at risk (47 from endemic area, and 30 from non-endemic area). Highly sensitive and specific serodiagnosis by both immunoblot and ELISA (Ito *et al.*, 1998, Am. J. Trop. Med. Hyg., 59, 291-294) has revealed that 12 (67%), 20 (20%), 0 (0%) and 13 (12 from endemic and 1 from non-endemic areas) (17%) cases were serologically confirmed cysticercosis, respectively. It has been known that three districts in Irian Jaya Province have already been contaminated with taeniosis/cysticercosis (Wandra *et al.*, submitted for publication). We have found one case of cysticercosis from non-endemic area from another district. Follow-up study has revealed that most of the 13 serologically confirmed cysticercosis cases of people at risk have epilepsy, subcutaneous nodule(s), indeed. Therefore, we suppose that cysticercosis is spreading in Irian Jaya. We are interested in doing serological study of the local people in Papua New Guinea.

44 THE RESISTANCE OF TREATMENT AND SPECIFIC IgG4 ANTIBODY TITER IN HLA-DRB1*0901 POSITIVE PATIENTS WITH STRONGYLOIDIASIS

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Strongyloidiasis is difficult to be completely treated. To find host factors involved in response to treatment, patients were analyzed specific antibody titers and *HLA-DRB1* alleles. Higher titers of IgG4 antibody were observed in the non-cured group than in the cured group ($P<0.05$). IgG4 antibody titers were higher in the *DRB1*0901* positive group than in the negative group ($Pc<0.05$). These results suggest that *DRB1*0901* is a possible genetic marker for resistance to treatment that is associated with elevation of IgG4 antibody titer.

45 *STRONGYLOIDES STERCORALIS* CARRIERS IN OKINAWA, JAPAN

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A surprisingly high prevalence rate of *Strongyloides stercolaris* infection in Okinawa has been reported (Asato *et al.*, 1992). The impact of infection, however, on the health status of patients has not been well studied although the infection is generally considered opportunistic.

1. It is likely that the transmission of *S. stercolaris* does not exist because the age-prevalence has been decreasing constantly both in Itoman from 1983-1995 and in Osato from 1989-1995.
2. Using the health check data obtained in 1989-1992 from a health center, the results of chest X-ray, serum cholesterol, neutral fat, HDL, GI tract examination, urine protein, urine blood, BUN, cleatinin, uric acid, albumin, alkaline phosphatase, GOT, GPT, ZTT, serum bililbin, serum protein, blood sugar, haemoglobin, heamatocrit, RBC, WBC and its analysis, ESR, Rheumatoid factor, platelet, cholinesterase, LDH and gamma-GTP were compared between *S. stercolaris* carriers and non-carriers. The differences were found only in the number of WBC, eosinophile and lymphocyte. They were significantly higher in carriers than in non-carriers.
3. A relationship between HTLV-1 infection and disseminated strongyloidiasis has been reported. Among the *S. stercolaris* carriers, total IgE level was compared between HTLV-1 carriers and HTLV-1 non-carriers. The IgE level was higher in males than in females, and tended to be higher in HTLV-1 non-carriers both in males and females.
4. In the record of the cause of death from 1990-1994 in Okinawa *S. stercolaris* infection was reported, as a cause of death, only in 3 cases among 21,608 deaths.

46 A CASE OF GENITAL ELEPHANTIASIS DUE TO POSSIBLE FILARIA INFECTION

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In Japan, elephantiasis is a very rare disease characterized by the aspect of lesional skin which is similar to that of the elephant. The latest case of filaria-induced elephantiasis was reported in 1982 by Shirahama *et al.* in Japan. A 62-year-old man, born in Okinawa, had a history of fever with shivering from age of 25 to 30 years at a rate of one time a week followed by swelling of the genital region. Before the onset of the disease he had been in Bolivia where filariasis prevailed. Ten years before visiting our hospital, surgical treatment was performed elsewhere. However, a few months after the operation the same eruption was recurred. When he visited our hospital, the skin of his penis and scrotum was rough, verrucous, thickened and colored dark brownish. Milky discharge from the scrotum was observed. Histopathologic examinations revealed that acanthosis with hyperkeratosis, fibrosis of the dermis and dilatation of lymphatic vessels. Lymphography from the left third toe-finger revealed that obstruction of thoracic duct and reflux of contrast medium on kidney and scrotum. Repeated examinations for microfilaria in the midnight urine were negative. Based on clinicopathological and epidemiological studies, the diagnosis of genital elephantiasis was established. The lesional skin was resected and free skin graft was done onto the penis shaft. Skin defect on scrotum was suturred. The postoperative course was uneventful, except for a small ulcer formation on the scrotum at the operation wound with bacterial infection, which had been improved in a few weeks.

