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AN IMPROVED SINGLE-STEP SCREENING METHOD FOR GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY

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Abstract: We have established a new simple and rapid screening method for glucose-6-phosphate dehydrogenase (G6PD) deficiency. The principle of this method is the formation of blue formazan with the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) produced by G6PD absorbed on a DEAE-Sephadex anion exchanger. The whole procedure is performed in a microcentrifuge tube and it can be completed in less than 30 min without any special equipment other than micropipettes. Our method is particularly suitable for field detection of G6PD-deficient subjects prior to administration of primaquine *in situ*.

Key words: G6PD deficiency, screening method, acute hemolytic anemia, primaquine

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most common hereditary disorders, which is prevalent in malaria endemic areas, probably because G6PD-deficient erythrocytes are resistant to *Plasmodium falciparum* infection (Luzzatto and Mehta, 1995). G6PD deficiency may cause various types of hemolytic anemia, most typically an acute hemolytic attack after taking certain oxidative drugs such as primaquine.

Primaquine has been widely used for the radical treatment of *Plasmodium vivax* malaria. In addition, its gametocytocidal action is effective to cut the transmission of *Plasmodium falciparum* gametocytes in endemic areas (Matsuoka *et al.*, 1987; Doi *et al.*, 1989). Primaquine-induced hemolytic crisis in G6PD-deficient individuals is a serious problem in the chemotherapeutic malaria control activities. Thus, it is important to screen out G6PD-deficient individuals before starting the operation (Ishii *et al.*, 1994). Malaria control activities are often carried out in the field where no electricity is available. In addition, many patients travel over great distances from their villages and both diagnosis and initial administration of primaquine must be completed on the same day. Under such conditions, it is necessary to complete the whole screening procedure from collect-

ing blood to interpreting the results in less than one hour without any electrical equipment. Current screening tests for G6PD deficiency including a fluorescent method (Beutler, 1966; Beutler and Mitchell, 1968) and previous formazan methods (Fairbanks and Beutler, 1962; Fujii *et al.*, 1984) do not fully meet the requirements.

We describe here an improved single-step formazan method suitable for rapid screening for G6PD-deficient subjects in the field. Our procedure can be completed within 30 min without any special equipment.

MATERIALS AND METHODS

Chemicals

Glucose-6-phosphate (G6P) and nicotinamide adenine dinucleotide phosphate (NADP⁺) were purchased from Boehringer-Mannheim (Germany) and DEAE-Sephadex A-50 was from Pharmacia (Uppsala, Sweden). 3-(4,5-Dimethylthiazolyl-1- β -2,5-diphenyltetrazolium bromide (MTT) was from Dojin (Kumamoto, Japan) and phenazine methosulphate (PMS) was from Sigma (St. Louis, MO). Other reagents were of analytical grade.

Preparation of mixtures

DEAE-Sephadex A-50 was equilibrated in 0.1 M

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Table 1 Reaction mixtures

	Standard method (μ l)	GSSG method (μ l)
DEAE-Sephadex	200	200
Substrate mix	200	200
MTT-PMS mix	200	200
4.8mM GSSG	—	200
H ₂ O	200	—
Whole blood	5	5

Tris-HCl, 10 mM MgCl₂, pH 6.5. The substrate mix contained 5 mM G6P, 0.4 mM NADP⁺, 0.2 % Saponin in H₂O and the MTT-PMS mix contained 0.025 % of each reagent in H₂O.

Procedure

All the reactions were carried out in a 1.5 ml microcentrifuge tube (Eppendorf) at room temperature. A tube containing 200 μ l each of DEAE-Sephadex A-50 gel, the substrate mix, the MTT-PMS mix and distilled water was prepared (Table 1). The reaction was started by adding 5 μ l of whole blood to the tube and mixing by shaking several times. The tube was then left to stand. A gel bed formed immediately by natural sedimentation and was clearly separated from the upper reddish aqueous layer. After 20 min incubation, the development of blue color on the gel with patient's blood was compared with that with control blood.

RESULTS

Fig. 1 shows the blue color development. With normal control samples, color development was apparent after 20 min incubation and the intensity reached a maximum after 40 min. We tested several G6PD-deficient samples with various residual activities. Samples with less than 30% residual activities showed very slow color development, and could easily be distinguished from normal samples until after 24 h of incubation. However, G6PD-deficient samples with higher residual activities showed more rapid color development and it was difficult to differentiate them from normal samples after more than 12 h incubation. Adding oxidized glutathione (GSSG) to the reaction mixture ("GSSG method" in Table 1) reduced such ambiguity in interpreting the results after prolonged incubation periods (Beutler and Mitchell, 1968). The optimal amount of blood for addition to the reaction mixture was 5-10 μ l. Although amounts up to 20 μ l were acceptable, adding more blood caused difficulty in interpreting the results.

We also found that dried blood blotted on regular

filter paper or a cation-exchange cellulose paper (Whatman, P 81) could be used as samples. After adding a piece of filter paper with dried blood to the reaction mixture, the tube was shaken several times and stood for 5 min to make remove blood from the paper. The tube was then inverted to make the filter paper attach on the reverse side of the cap and was then kept upright as usual. When testing a large number of samples, the reaction could be done in 96-well microtiter plates in place of microcentrifuge tubes using 1 μ l of blood with a one tenth reduction in reaction volume.

The stability of the prepared mixtures was examined. After one year storage of DEAE-Sephadex at room temperature, the MTT-PMS mix kept in a dark bottle at 4°C, and the substrate mix at -20°C with frequent freezing and thawing, respectively, we found no changes in their efficiency. The substrate mix and the MTT-PMS mix were also stable at room temperature for several days.

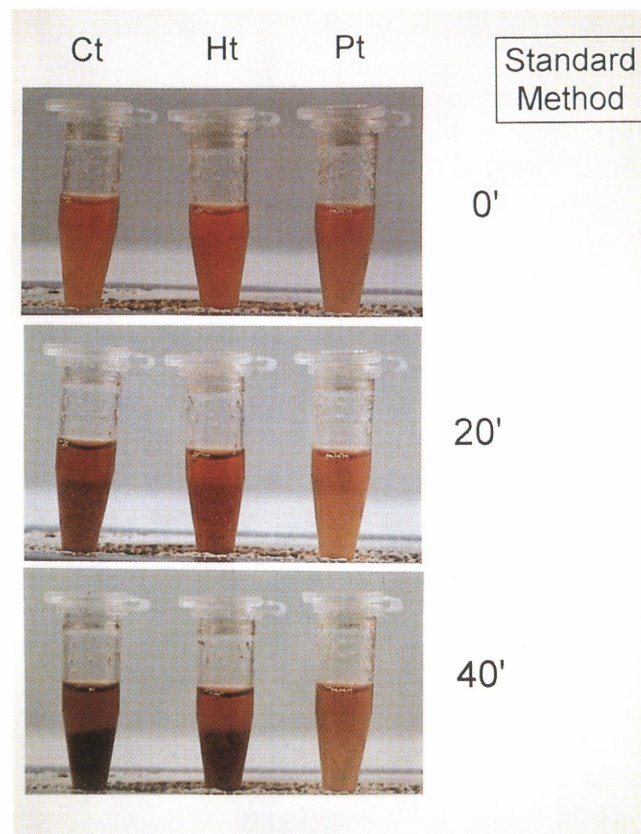


Figure 1 Blue color development in reaction tubes with blood samples from a normal control subject (Ct), a G6PD-deficient patient (Pt) and a heterozygous female (Ht).

DISCUSSION

A number of methods for rapid diagnosis of G6PD deficiency have been described (Beutler *et al.*, 1955; Brewer *et al.*, 1960; Bernstein, 1962; Fairbanks and Beutler, 1962; Beutler, 1966; Beutler and Mitchell, 1968; Fujii *et al.*, 1984). Among these, a fluorescent method (Beutler, 1966; Beutler and Mitchell, 1968) and some formazan methods (Fairbanks and Beutler, 1962; Fujii *et al.*, 1984) have been adopted. The spot test developed by Beutler and Mitchell (1968), which depends upon the fluorescence of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as an indicator of G6PD activity, was recommended as a standard screening method by the International Committee for Standardization in Hematology (Beutler *et al.*, 1979). Although the procedure is reliable, simple and can be completed in less than 30 min, it requires ultraviolet (UV) light to examine the results, which might be a significant drawback in applying the procedure to the rapid detection of G6PD-deficient subjects in the field.

The tetrazolium dye MTT forms blue colored formazan when reduced in the presence of a hydrogen carrier such as PMS. This reaction can be used as an indicator of G6PD activity by monitoring production of the potent reducer NADPH. Although the principle is straightforward, the reaction cannot be applied directly to blood samples because hemoglobin reacts nonspecifically with MTT and its dark red color strongly interferes with interpretation of the result. Several attempts have been made to overcome this problem, including absorption of either G6PD on anion-exchange cellulose paper (Fairbanks and Beutler, 1962) or hemoglobin on cation-exchange cellulose paper (Fujii *et al.*, 1984). Although these methods have the advantage of requiring no UV light when examining the results, the procedures might be too laborious and time-consuming to be carried out in the field.

Our present method also depends on formazan formation. G6PD in blood is absorbed on DEAE-Sephadex beads and separated from hemoglobin in the aqueous layer by natural sedimentation. Since the reaction progresses only in the gel, the nonspecific reaction of hemoglobin with MTT and the interference in the color development can be kept at a minimum. The bluing of the gel is apparent after 20 min incubation with normal samples and can readily be distinguished from that with G6PD-deficient samples. The whole single-step procedure can be completed in a microcentrifuge tube in less than 30 min without any special equipment other than micropipettes. In comparison with other procedures,

our method has the great advantage of its rapidity and simplicity with similar reliability, although it might not be very useful for some genetic surveys which require dealing with thousands of samples at a time. These features make our method particularly suitable for field detection of G6PD-deficient subjects prior to administration of primaquine *in situ* as well as for ordinary laboratory tests.

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APPLICATION OF THE GELATIN PARTICLE INDIRECT AGGLUTINATION TEST IN THE SERODIAGNOSIS OF HUMAN OPISTHORCHIOSIS

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Abstract: *Bithynia funiculata* snail antigens were partially purified by Sephacryl S-200 gel filtration chromatography. Four fractions of *B. funiculata* snail antigens were obtained—fraction 1 (F1), fraction 2 (F2), fraction 3 (F3) and fraction 4 (F4), which F1 was chosen for the study since this is the most abundant and most reactive fraction. Levels of antibodies in sera of 85 patients with opisthorchiosis, 15 normal healthy individuals and 72 patients with other helminthic infections were assayed against crude antigens from *O. viverrini* adult worms and the F1 fraction using the gelatin particle indirect agglutination test (GPAT). For both antigens, the mean reciprocal titer in the sera of opisthorchiosis patients was significantly higher than sera from patients with other helminthic infections and normal healthy individuals ($p < 0.0001$). In an attempt to search for another antigen in place of *O. viverrini* antigens for the serodiagnosis of opisthorchiosis, the sensitivity and specificity of the two antigens were compared. It was shown that differences in the sensitivity and specificity were not evident. This study indicates that antigens from *O. viverrini* adult worms were the most suitable antigens for serodiagnosis of opisthorchiosis by GPAT despite being crude antigens. However, antigens from partially purified *B. funiculata* snails (F1) should prove to be useful for serodiagnosis of this disease since large amounts of antigens can be obtained with relative ease.

INTRODUCTION

Opisthorchiosis caused by *Opisthorchis viverrini* is still a major public health problem in Thailand where there are at least 8.6 million Thais harbouring this parasite, particularly those residing in the northeastern part of the country (Jongsuksuntigul *et al.*, 1992). Owing to the geographical location and similar eating habits, several millions more are likely to be infected in neighboring Lao PDR, Cambodia, Vietnam and China. The people acquire the infection by consuming raw infected cyprinoid fish including undercooked fish which is a common practice among the people of the northeastern and the northern parts of Thailand. The disease is usually chronic in nature; patients with light or moderate infections rarely produce significant symptoms. However, heavy infections are associated with considerable morbidity, overt signs and symptoms, hepatobiliary abnormalities and even death (Pungpak *et al.*, 1985;

Elkins *et al.*, 1990).

Current methods for the diagnosis of opisthorchiosis are based on the demonstration of eggs in faeces of suspected individuals. Although such an examination is reliable and permits species identification of the parasites in question, it is useful only when the intensity of infection is high and reliable only in the hands of experienced personnel. However, a false negative result is not uncommon in cases with light infection or biliary obstruction. Previous efforts to develop immunodiagnostic methods for opisthorchiosis were largely unsatisfactory due to the specialized materials and reagents required and the problem of cross reactions with other helminthic infections still remain to be resolved (Sirisinha *et al.*, 1991). Although an encouraging attempt was made employing a more defined antigen (Poopyruchpong *et al.*, 1990), it could not be made available in mass quantities for routine use due to the lack of an adequate supply of the parasite's antigens. In the attempt to

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overcome this problem, the phenomenon of antigen sharing between a parasite and its snail intermediate host is currently receiving considerable attention. Several investigators have studied the possibility of using these shared antigens from snail intermediate hosts instead of those from the parasites themselves in various immunodiagnostic techniques such as complement fixation test (Fairley, 1919); immunoelectrophoresis (Capron *et al.*, 1965); hemagglutination test (Jackson, 1976; Chieffi *et al.*, 1982); enzyme-linked immunosorbent assay and enzyme-linked immunoelectrophoretic blot (Rivera-Marrero and Hillyer, 1985). In the present study; attempts to purify and produce sufficient amounts of antigens for immunodiagnosis has produced partially purified antigens from *B. funiculata*, the snail intermediate host of *O. viverrini*, which were employed in the indirect agglutination method. The reasons are that large amounts of material can be obtained rapidly and at a comparatively lower cost than *O. viverrini* antigens which require maintenance of a complex life cycle. Moreover, the indirect agglutination test is rather simple and can be performed rapidly without specialized equipment, reagents or facilities, thereby giving us a chance to explore into areas where only scanty information is being published.