47 DETECTION OF CIRCULATING *WUCHERERIA BANCROFTI* ANTIGEN, FILARIA SPECIFIC IgG AND IgG4 IN CHYLURIA CASES IN OKINAWA

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The diagnosis of filarial chyluria is made simply based on patients' history of living in the past endemic areas of filariasis. In this study, we studied (1) if we can obtain immunological evidence to support the filarial origin of chyluria, and (2) if there is any immunological method useful to make a diagnosis of filarial chyluria.

Serum samples from 16 chyluria patients in Okinawa were tested. Sera from 14 healthy Japanese were negative controls, and 7 sera from Sri Lankan carriers of microfilariae (mf) were positive controls. Circulating *W. bancrofti* antigen was assayed by ELISA using Og4C3 monoclonal antibody. Filaria-specific antibodies were detected using *Burgia pahangi* antigens. Antigens of *Dirofilaria immitis, Anisakis simplex* and *Strongyloides ratti* were used to absorb antibodies cross-reactive with these antigens.

Absorption with combined antigens of *D. immitis* and *Anisakis* reduced cross-reactive antibodies, and clearly separated mf positive Sri Lankans, chyluria patients and normal controls. If the average antibody level of the healthy control +3SD is regarded as a cut-off point, 7 of 14 chyluria patients could be diagnosed as filarial. Antibodies of IgG4 subclass, which cross-reacted very little with other parasites antigens and associated with active filarial infection, were detected in one chyluria petient. In conclusion, for the diagnosis of filarial chyluria, (1) measurement of *B. pahangi*-reactive IgG4 or (2) measurement of *B. pahangi*-

reactive IgG after absorption of cross-reactive antibodies with *Anisakis* and *D. immitis* antigens would be useful.

48 ENHANCEMENT OF IL-10 PRODUCTION BY TREATMENT WITH rIL-3 IN *TRICHINELLA SPIRALIS*-INFECTED MICE

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IL-3 has been characterized as a hemopoietic growth factor. Recently *in vitro* studies have shown that IL-3 can modulate production of Th2 cytokines, IL-4, IL-10 and IL-13. In addition, IL-3 has been shown to be involved in adult worm expulsion of *Strongyloides venezuelensis*. Infection with *Trichinella spiralis* induces Th2-type responses such as mastocytosis and IgE production in mice. However, there is little knowledge about an interaction between IL-3 and Th2 cytokines in helminth-infected mice.

Male C3H/He mice were injected intraperitoneally with rIL-3 before infection with 400 muscle larvae. Spleen cells and mesenteric lymphnode (MLN) cells were recovered on days 0, 5, and 14 after infection. After 48 hr cultivation under anti-CD3 or larval antigenic stimulation, IL-10 in supernatant was measured by ELISA. IL-10 was produced by MLN cells in early stage of infection and its production was more in rIL-3-treated mice than in nontreated mice. The major producer was a CD8-T cell population. When cells were cultured with muscle larval antigens, MW>50 kDa antigens induced IL-10 but neither 30 kDa-50 kDa nor <30 kDa antigens. The results suggest that IL-3 might be one of the regulators for Th2 cytokine production in infected animals.