MATERIALS AND METHODS

Subjects

Group A consisted of 85 serum specimens from individuals with opisthorchiasis residing in Prachinburi Province, confirmed by finding the characteristic eggs in faeces using Cellophane thick smear method. Group B consisted of 15 serum specimens from apparently healthy individuals residing in non-endemic areas who did not renew any parasitic infections upon faecal examination. Group C consisted of 72 serum specimens from individuals infected with other helminths and who were also negative for *O. viverrini* eggs. These helminthic infections are as follows: paragonimosis 8, echinostomosis 1, taeniosis 15, cysticercosis 4, sparganosis 1, gnathostomosis 7, angiostrongylosis 4, trichinosis 6, larval toxocarosis 2, ascariosis 4, trichuriasis 3, capillaritis 1, strongyloidosis 10 and hookworm infection 6. All serum specimens were frozen at -20°C until used.

O. viverrini antigen

Adult worms of *O. viverrini* were obtained from infected adult golden Syrian hamsters, *Mesocricetus auratus* and the antigen was extracted by grinding the worms in distilled water on ice. The homogenate was further sonicated (Sonicator Ultrasonic Processor,

Model XL 2020-010, Heat System, Inc., USA; Standard probe No. 419) and centrifuged in an automatic ultracentrifuge (Hitachi Centrifuge, Model SCP 85H2, Hitachi Koki 30., Co., Ltd., Japan) at 15,000 rpm at 4°C for 1 hr, after which the soluble extract was collected.

B. funiculata antigen

Uninfected *B. funiculata* snails were gently crushed and the shell removed under a stereomicroscope. Each snail was washed with normal saline solution (NSS) for several times and then examined for parasitic infections under the microscope. Only parasite-free snails were lyophilized and manually homogenized with the addition of alumina using a glass pestle and mortar on ice. Further procedures were mostly carried out as previously described above except for the speed of centrifugation which was increased to 30,000 rpm at 4°C for 1 hr. In order to minimize cross reactions, further purification was performed by using Sephacryl S-200 gel (Pharmacia, Fine Chemical, Sweden) filtration chromatography with phosphate buffered saline solution (0.15 M PBS, pH 7.2) as eluent, with a collection rate of 120 drops per tube. The fractions belonging to the same peaks were pooled, concentrated by Amicon (AM 10, Pharmacia, Uppsala, Sweden), and stored at -20°C .

Protein determination

The protein content of all sample extracts were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Gelatin particle indirect agglutination test (GPAT)

The optimal concentrations of prepared antigens (*O. viverrini* adult worms and partially purified *B. funiculata* snails) were preliminary titrated and then added to artificial gelatin particles (Fujirebio, Inc., Tokyo, Japan). Each antigen was coupled to the particles by means of tannic acid (Merck, Darmstadt, Germany) according to the method of Sato and Ryumon (1990) with some modifications as follows: the particles were washed 3 times by centrifugation at 2,500 rpm for 5 min with a 2-fold excess of 0.15 M NSS and adjusted to a 5% suspension with PBS (0.15 M, pH 7). An equal volume of 5 $\mu\text{g/ml}$ tannic acid solution diluted in PBS was added to the suspension and incubated in a 37°C water bath for 15 min with agitation every 5 min. The tanned particles were washed with cold NSS 3 times and resuspended as a 5% suspension in PBS. The suspension was then poured into tubes each containing half a volume of optimal antigen dilution. The mixture was incubated in a 37°C water bath for 1 hr with agitation every 5 min. Meanwhile, control particles were prepared by adding PBS instead of the antigens. The particles were washed with cold NSS 3 times and finally made

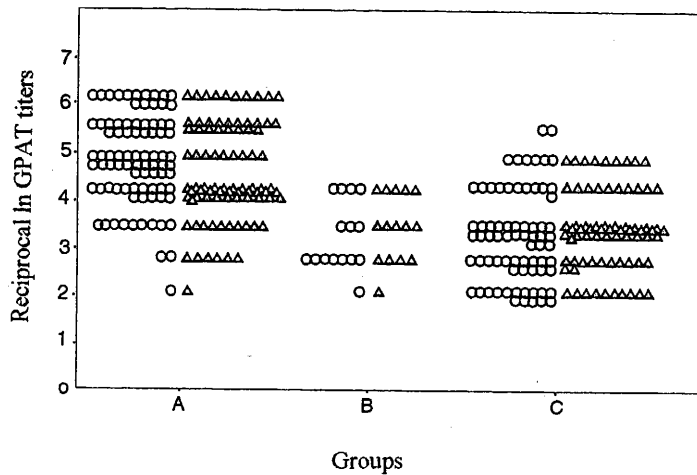


Figure 1 Distribution of GPAT antibody titers against *O. viverrini* adult worms (O) and F1 (Δ) in 85 opisthorchiosis patients (Group A), 15 normal healthy individuals (Group B) and individuals with other helminthic infections (Group C).

into a 1% suspension with inactivated normal rabbit serum (NRS). These coated gelatin particles were then ready for use in the test.

The test was performed in an U-shaped bottom microtiter plate (Falcon, Becton Dickinson and Co., USA) as described elsewhere (Sato and Ryumon, 1990; Yamashita *et al.*, 1994). One drop containing 25 μ l of 1% NRS in PBS was placed into each well using an automatically calibrated syringe dispenser (Nichiryu, Model 8100, Nichiryu Co., Japan). The test serum was dispensed into the wells starting at 1:4 dilution then a 2-fold serial dilution was carried out upto 1:2,048 dilution. A control antiserum of known titer as well as a negative control was included with each test run. Finally, 25 μ l of antigen-coated particles suspension was added to all wells except those of the second column of each microtiter plate where control particles were placed instead. The plates were thoroughly shaken in a micromixer for 2 min to completely suspend the particles. The particles were then allowed to settle for at least 3 hr at room temperature and the patterns formed at the bottom of each well were examined. The negative and positive agglutination patterns were scored in accor-

dance with Campbell *et al.* (1974): particles which settled smoothly at the bottom and center of the well indicated a negative result, while particles that diffused film agglutinated and formed a covering the bottom of the well including the edges where the film is either folded or somewhat ragged indicated a positive result. The estimation of agglutination titers was determined using the highest serum dilution that gave a positive reaction.

Statistical analysis

Sensitivity, specificity, and positive and negative predictive values of the tests were determined according to the method of Galen *et al.* (1980). Student's *t* test was used to evaluate the statistical significance of the data of Group A against those of Groups B and C.

RESULTS

Kinds and amounts of antigens prepared as antigen-coated particles

The extract of *B. funiculata* was separated by Sephacryl S-200 gel filtration chromatography into 4 fractions; namely F1, F2, F3 and F4, respectively.

Table 1 Summary of sensitivity, specificity and predictive values of the GPAT when *O. viverrini* adult worms and F1 were used as antigens

Antigens	Amount (μ g/ml)	Cut-off value for positivity (dilution)	Sensitivity (%)	Specificity (%)	Predictive values positive	Predictive values negative
<i>O. viverrini</i>	600	1:50	85.9	73.6	76.0	84.2
F1	330	1:60	80.0	72.4	73.9	78.8

However, F1 appeared to be available for the test as the amounts were in non-limited supply for sensitizing the gelatin particles. Hence, two concentrations of each antigen were prepared as follows: for *O. viverrini* adult worms and F1, 600 and 330 $\mu\text{g/ml}$, respectively.

Indirect agglutination titers of patients' sera against antigens from *O. viverrini* adult worms and partially purified *B. funiculata* snails

Both antigens prepared from *O. viverrini* adult worms and F1 were used to detect the antibody titers of 3 groups of serum specimens. The distribution of reciprocal GPAT antibody titers in the study groups for both antigens were shown in Fig. 1. The mean antibody titers determined by both *O. viverrini* adult worms and F1 in Group A were significantly higher than those in Group B and Group C ($p < 0.0001$). The mean antibody titer of normal healthy individuals upon GPAT evaluation against antigens from *O. viverrini* adult worms was determined to be 3.23 ± 0.68 . A cut off value for positivity was then established at 1:50 dilution, In titer 1: 3.91, (X+SD). Using this cut-off value, 73 out of 85 serum specimens (85.9%) of Group A were positive and 11 out of 15 serum specimens (73.3%) of Group B were negative. Of the 72 serum specimens from Group C, 53 serum specimens (73.6%) were negative. These were those with echinostomosis, cysticercosis, sparganosis, trichinosis, ascariasis, trichuriasis, capillariosis and hookworm infection, while the rest of the 19 were cross-reactive at this cut-off titer. These were those serum specimens from people infected with paragonimosis (5/8), taeniosis (6/15), gnathostomosis (2/7), angiostrongylosis (1/4), larval toxocariosis (1/2), and strongyloidosis (4/10). The sensitivity, specificity, and positive and negative predictive values were 85.9%, 73.6%, 76.0% and 84.2%, respectively. The mean antibody titer of normal healthy individuals against F1 was 3.42 ± 0.67 . A cut off value for positivity was then established at 1: 60 dilution, In titer 1: 4.09, (X+SD). Using this cut-off value, 68 out of 85 serum specimens (80.0%) of Group A were positive while 10 out of 15 serum specimens (66.7%) of Group B were negative. Group C had 53 out of 72 serum specimens (73.6%). These were those specimens from people with larval toxocariosis, trichuriasis, capillariosis, but which were also cross-reactive at this cut off titer with sera from patients with paragonimosis (3/8), echinostomosis (1/1), taeniosis (1/15), cysticercosis (1/4), sparganosis (1/1), gnathostomosis (2/7), angiostrongylosis (1/4), trichinosis (3/6), ascariasis (4/4), strongyloidosis (1/10) and hookworm infection (1/6). The sensitivity, specificity, and positive and negative predictive values were 80.0%, 72.4%, 73.9% and

78.8%, respectively. The overall results of the assay are summarized in Table 1.

DISCUSSION

After being processed through gel filtration on Sephacryl S-200, the F1 was chosen to be suitable for the sensitization of gelatin particles since large amounts were readily obtained. Besides, it was found that the F1 was the most reactive fraction against the sera samples from opisthorchiosis patients by ELISA (Waikagul., personal communication), thus further study of this fraction was attempted as to whether it, instead of *O. viverrini* antigens, can be used for the serodiagnosis of opisthorchiosis. The relationship between the GPAT antibody titer to each of the two antigens—*O. viverrini* adult worms and F1 in 172 individuals with and without *O. viverrini* eggs in their faeces were compared. When the cut-off for GPAT reading was made at the serum dilution of 1:50 and 1:60, the sensitivity of antigens from *O. viverrini* adult worms and F1 were 85.9% and 80.0%, respectively. A slightly lower sensitivity was obtained with the F1 which was similar to a previous study reported by Rivera-Marrero and Hillyer (1985) who used antigens from *B. glabrata* instead of from *S. mansoni*. This was probably due to the poorer quality of the F1 in detecting *O. viverrini* infected sera as the stimulated antibodies of the infection were actually produced by *O. viverrini* worms themselves. Although both antigens exhibited rather high sensitivity, their specificity were relatively low indicated by the large number of reactive sera from patients with other helminths which were likely not to be different from each other (73.6%, 72.4% for *O. viverrini* adult worms and F1, respectively). The cross reactions of some sera may be explained by the fact that the sera samples were obtained from people in both non-endemic and endemic areas, thereby the infected persons with other helminths may also have a very low *O. viverrini* infection where the *O. viverrini* eggs cannot be detected by faecal examination. The reactive intensity of antibodies from other helminthic infections may result in binding with the antigens, and not with the antibodies from mixed infection of *O. viverrini* worms. The quality of the antigens employed is also quite important, where fewer cross reactions were previously observed using antigens prepared by isolating and absorbing out the *Fasciola hepatica* cross reacting antigens. These made the snail antigens comparatively more specific than *S. mansoni* antigens (Rivera-Marrero and Hillyer, 1985). The occurrence of false positive results in the group of individuals with negative faecal examination was probably due to the presence of nonspecific epitopes

in the antigens which are sensitive enough to detect even 0.02–0.04 μg of corresponding antibodies by the indirect agglutination test (Campbell *et al.*, 1974). Although the main advantage of the GPAT is its sensitivity but care must be exercised to control nonspecific reactions. When it is used routinely, a suitable serum dilution (1: 60), a gel particle control, a control antiserum of known titer and a negative control should be included with each test run. This study also confirmed previous works (Sato and Ryumon, 1990; Yamashita *et al.*, 1994; Yang *et al.*, 1994; Kobayashi *et al.*, 1995) that GPAT is much simpler because it is a one-step reaction between the antigen-coated particles and the test serum, and is indeed simple for an ordinary laboratory or for a field survey to carry out for the mass screening of opisthorchiasis. Furthermore, the test requires no specialized equipment and reagents, and the processing time is much shorter than in other tests as the results can be obtained within approximately 3 hours.

When antigens prepared from *O. viverrini* adult worms and F1 were used and assayed against the representative sera from patients with opisthorchiasis, both antigens exhibited comparable sensitivity and specificity which were not so different from each other. It thus appears that the F1 could probably serve as an alternative source of antigens when used for the GPAT. Attempts are being made to improve both the sensitivity and the specificity, and includes simplifying the protocol before conducting a large-scale field trial for the next study.

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PROCEEDINGS OF XXXVIII ANNUAL MEETING OF JAPANESE SOCIETY OF TROPICAL MEDICINE

5-7 November 1997, Utsunomiya

President

Akira Ishii

Professor, Department of Medical Zoology, Jichi Medical School

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Satellite symposium:

1 A MAJOR IMMUNE RESPONSE DURING MALARIA INFECTION: ACTIVATION OF EXTRATHYMIC T CELLS IN THE LIVER

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Since *Plasmodium* (*P*) is an intracellular pathogen for hepatocytes and erythrocytes, it is suspected that conventional T cells and immunoglobulins are hardly effective for protection. We recently demonstrated the existence of extrathymic T cells in the liver. These T cells comprise self-reactive clones and abnormal self-cells might be their targets. Such abnormal self-cells include tumor cells, virus-infected cells, and listeria-infected cells. We investigated herein how extrathymic T cells were activated in malaria infection. When mice were infected with lethal or sublethal strains of *P. yoelli*, lymphocytosis was induced in the liver and spleen a week after infection. The major expanding lymphocyte subset was IL-2R β ⁺CD3^{int} cells (i. e., extrathymic T cells). In mice infected with the lethal strain, they died within a week, showing granulocytosis. Mice infected

with the sublethal strain did not show granulocytosis and the switching from $\alpha\beta$ T to $\gamma\delta$ T cells among CD3^{int} cells occurred at the restoration phase. We then examined the blood of human malaria patients. In a patient with cerebral malaria, he had liver dysfunction, severe granulocytosis, and a slightly elevated level of extrathymic T cells. These T cells could be identified as CD56⁺T or CD57⁺T cells (i. e., NKT cells). They shared the same properties with murine NKT cells, including CD4⁻8⁻ cells and $\gamma\delta$ T cells. The patients who visited to hospital with symptoms had no granulocytosis, inversely, had an increased level of extrathymic T cells such as CD56⁺T, CD57⁺T, and $\gamma\delta$ T cells. These results suggest that the activation of extrathymic T cells are a major immune response in malaria infection.