49 CONSTRUCTION OF cDNA LIBRARY OF *TRICHINELLA SPIRALIS* MUSCLE LARVAE AND THE GENE PRODUCTS

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We constructed cDNA library of *Trichinella spiralis* muscle larvae and some of gene products were obtained. Such products are supposed to be indistinguishable in some points: as a well characterized antigen for immunodiagnosis, as a vaccine that induce protective immune response, and as a bioactive substances that may play an essential role in transformation of infected muscle cells. Messenger RNA was isolated from *T. spiralis* muscle larvae by using Purification Kit (Pharmacia Co. Ltd.), and cDNA was synthesized by using TimeSaver cDNA Synthesia Kit and introduced to λ ZAPII vector of Stratagene. Complementary DNA library was constructed by *in vitro* packaging using Gigapack II Gold, which resulted in 5×10^5 pfu/μg. This cDNA library was immunoscreened against infected sera with *T. spiralis*, resulting in positive 10 clones. Three clones were sequencesed: one clone was ca 1.3 Kbp, and likely same as *T. spiralis* hypothetical ORF 9.10 mRNA (Despommiert *et al.*). A clone of ca 1.4 Kbp shared about 60-80% homology with protease inhibitors.

50 IS THE *SCHISTOSOMA INDICUM* GROUP MONOPHYLETIC?

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Four *Schistosoma* species, *S. indicum, S. spindale, S. incognitum* and *S. nasale* found in India and Southeast Asia develop in snails of the family Lymnaeidae, and tentatively referred as to the *S. indicum* group, having terminal -or subterminal- spined eggs (Rollinson and Southgate, 1987). However, *S. incognitum* does not possess a true terminal spine to its egg and its intermediate host belongs to the genus different from that of the other three species. In the present report, phylogenetic relations between *S. spindale* and *S. incognitum* and their relations with other *Schistosoma* groups were investigated using PCR-amplified nucleotide sequences. For *S. spindale,* naturally infected snail species of Indoplanorbis were collected in Malaysia and maintained in the laboratory of IMR. For *S. incognitum,* Rattus rattus were trapped in Jakarta, Indonesia to obtain adult worms. Adult worms for each species were grouped to extract DNA. Amplified regions are the 2nd internal transcribed spacer (ITS2) and the cytochrome c oxidase subunit 1, CO1. Primers, PCR conditions, and computor analyses were described elsewhere (Agatsuma *et al.,* 1997; Blair *et al.,* 1997). The Neighbour Joining method was performed to construct phylogenetic trees for the two DNA regions. Both of the trees showed that *S. incognitum* was not clustered with any species groups, and *S. spindale* was included into an African cluster, or specifically the *S. haematobium* group. Affinity between *S. spindale* and *S. haematobium* has been also obtained by the S18 rRNA gene tree analysis using the Fitch-Margoliash least squares method (Johnston *et al.,* 1993). The present result clearly indicates that the *S. indicum* group is not monophyletic but paraphyletic.

51 SONOGRAPHIC ASSESSMENT OF BOTH URINARY TRACT AND HEPATIC MORBIDITY DUE TO *SCHISTOSOMA HAEMATOBIUM*

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Since present aim of the global control of schitosomiasis is to reduce morbidity rather than to reduce transmission or even eradicate the disease, ultrasonography has widely been used in a number of epidemiological studies in schitosomiasis endemic areas. The method has proved to be feasible and useful to examine the morbidity. Pathology due to *Schistosoma haematobium* is examined exclusively for the urinary tracts so far. However, recently periportal fibrosis of liver has also been described in *S. haematobium* infection by using ultrasonography. We commenced the ultrasonographic studies to examine the extent of liver pathology, in addition to urinary tract morbidity in an area where schistosomiasis haematobia is highly endemic and no intervention was introduced so far.

The investigations were conducted in the Tserezani village, Coast province, Kenya. In February 1998, 225 and 222 school children were examined for liver and urinary tract respectively by ultrasonography. Urinary bladder morbidity was graded by the classiaication system proposed by Medhat, A. *et al.* (1997). The body height-dependent reference values published in Senegal (Yazdanpanah, Y. *et al.,* 1997) were applied for evaluating the periportal fibrosis. Urine passed by each subject at midday was microscopically examined for eggs by the nucleopore filtration method.

Pathological lesions of urinary bladder were observed in 160 (72%) subjects. Among these, 75 (34%) subjects had ultrasonographic score of grade III, while only 6 (2.7%) subjects had kidney congestion. Peripor-

tal fibrosis were shown in 54 (24%) subjects. The comparative analysis using logistic regression model shows that both urinary bladder morbidity and periportal fibrosis are associated with presence of eggs in urine.