2 MITOCHONDRIA FROM MALARIA PARASITE: A TARGET OF CHEMOTHERAPY

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Understanding of the characteristic of human-parasitic stage of *Plasmodium* must contribute significantly to the development of novel strategy against malaria. Energy metabolism of the parasite is one of the target for the chemotherapy because that of the *Plasmodium* seems to be different from human host¹⁾. The favourable effect of low O₂ level on the *in vitro* cultivation of *P. falciparum* using human erythrocyte, which is now recognized as model of erythrocytic stage in human host, suggests that the parasite is microaerophilic in its erythrocytic stage. Thus, mitochondria with different function from human host seem to be critical for energy metabolism of *P. falciparum*. For example, hydroxynaphthoquinone which is the derivative of mitochondrial electron transporter coenzyme Q (ubiquinone) has been recognized as promising antimalarial compounds,

although the mechanism of the action of this compound is unknown. Furthermore, study by Hammond showed that mitochondria of *Plasmodium* are also critical for the pyrimidine biosynthesis. However, only little information has been available about *Plasmodium* mitochondria because of difficulty in preparing intact mitochondria from the parasite, although the presence of functional mitochondria during the erythrocytic stage was confirmed by the characterization of isolated mitochondria reported by Fry. Therefore, we have started molecular biological approach to *Plasmodium* mitochondria for the first step of the study.

I) Cytochrome *c* oxidase III (COIII) gene of *Plasmodium vivax*

In malaria parasites, there is a 6 kb DNA element which encodes cytochrome *c* oxidase subunit I, subunit

III (COIII), apocytochrome *b* and fragmented ribosomal RNAs genes. Previously, we have amplified a partial DNA fragment of the COIII gene of *Plasmodium vivax*, the human malaria parasite, using PCR primers derived from *P. falciparum* sequence². In this study, we amplified two other DNA fragments of *P. vivax* using PCR primers derived from either *P. falciparum* or *P. vivax* sequences to cover the whole COIII gene region. The possible open reading frame in the determined sequence is 792-nucleotides long. The complete COIII sequences of *P. vivax* and *P. falciparum* are 71% nucleotide and 73% amino acid identical, while the *P. vivax* and *P. yoelii* sequences are 81% nucleotide and 82% amino acid identical. We have detected RT-PCR products using COIII gene specific primers and oligo(dT)-or random-primed cDNA from poly(A)⁺RNA from the erythrocytic stage of *P. vivax*. The results suggest that the COIII gene of *P. vivax* is functional in its erythrocytic stages and that the COIII gene transcript has the 3'-poly(A) sequence. The recent results of crystallographic studies on bacterial and bovine cytochrome *c* oxidase revealed the 3 dimensional structure of this subunit. The sequence homology of COIII of *Plasmodium* to those of other organisms suggested the presence of similar, but not identical structure. The deletion specific for *Plasmodium* COIII were found in the N-terminal region, helix I and hydrophilic region between helices III and IV. Such difference especially from the counterpart of human, the host organism, may relate to the different interactions with other components involved in electron transport².

II) Complex II (succinate-ubiquinone oxidoreductase/fumarate reductase)

Complex II is well known marker enzyme for mitochondria and functions as succinate dehydrogenase (SDH) in the respiratory chain of aerobic energy metabolism. In addition, complex II of many parasites exhibit high fumarate reductase activity (FRD), reverse reaction of SDH, and play a key role as a terminal enzyme in the anaerobic electron-transport chain, the NADH-fumarate reductase pathway. Complex II is generally composed of four polypeptides and appears to be highly conserved enzyme complex. The largest flavoprotein

subunit (Fp) with a molecular weight of about 70 kDa contains covalently bound flavin (FAD; flavin adenine dinucleotide) as cofactor, and the second largest subunit with a molecular weight of about 30 kDa contains three distinctive iron-sulfur clusters, S-1 [2Fe-2S], S-2 [4Fe-4S], S-3 [3Fe-4S], and is referred to as the iron-sulfur-subunit (Ip). These subunits forms rather hydrophilic catalytic portion of the enzyme complex, transferring reducing equivalents from succinate to water-soluble dyes such as DCIP (SDH) or reduced methyl viologen to fumarate (FRD). In the present study, the amino acid sequences of Fp and Ip subunit in *P. falciparum* mitochondria were deduced from genomic clones, and compared with those of other parasites such as *A. suum* as well as that of human host. The deduced amino acid sequences show that N-terminal domains of both Fp and Ip are rich in lysine, threonine and serine residues, which is typical characteristics of mitochondrial targeting sequence. Comparison to other species shows the basic structure including domains essential for enzyme-activity is well conserved. However, detailed sequence comparison also reveals unique sequence in *P. falciparum* Fp; one is deletion near C-terminus and the other is insertion near the domain interacting with AMP portion in the FAD. Sequences analyzed so far show that the deletion near C-terminus is common only amongst complex II of unicellular organisms; bacterial-, yeast mitochondrial- and that of *P. falciparum*. Insertion near the AMP-interacting domain is found in genome-DNA of four *P. falciparum*-isolates (K1, FCR3, THAI-K and SOLOMON-A). RT-PCR confirms the presence of this insertion sequence in mRNA, indicating the expression of this inserted region as peptide in the final product.

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3 CHEMOTHERAPY AND DRUG RESISTANCE OF FALCIPARUM MALARIA IN THAILAND

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In Thailand *Plasmodium falciparum* has become resistant to almost all available antimalarials. This is a particular problem in the border areas where most malaria cases are recorded. For years chloroquine and Fansidar have ceased to be effective. In recent years new drugs such as mefloquine and halofantrine have also lost their efficacy. Today, the treatment of uncomplicated infections relies on the combination of mefloquine and the Qinghaosu derivative artesunate. The adjunction of a 3 day course of artesunate to high dose mefloquine is still over 95% effective and seems to interrupt the transmission of falciparum malaria. Quinine the

drug of choice of severe and complicated malaria is also losing efficacy. This evolution brings closer the prospect of untreatable malaria. The spread of multi-drug resistant parasites is probably accelerated by the population flows of migrant workers from neighboring countries to and within Thailand. This poses a real threat not only to the local populations but also to the world at large and especially to Africa, the most affected continent. There is an urgent need for a new strategy for the use of antimalarial drugs and to address the question of migrant populations.

4 BIOLOGY OF MALARIA PARASITE AND MOSQUITO VECTOR

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Experience of past malarial control strategies, of current research effort, and of predictive mathematical modelling suggest strongly that future attempts to limit malaria should include strategies targeted to the parasite in the mosquito vector. Such strategies will include environmental management, suppression of vector-man contact, and measures directed specifically against the parasite in the vector. To date the only effective antiparasitic measures available were based upon the use of gametocytocidal or sporotocidal drugs. Over the past 5 years there has been a reawakening of scientific interest in the development of drugs, vaccines and other strategies against the parasite in the mosquito vector. This interest has led to a greater understanding of the biology of the interaction of the malarial parasite with its mosquito vector.

This review will address these interactions at 3 levels.

1. The activation of the gametocyte from its quiescent state (Go) in the peripheral blood such that it rapidly undergoes gametogenesis and fertilization.
2. The interactions of the zygote/ookinete with the mosquito bloodmeal and the mosquito midgut. These interactions contribute to the survival of the parasite in the 'hostile' environment of the midgut, and the specific signalling required to ensure the successful escape of the ookinete from the midgut and its subsequent differentiation into an oocyst.
3. The migration and survival of the differentiating malarial sporozoite from the oocyst, through the haemocoel to the salivary glands.

Prize Winner's lecture

JSTM (Japanese Society of Tropical Medicine) Young
Investigator Award

**INDUCTION OF ANTI-MALARIAL TRANSMISSION BLOCKING IMMUNITY WITH A
RECOMBINANT ANTIGEN OF *P. BERGHEI* PRODUCED IN SILKWORM LARVAE
USING THE BACULOVIRUS EXPRESSION VECTOR SYSTEM**

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I have studied Pbs21, a major ookinete surface protein of *Plasmodium berghei*, for the development of a model transmission blocking immunogen. In the mouse, recombinant Pbs21 expressed in the *Escherichia coli* expression system (EcrPbs21) is not as effective in inducing transmission blocking antibodies as native Pbs21 (nPbs21), possibly because of differences in post-translational processing between EcrPbs21 and nPbs21 (Matsuoka *et al.* Parasite Immunology 16: 27-34 1994). In an attempt to improve the efficacy of the recombinant molecule, I used a baculovirus expression vector system in the silkworm *Bombyx mori*. Following an injection of recombinant baculovirus containing Pbs21 cDNA, *B. mori* larvae produced recombinant Pbs21 (BmrPbs21) with a molecular weight indistinguishable from nPbs21. Fifty micrograms of BmrPbs21 could be purified from the hemolymph of each infected larva

using affinity chromatography. Immunization of BALB/c mice with BmrPbs21 induced high anti-BmrPbs21 and anti-ookinete antibodies but low anti-EcrPbs21 antibody. In contrast, EcrPbs21 induced high anti-EcrPbs21 antibody but low anti-BmrPbs21 and anti-ookinete antibodies. This suggests that most B-cell epitopes on nPbs21 are conformational and that many of the linear epitopes in EcrPbs21 are not normally exposed in nPbs21. Oocyst formation in *Anopheles stephensi* mosquitoes, which fed on mice immunized with purified BmrPbs21 and infected with *P. berghei*, was blocked by 85.5-97.1%. These results suggest that the baculovirus-silkworm system produces useful quantities of recombinant Pbs21 which in limited studies is structurally and immunogenically indistinguishable from the native molecule (Matsuoka *et al.*, Vaccine 14: 120-126, 1996).

President's lecture

MALARIA: MICRO AND MACROSCOPIC RESEARCHES; AN APPRAISAL

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For the first time in the human history, World Health Organization had launched a global malaria control program in 1957. Malaria eradication program (MEP) with its initial brilliant success in 1960's met with difficulties such as environmental problem of DDT, appearance of insecticide resistant anopheline mosquitoes and subsequently had to resort to malaria control program (MCP). The world today do not have sufficient measures to combat the resurgent of malaria. The malaria parasite competes with man for survival. Scientists of the world are striving in malaria research and yet a suitable vaccine against malaria has not been accomplished. We are to compete in ideas and finally in results. Researches in microscopic world have been blooming in the world utilizing biochemical and molecular biological methodologies. In Japan also there are many researchers who are now involved in research in molecular biology supported by a new research grant. On the other hand, research works in macroscopic world are not enough and rather lacking in Japan. There is a necessity to have a well balanced development of researches both in micro and macroscopic field. Even if any protective malaria vaccine were available at hand, there is a report suggesting possible future failure of community protection of malaria based on model prediction. We have been attempting selective age-group chemotherapeutic malaria control with mass examination and treatment using not only chloroquine but also gametocytocidal primaquine based on the results obtained in Indonesia and the Solomon Islands. Possible adverse effect of hemolysis with primaquine was excluded by detecting G6PD deficiency with Fujii method. In North Sumatra, Indonesia where they have mesoendemic malaria we could bring down both parasite rates and spleen rates after our interventive operations. In Guadalcanal, the Solomon Islands, where they suffer from holoendemic malaria, we chose three villages namely; village A treated with chloroquine (ch) and primaquine (pr), village B with ch only and village C with ch and pr with bed net. We visited 1-2 times a year on a mobile unit with necessary equipments as a selective primary health care activity. Results were not favorable as in Indonesia due to reinfection as a consequence of intense transmission of holoendemic malaria,

however villages treated with primaquine showed significantly lesser parasite rates. We tried to predict the result with computer simulation based on a mathematical model. The model (Ishikawa) was a modified model of DMT-Collet. It showed that vectorial capacity of the vector mosquito has the greatest influence on the malaria transmission and even if the parasite rate decreased it will return to even higher level than before after 1-2 years. If we could reduce the vectorial capacity of the vector mosquito by any possible measure such as bed net, we would be able to maintain a decreased level of parasite rate for 4-5 years with chemotherapeutic operation once a year. Chloroquine has reduced the mortality of malaria. However it would be an endless effort to diagnose and treat cases by constructing clinics with tremendous financial investment unless we could block transmission of malaria. We have shown that using a gametocytocidal drug primaquine we could decrease parasite rate especially that of *P. falciparum*. However, it is still necessary to develop malaria vaccine as a laboratory microscopic research to prevent the decrease of immunity against malaria in the community. We propose that even now it would be possible to control malaria by introducing selective age group chemotherapeutic control with addition of primaquine to kill gametocytes especially in mesoendemic region. In holoendemic region it would be necessary to reduce vectorial capacity of the vector mosquito by the use of vector control such as bed net. This could be conducted in villages by using mobile unit in selective primary health care system.