In conclusion, a relatively high morbidity due to *S. haematobium* infection in an area where no intervention has been done was demonstrated.

52 CURRENT STATUS OF BOAR-MEAT TRANSMISSION OF PARAGONIMIASIS IN JAPAN

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Human paragonimiasis has been reported in most parts of Japan. In the early 1950s, a total number of patients with the lung flukes in Japan were estimated from three to five hundred thousand. The inhabitants of endemic areas had a custom of eating crabs, *Eriocheir japonicus* or *Geothelphusa dehaani,* the second intermediate hosts of the lung flukes. Human infection mainly occurred an accidental transfer of the encysted larvae to the mouth through the handling of crabs when preparing them for food. In parallel with the recent socio-economic changes, the prevalence has dramatically decreased with the health education, the use of effective drugs and changing people's eating habits. On the other hand, sporadic cases of paragonimiasis due to another infection mode, boar-meat transmission, have been found in the southern part of Kyushu Island since the middle of 1970s. It has been demonstrated that wild boars in the endemic area harbored larval forms of *P. westermani* in their musdles, and they can easily mature in the definitive host. Many outbreaks in recent years have been associated with eating the flesh of boar as wild game in Kyushu.

1) During the last three years, a total of 32 cases were diagnosed by serological tests as paragonimiasis in our laboratory. Among them 23 cases were from Kyushu and the half (11) of them were associated with eating the flesh of wild boar.
2) The incidence of *Paragonimus* spp. in boars was assessed. 100-200 g of muscle taken from each boar was examined for the larvae. One of 67 wild boars from Kyushu was found to be infected with the larvae. In addition, a total of 106 serum samples from different regions were checked specific antibody by ELISA using *P. westermani* antigen. Out of 59 collected from Kyushu, 44 (75%) were reacted positively. Out of 39 collected from the middle part of Honshu Island (Kinki area), 16 (41%) were positive. In contrast, no positive case (8 examined) was found from domestic boars.
3) Boar hunters have to be considered at risk for boar-meat transmission of paragonimiasis. We made inquiries the hunters about an eating custom of the flesh of boars. Four groups of boar-hunters from different regions in Kyushu replied this questionnaire. More than sixty percent (44 examined) of hunters have experienced in eating the flesh of boar in the raw (sashimi).

53 MORPHOLOGICAL VARIATIONS IN NEXT GENERATION OF *FASCIOLA GIGANTICA* FROM ZAMBIA

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Purpose: Research individual genetic variations in the morphology in *F. gigantica.*

Materials and Methods: 1. Collect *F. gigantica* from Lusaka, Zambia. 2. Obtain eggs from a *F. gigantica.*

3. Cultivate at 30°C for about 2 weeks. 4. Infect *Lymnaea ollula* with the hatched miracidia. 5. Keep *L. ollula* at 25-30°C for 30-40 days. 6. Store the metacercaria in vinyl bag at a low temperature. 7. Administration of the metacercaria to 6 wild goats from Madara isld., Saga, orally. 8. Obtain adult flukes from biliary ducts 129-130 days after infection. 9. Measurement of body length and breadth.

Results: body length×body breadth=32-43×11-15 (average 36.4±3.0×12.7±1.2)

coefficient of correlation; -0.509

Consideration: Results may be considered as follows:

1) Variation of body length and body breadth in next generation from a *F. gigantica* have wide range, but range is smaller than the individual variation measured by other researchers.

2) There is a negative correlation between body length and body breadth, and the method of preparing specimens yield a difference between both values.

54 A CASE OF CYSTIC ECHINOCOCCOSIS IN A JORDANIAN PATIENT: TREATMENT WITH ALBENDAZOLE

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A case of cystic echinococcosis (CE) identified in a Jordanian male is presented. He has come to Japan as a JICA participant and was subjected to abdominal ultrasonography because of microscopic hematuria, which incidentally revealed an intrahepatic cystic lesion of 6-7 cm in diameter with several daughter cysts in its inside. Substantially the same cystic lesion was revealed by CT scan, which was surrounded by a capsule with some calcification. Serological tests with immunoblot using antigen extracts from both *Echinococcus granulosus* and *E. multilocularis* revealed a positive band for antigen B from *E. granulosus* but none for Em 18 from *E. multilocularis,* a pattern that is compatible with CE. Because of his special situation as a JICA participant, surgical treatments were not adopted and he was put on the albendazole treatment which consisted of 28 days of drug administration and 14 days of cessation. The results of successive imaging procedures clarified the amelioration of the CE after the start of chemotherapy, as revealed by the disappearance of the daughter cysts and the accumulation of echo-dense materials. After three cycles of albendazole he has left Japan as had been scheduled previously.

In Jordan the positive rate for antibodies against *E. granulosus* were reported to be 2.4% and 5.8% among the general population and those attending outpatient clinics, respectively. Another study done in those areas showed that 13% of sheep and cattle carry the larval worms, and 14% of dogs carry the adult worms. Physician should be alert to the possibility of CE when encountering intrahepatic cystic lesions in individuals from endemic countries. Application of excellent serological methods is recommended to confirm the diagnosis.

55 QUESTIONARY TEST AND PREVALENCE OF INTESTINAL HELMINTIC INFECTIONS IN BARRU, SULAWESI, INDONESIA

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A questionnaire with parasitological study was carried out on the inhabitants of 4 villages of Barru District, Sulawesi Is., Indonesia from 1994 to 1995. A questionnaire was made on life style and sanitary conditions. In 482 houses of 4 villages, interview for items of questionnaire sheets was made to owner, housekeeper and children of same family by us with staffs of Barru Health Center. In Pancana and Lalolang, 37.7% and 50% of men in the inhabitants surveyed were fishermen, while in Lompo Riaja and Pattappa, 38.6% and 65.5% were farmers. The highest proportion of official workers was 33.7% in Lompo Riaja. Educational level was low, and 88.4% of Pancana, 90.4% of Lalolang, 62.1% of Lompo Riaja and 91.2% of Pattappa had elementary or under elementary school education. While in Lompo Riaja, 30.8% of the inhabitants had graduated senior high school or university. The percentage of own latrine was 30.3% in Pancana, 13.2% in Lalolang, 31.9% in Pattapa and 60% in Lompo Riaja which is significantly highest rate. The 5.4% to 11.4% of the latrines overflowed in rainy season. The people without latrine usually defecated mainly in rice field (farm), seaside or riverside.

A total of 654 fecal samples were examined by modified Kato-Katz thick smear method. Five nematode species, *Ascaris lumbricoides, Trichuris trichiura, Necator americanus, Strongyloides stercoralis* and unidentified Rhabditoids of free-living nature were detected. Cestode, *Hymenolepis nana,* infection was proven. All of hookworm examined by modified Harada-Mori culture technique were *N. americanus.*

The prevalence of *Ascaris* and *Trichuris* infections was significantly lower in persons who had graduated senior high school or university. The prevalence of hookworm infection was not different between the persons with latrine or without it, while in the prevalence of *Ascaris* and *Trichuris,* there was significant difference. In persons without own latrine, 62.1% of the persons had *Ascaris* and 44.9% had *Trichuris,* and most of them use seaside for evacuation. The difference in *Ascaris* and *Trichuris* hookworm infections was not shown between the persons with helminthic medicine or without it.

56 AN EPIDEMIOLOGICAL STUDY OF MALINGNANT SKIN TUMORS IN WESTERN KENYA AND NAGASAKI, JAPAN

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The skin provides protection against ultraviolet light and mechanical, chemical and thermal insults and acts as a barrier to invasion by micro-organisms, but, on the other hand, the skin is directly damaged by these harmful factors. In this report, we discuss the results of a comparative epidemiological study on malignant skin tumors in western Kenya, East Africa and Nagasaki, Japan. The cases of the atomic bomb survivors in Nagasaki were excluded. The most common malignant skin tumor in western Kenya was squamous cell carcinoma (SCC), followed by malignant melanoma (MM), basal cell carcinoma (BCC), skin appendage tumor (AT) and Bowen's disease (BD). In Nagasaki, BCC was the most common tumor, followed by SCC, BD,