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Invited lectures

1 THE SPf66 MALARIA VACCINE: LESSONS FROM THE THAI TRIAL

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The synthetic polypeptide malaria vaccine SPf66 was tested in malaria endemic area on the Western border of Thailand 1,349 children age 2–15 years were recruited and vaccinated with either GMP made SPf66 or Engerix-B vaccine. They were followed daily for 15 months after the third dose. The study had 90% power to detect a 30% reduction of first clinical *P. falciparum* malaria episodes when compared to the control group. The SPf66 vaccine was safe and immunogenic but gave no protection. Further studies with SPf66 are being carried out in Tanzanian children. This trial was the most detailed and carefully conducted malaria vaccine

study carried out. It followed 2 years on detailed epidemiology at the study site. Despite this, the study failed to demonstrate any protective effect of the vaccine against *Plasmodium falciparum*. This is in contrast with the results of previous trials conducted in South America and Africa. Possible explanations for these conflicting results will be discussed. In Thailand where the race against the spread of drug resistance is being lost, this result implies that the control of malaria relies more than ever on early diagnosis and treatment and on vector control.

2 THE REGULATION OF INFECTIVITY OF MALARIAL PARASITE TO THE MOSQUITO VECTOR

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The mature sexual stages of malaria, the gametocytes, that circulate in the peripheral blood of the human/vertebrate host are exclusively responsible for the transmission of *Plasmodium* to the mosquito vector. However the infectivity of malarial infected hosts to the mosquito is not directly related to the number of gametocytes ingested into the mosquito bloodmeal. Recent studies have shown that gametocyte infectivity is regulated by specific and non-specific factors that are produced by the vertebrate host in response to malaria (or other) infections; by factors produced by the mosquito vector, by factors produced by the parasite itself, and by interactions between two or more of the above. Examples of host factors include, specific antibodies, cytokines and related non-specific killing mechanisms (e. g. macrophages and nitric oxide), and simple physiological changes in the host blood (e. g. HCO₃ concentration and pH). Examples of mosquito factors include signals to exflagellation (gametocyte activating factor), the bifunctional midgut proteases, and the peritrophic matrix. The parasite responds in a dynamic way to the above factors exploit-

ing some to act as developmental cues for the regulation of development, or by singularly designing its own differentiation to circumvent otherwise deleterious events. This delicate equilibrium can be disturbed by specifically targeted intervention strategies (e. g. transmission blocking vaccines and sporontocidal/gametocytocidal drugs), but recent studies have shown there is the possibility of previously unforeseen interactions following the treatment of patients with schizonticidal drugs (e. g. chloroquine) that result in the active enhancement of transmission of the parasite to the mosquito vector. Together these studies highlight the paramount importance of understanding the biology of the malarial parasite in the living hosts (man *and* mosquito) if we are to develop and apply control strategies effectively. In the immediate future, the data presented suggest we should reconsider how we currently use chloroquine for malaria treatment, and in the longer term we must evaluate all new antimalarials against all stages of the life cycle if we are to design effective methods for their use in the field.

3 POLIOMYELITIS ERADICATION PROGRAMME IN THE WHO WESTERN PACIFIC REGION

SHIGERU OMI

Communicable Disease Prevention and Control, WHO Regional Office
for the Western Pacific

Expanded Programme on Immunization in developing countries started in mid 1970s and since then immunization coverage has been improved for EPI six target diseases (measles, poliomyelitis, tuberculosis, tetanus, whooping cough and diphtheria). In 1988 routine immunization coverage as a whole reached 80% at global level. As the immunization coverage has improved, the incidence rate of those six target diseases has declined dramatically. Based upon these achievements, the World Health Assembly passed a resolution to eradicate poliomyelitis globally by the year 2000.

In the Western Pacific Region, to meet this target three major strategies were adopted:

- (1) to sustain the routine coverage to the level of 80%;
- (2) to conduct subnational immunization activities such as National immunization Days to interrupt transmission of the poliovirus;
- (3) to institute acute flaccid paralysis (AFP) surveillance followed by virological confirmation.

Thanks to the strong government commitment and support from partner agencies including that of JICA, the number of cases reported fell drastically from more than 5,000 in 1990 to 9 wild virus associated cases in 1997. These nine cases are reported from Mekong Delta of Viet Nam and Cambodia. In order to eliminate the remaining foci of poliovirus transmission those countries conducted, in addition to National Immunization Days, High Risk Response Immunization activities in May-June 1997 to reach the previously unimmunized children, using the "boat-to-boat" or "house-to-house" strategy. The Region as a whole has had no case since the last case from Cambodia which had onset on 19 March 1997. To make sure that the poliovirus is totally eliminated from the Region, the Regional Commission for Certification of Poliomyelitis Eradication has been established. The Commission has developed criteria that have to be met by all the Member States in the Region before certification can be declared.

Special lecture

**NEW HEALTH STRATEGY OF THE WORLD HEALTH ORGANIZATION
IN THE WESTERN PACIFIC REGION**

S. T. HAN

World Health Organization Regional Office for the Western Pacific

As one of six regional offices of WHO, the Western Pacific Regional Office is mandated to improve the health status of people in the Western Pacific Region. Since the establishment of the Regional Office, many communicable diseases in the Region have shown decreases in incidence and prevalence. In particular, in 1997 as of October, only nine wild poliovirus associated poliomyelitis cases had been reported, compared with 6,000 cases in 1990. In addition to changing disease patterns, WHO has to take account of the broader issues affecting health status, including urbanization, increasing populations of older persons and increases in diseases associated with lifestyles. One of the most important current issues is emerging and re-emerging communicable diseases. In the last two decades, more than 30 new and highly contagious diseases have been identified worldwide (e. g. HIV/AIDS, virulent Ebola type viral haemorrhagic fever). At the same time, some diseases we thought had been controlled have re-emerged (e. g.

tuberculosis, diphtheria, cholera, plague, dengue fever). Because of this, a seventh regional priority, management and control of emerging and re-emerging diseases, was added to the existing regional priorities at the forty-seventh session of the Regional Committee in 1996. The other regional priorities are: development of human resources for health, eradication and control of selected communicable diseases, health promotion, environmental health, exchange of information and experience, and strengthening management. To meet these new challenges, we need new approaches to health and human development in the 21st century. The policy of the Regional Office for the Western Pacific is articulated in the document, *New horizons in health*. This adopts a holistic approach and emphasizes promotion and protection of health and actions that can be taken by individuals in communities supported by sound public policies.

Symposium: Hepatitis and hepatocellular carcinoma in Asia

1 HEPATITIS VIRUSES: SUBTYPES AND VARIANTS

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Every isolate of extant viruses has his/her own history of evolution, but his/her family tree may be at least partly shared by someone else. For example, isolates-1, -2, -3 belonging to separate subtypes (genotypes) of same viral species must have shared a common ancestor some hundreds, thousands, or more years ago (another common ancestor for isolates -4, -5, -6 derived from separate viral species of same virus genus is much older). Diversification of an ancestral virus into a spectrum of mutually different progenies we see today is due to errors in replication of viral genome—mutations—over a large scale of time that has elapsed until now. Selected/survived mutants (even “wild-type” is also a mutant if viewed from its ancestral virus) conform to the set of subtypes (or genotypes), exemplified by the four major HBsAg subtypes (*adr/adw/ayr/ayw*) of hepatitis B virus (HBV) and much greater number of genotypes (1a/1b/1c/2a/2b/2c/2d/2f/3a/3b...) of hepatitis C virus (HCV). Examining such subtypes of viruses is of various interests. For example, HBV subtypes are of values in understanding historical migration of human populations worldwide, because HBV subtype functions as a maternal genetic marker as mitochondrial DNA does (HBV is vertically transmitted from generation to generation mostly by matern-