MM, extra-mammary Paget's disease (PD) and AT. BCC and BD were rare skin tumors and no case of PD was found in western Kenya. Although the most common primary site of SCC in western Kenya was the lower extremities•foot, followed by the penis and scalp•neck, the face was the most common site, followed by the shoulder•upper extremities•hand and lower extremities•foot in Nagasaki. Most SCCs developed from antecedent lesions such as burns, scars and chronic ulcers in western Kenya. Over 80% of MN cases occurred in the lower extremities•foot in western Kenya and 45% of the cases in Nagasaki. The higher prevalences of SCC in the penis were found among the ethnic groups, who have no custom of male circumcision in western Kenya. These findings suggest that the inhabitants in western Kenya have a higher resistance against ultraviolet light than the peoples in Nagasaki and natural and social environments and ecological situations, including specific customs, influence the occurrence of these malignant skin tumors.

57 A STUDY OF PUBLIC OPINION SURVEY OF JAPANESE RESIDENTS LIVING IN FOREIGN COUNTRIES REGARDING THEIR HEALTH

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Health problems on Japanese living abroad were surveyed by questionnaires in order to provide the better health education.

Questionnaires regarding their life and health had been distributed to Japanese residents through the Japanese societies from January 1995 to November 1997. Total 953 answers in 19 countries were returned and analyzed.

Infectious diseases were the most common illness which Japanese were afraid of in developing countries, while "adult diseases" were less common. In contrast, "adult diseases" were more common in developed counries than infectious diseases. Mental stress was mentioned in 30-40% of the whole subjects, and practical troubles were described in 60-70% of them. The difference of the frequency of abnormal findings at health checkup before leaving Japan was not statistically significant between developing countries and developed countries. Immunization before leaving, annual health checkup, and caution for foods and water, which are the preventive measures for health problems, were mentioned more frequently in those from developing countries than those from developed countries.

A variety of self-defensive measures have been performed to prevent infectious diseases by Japanese residents especially in developing countries. However, "adult diseases" and mental stress were still serious health problems without any effective countermeasures in both developed and developing countries. Therefore, preventive measures for such problems are required.

58 A CASE OF *AMBLYOMMA TESTUDINARIUM* BITE

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A 73 year-old man, who lived in Tochigi prefecture, presented with a bean-sized brownish tick on his right shoulder. He has been to the mountain in Shizuoka 19 days before, where he seemed to have been attacked. The lesion was excised with the surrounding skin. The parasite was identified as an adult female tick named *Amblyomma testudinarium*.

A. testudinarium widely distributes over the South Asia, Southeast Asia and the southern and western part of Japan. The patient with tick bite of *A. testudinarium* is, however, very rarely found in Tochigi which is located in the middle-northern part of Japan.

59 SYSTEM OF DEFENSE FROM ULTRAVIOLET RAYS IN WILD MAMMALS

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Wild mammals possess systems of life protections from the ultraviolet rays (VU). We make clear the defensive ability and relationship between it and environmental conditions. The hair of some species of wild mammals has a two color structure. It is black near the body, and is body color the end. The black part of the hair (black layer) may be effective in absorbing UV and infrared radiation. Therefore we investigated the effects of black layer on skin protection to UV. The study used Yellow rats (*Citellus dauricus*, N=9), Squirrels (*Eutamias sibiricus*, N=12), Jirds (*Meriones unguiculatus*, N=15) and Pikas (*Ochotona curzoniae*, N=17) with black layers, and Wistar rats (N=12) for controls.

Experiment I: Transmitted UV were measured through hair and skin tissue, black layer and skin tissue, and skin tissue. Experiment II: For pretreatment, the back hair of animals was cut in three ways; all hair was cut, and part of the body color hair was cut, and no hair was cut. The animals were irradiated by UV-B at 2.013 mW/cm^2 for two days, and the cell degeneration of skin and subcutaneous tissues were then analyzed pathologically.

1) Transmittance of UV through the black layer is significant low, and the damage of skin tissue in rats compared with other animals was hard. Because black layer absorbed UV efficiently. This result suggests that the black layer has an effective role to protect the skin tissue from UV.

2) The hair length depends on environmental conditions in particular temperature, and transmittance of UV through the black layer is low in longer hair. So efficiency of defense from UV is difference among species depended on their living environmental conditions.

3) Yellow rats live in where ambient temperature changes widely in a day. The hair length of them is short, but epidermis is thick compared with other species. Long hair induce increase core temperature, so thick epidermis protect skin tissue from UV.

These wild mammals possess the protecting ability with black layer of hair and/or thick epidermis to protect skin from UV, and environmental conditions influence the protecting ability.

60 SUPPRESSION OF THE SWEAT GLAND SENSITIVITY TO ACETYLCHOLINE APPLIED IONTOPHORETICALLY IN TROPICAL AFRICANS COMPARED TO TEMPERATE JAPANESE

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Tropical inhabitants possess the ability of heat-tolerance through permanent residence in the tropics. Previously, we have shown that tropical African and Thai subjects regulate the core temperature with less amount of sweat against heat compared to temperate Japanese subjects and that suppression of sweating in tropical subjects was attributed to suppression in both central and peripheral sudomotor mechanisms. To elucidate the peripheral mechanisms of the suppressed thermal sweating in tropical natives, sweating responses to acetylcholine (ACh), a primary transmitter of the sudomotor innervation, were compared between Japanese and Africans. All experiments were carried out in a climatic chamber (24±0.5°C, relative humidity 40±3%) at 2-5 p.m. between October 1997 and June 1998 in Nagasaki, Japan. Upon arrival into the climatic

chamber, the subject wore light indoor clothing and sat on a chair for 60 min before the experiment. Quantitative sudomotor axon reflex test, QSART (Low *et al.*, 1983; Lee *et al.*, 1997), was performed to quantitatively evaluate glandular ACh-sensitivity. ACh was iontophoretically administered on the forearm. Directly activated and axon reflex-mediated sweat responses were evaluated by quantitative sudomotor axon reflex test. The sweat onset-time was 0.72 min shorter ($P<0.01$) and the sweat volumes were 72%-110% higher ($P<0.01$) in the Japanese than the Africans. Iodine-impregnated paper method revealed that sweat gland density was 50.6% higher ($P<0.001$) and sweat gland output per single gland was 20.4% larger ($P<0.001$) in the Japanese compared to the Africans. The Japanese showed the a 0.17°C higher oral temperature and a 0.30°C higher forearm skin temperature compared to the Africans ($P<0.05$, respectively) at rest under a thermoneutral condition. ACh iontophoresis did not produce any influences on oral temperature, but increased the local skin temperature in both the Japanese and the Africans. These results indicate that suppressed thermal sweating in Africans is, at least in part, attributed to the suppressed glandular sensitivity to ACh through both recruitment of sweat glands and sweat output per each gland.

61 INSTALLATION AND MAINTENANCE OF PROTECTIVE NETS AGAINST VENOMOUS JELLYFISH ON THE BEACHES OF OKINAWA

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Venomous marine animals inhabiting the coastal areas of Okinawa islands are known to be hazardous or even fatal to swimmers or divers particularly in the summer season when marine recreational activities are at their peak. Most of the incidents are caused by Coelentrata, particularly *Chiropsalmus quadrigatus,* as seen in the two fatal cases since last year. This species of jellyfish inhabit coastal areas ranging from the Ryukyu *Archpelago* to Southeast Asian countries. To avoid such marine animal-related fatal accidents, several beaches in Okinawa have installed protective nets against the jellyfish invasion. The following report is based on our recent surveys regarding the current status of installation and maintenance of protective nets in the island of Okinawa.

(a) Time and location: through August to September 1998, at 8 beaches equipped with the nets (including the main island of Okinawa and neighboring islands).
(b) Method of survey: (1) interviewing with those who are in charge of the management of the protective nets, and (2) observation of the nets by diving.

In the northeastern coast of Australia, the beach managers have installed nets against *Chironex fleckeri* that belong to the Box-jellyfish and the nets are found to be useful for the prevention of jellyfish invasion. *C. quadrigatus* is the same species as *C. fleckeri,* can be observed during a period from June through September in Okinawa, and are known to cause sting accidents to swimmers on the beaches.

The net was first installed in the Sunset-beach (Chatan town) in 1989, but accidents continued each year. After making several constructional improvements to the net e.g., (1) adjusting the gap between the net and water surface, (2) narrowing the gap between the bottom of the net and seabed, (3) strengthening the both ends of the net, the number of annual incidents drastically decreased in comparison with numbers of accidents reported in previous years (5 reported cases in 1993).

Introduction of such improvements to the nets almost completely eliminated the inside-the-net accidents. However, the following concerns have been raised over how to increase the efficiency of protection: 1) the seaweed sticking to the mesh, 2) the size of the mesh, 3) the height of nets, 4) the fixed part of net's both ends, 5) the gap between the net and bottom of the net and the water surface and the seabed, 6) the rift of the mesh, 7) the way of monitoring and recording, 8) countermeasures in typhoon seasons.

At some beaches, it was found that the omission of cleaning nets caused extensive seaweed growth. A small tidal current, as is often seen at an artificial beach, is known to offer a favorable habitat for this species of jellyfish.

62 MOLECULAR CHARACTERIZATION OF THE JAPANESE ENCEPHALITIS VIRUS REPRESENTATIVE IMMUNOTYPE STRAIN JaGAr01

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We determined the full genomic sequence of the Japanese encephalitis (JE) virus JaGAr01 strain and its predicted amino acid sequence. Nucleotide sequence comparison with nine fully sequenced JE strains shows a homology range from 89.62-99.49%. Amino acid sequence homologies range from 96.85-99.74%. Comparison of amino acid sequences shows a unique amino acid, arginine, for JaGAr01 at position 123 of the E-protein, while the eight other strains had serine. Secondary structure at 3' end of genomic RNA predicted by free energy minimization shows a unique structure for JaGAr01 that includes an RNA segment that is conserved for all flaviviruses. The role of these facts may play in the replication and antigenic characteristics of JaGAr01. In addition, phylogenic analyses of the E-protein of JaGAr01 together with 35 other JE strains show diversity in amino acid characteristics between the prototype strains Nakayama, JaGAr01 and Beijing-1. Phylogenic trees computed by maximum likelihood and parsimony analysis of nuceic acid sequences show Nakayama and Beijing is one cluster different from JaGAr01.

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CONTENTS

Original article

Eamsobhana, P., Watthanakulpanich, D., Punthuprapasa, P., Yoolek, A. and Suvuttho, S.
Detection of Antibodies to *Parastrongylus cantonensis* in Human Sera by Gelatin Particle Indirect Agglutination Test ..... 1 - 5

Ando, K., Kuraishi, K., Nishikubo, K., Asami, T., Waighet-Kouadio, P., Matsuoka, H. and Chinzei, Y.
Sporozoite Invasion of *Plasmodium berghei*, Rodent Malaria Parasite, to the Salivary Glands of the Vector Mosquito, *Aedes stephensi* an Electron Microscopic Study ..... 7 -12

Jutavijittum, P., Jiviriyawat, Y., Yousukh, A., Toriyama, K., Itakura, H., Yano, M. and Hayashi, S.
A Seroepidemiological Study on Hepatitis B Virus, Hepatitis C Virus and Human Immunodeficiency Virus Infections in Northern Thailand 13-17

Short communication

Guevara, A.G., Ruiz C., J.C., Houghton, R.L., Reynolds, L., Sleath, P., Benson, D., Ouaissi, A. and Guderian, R.H.
Evaluation of a Recombinant Protein (rTc24) and Synthetic Peptides in Anti-*Trypanosoma cruzi* Positive Samples from Blood Bank Donors in Chagasic Endemic Areas of Ecuador 19-22

Proceedings of XXXIX Annual Meeting of Japanese Society of Tropical Medicine

Contents 23-26
Prize winner's lecture 27
President's lecture 29-32
Special lecture 33-37
Educational lecture 39-46
Symposium 47-102
Work shop 103-108
Panel presentation 109-144

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