infant route). On the other hand, evolution of mutants is also occurring contemporarily: it occurs even in a single infected host during the course of persistent infection. Mutability favors virus to survive against various selective pressures. A hallmark in this context is HCV, who undergoes successive mutations during persistent infection in individuals. Mutation in HCV genome occurs most frequently within the site called “hypervariable region (HVR)” that resides at 5' terminus of its envelope-coding region, E2/NS1. Missense mutations in HVR lead to emergence of quasispecies (a swarm of microvariants), each of which bears different antigenicity, allowing the virus to escape antibodies produced by hosts. Antibody-escape variants have also been observed in HBV, often in babies who were given hepatitis B immune globulin (HBIG) and vaccine to prevent mother-to-infant transmission of HBV. Escape-mutation is also found in regions other than structure-protein-coding regions. HBV undergoes mutations within its pol region, which favors for HBV to escape from drugs that suppress the activity of HBV DNA polymerase. Today's variants may become tomorrow's wild type. Man-made variants (e. g., vaccine-induced HBV mutants) are now emerging. We must keep watching what happens on viral genomes.

2 EPIDEMIOLOGY AND CONTROL OF HBV, HCV INFECTION IN JAPAN

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Today, serological diagnostic methods for each 6 hepatitis viruses ie; hepatitis A-G viruses are established And as a result of large-scale sero-epidemiological survey has been done for each hepatitis virus, it has become clear that our control efforts should be concentrated mainly on hepatitis B virus (HBV) and hepatitis C virus (HCV) infections in Japan. As to HBV infection, post-transfusion hepatitis B has been successfully prevented after the additional introduction of anti HBe screening in 1989 by HBsAg screening test.

And mother-to-infant (vertical) transmission of HBV has also been successfully controlled since the nation-wide preventive measure has started in 1985. As the result of reduction of the infection sources in the next generation and due to good sanitary condition prepared in our society, another routes of horizontal transmission of HBV except for sexual transmission has become very rare. With regard to HCV infection, post-transfusion hepatitis C has almost been eradicated after the introduction of highly sensitive and specific screen-

ing test ie; 2nd generation anti HCV detection system since February, 1992. And the results of large scale sero-epidemiological survey for HCV infection have taught us that the reproduction of HCV carrier due to not only horizontal but vertical transmission has very low incidence in Japan in recent years. Based on the sero-epidemiological information described above, it

has become clear that our efforts should be concentrated to establish the system of health care for HBV, HCV unconscious carriers exist in our society. Today, I would like to present total feature of sero-epidemiological background on HBV as well as HCV infection in Japan.

3 VIRAL HEPATITIS AND HEPATOCELLULAR CARCINOMA IN ASIA: CLINICAL FEATURES AND THERAPY

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Six hepatitis viruses have been identified to date: A, B, C, E, E, and G. Of these hepatitis viruses, A and E are transmitted orally and cause hepatitis A and E. Hepatitis A and E are seldom subclinical; in almost all cases, the typical symptoms of acute hepatitis appear followed by cure except in the rare instance of fulminant hepatitis developing, in which case death may result. Since they are self-limited diseases, therapy consists of only treatment of symptoms. Hepatitis B, C, D, and G viruses can cause persistent infection (carrier state) in addition to acute hepatitis. With hepatitis B contracted in infancy and hepatitis C regardless of age of onset, about 60% of patients become carriers. The typical symptoms of acute hepatitis, although not to the degree as those observed with hepatitis A, can be observed with hepatitis B and D, whereas hepatitis C is often asymptomatic and hepatitis G often goes unnoticed. Fulminant hepatitis is observed with hepatitis B and D. Carrier status with these hepatitis viruses can progress to chronic hepatitis and then to liver cirrhosis and hepatocellular carcinoma. This is particularly of concern with chronic hepatitis B and C. Hepatitis D is rare in Asia except for Taiwan, and hepatitis G has little association with disease progression.

Therapy for acute hepatitis is basically the treat-

ment of symptoms. Treatment with interferons (IFN) can be considered for some cases of hepatitis C. Antiviral therapy (IFN, Ara-A, lamivudine, etc.) is conducted for the treatment of chronic hepatitis B, but basically, this therapy relies on viral suppression by host immunological reaction by reducing the virus pool. Immunomodulators (corticosteroids, propagermanium, etc.) are also used, but they aim to achieve viral suppression by modifying host immunological reaction. Glycyrrhizin and ursodeoxycholic acid preparations are also used to suppress hepatitis reaction. In all cases, the results achieved are inadequate, but this is not considered a problem except in about 10% of cases since this disease tends to resolve naturally. Chronic hepatitis C is a progressive disease and is therefore of serious concern. IFN is used for the purpose of viral eradication. This is successful in about 30% of patients and in an additional 30%, suppression of hepatitis reaction is achieved. The suppression of hepatitis reaction leads to the delay in disease progression and hence the development of hepatocellular carcinoma. Glycyrrhizin and ursodeoxycholic acid preparations which also suppress hepatitis reaction are also used, and they generally achieve their goal in more than 80% of cases.

4 VIRAL HEPATITIS AND HEPATOCELLULAR CARCINOMA IN CHINA

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For an exact comprehension of the etiology of liver diseases in China, information based on our recent studies and current references was summarized. HBsAg was proved persistent in 9.7% of general population in China, in which a considerable variation was suggested in different regions and gender. The perinatal infection was believed as the most important transmission route of HBV, which was strongly supported by the evidence that 7.8% babies (<1yr) were HBsAg carriers. In our studies, HBsAg was verified in 10.8% of general population, 70% of Chronic Liver Disease (CLD) and 71.5% of Hepatocellular Carcinoma (HCC) patients in Sichuan Province, which were consistent with an overall results from nationwide researches, in which HBsAg was shown in 33% of Acute Hepatitis (AH), 74% of Chronic Hepatitis (CH), 78% of Liver Cirrhosis (LC) and 71% of HCC cases. On the other hand, anti-HCV was shown

in 3.2% of general population in a nationwide epidemiological study recently, while only one case was proved anti-HCV positive among 517 cases (0.19%) from countryside in our study. And also, anti-HCV was shown in 10-40% of the CLD and HCC patients in some reports, but 5.3% and 22.2% in Sichuan Province, in which more than half of them are co-infected with HBV. In conclusion: (1) Both HBV and HCV are hyperendemic in China, (2) HBV is the main cause of viral hepatitis and HCC in China, (3) More than half of HCV infection are co-infected with HBV, (4) HCV infection is not so prevalent in some rurals. Not only the prevention of HBV infection is an absolute precedence to make viral hepatitis under control, but also efficient steps to prevent the transmission of HCV are urgently needed in China right now.

5 NATURAL HISTORY AND MANAGEMENT OF CHRONIC HEPATITIS C

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Hepatitis C is a big public health problem worldwide. The prevalence of anti-HCV in most countries is between 0.4% to 1.0% while in Indonesia it is 0.5% to 3.4%. The natural history of hepatitis C is still an uncertain issue. While it is very important to know what will be the natural history of the disease, so that the doctors are able to inform the carriers and what will be the right decision for treatment. Follow up studies of hepatitis C reveals that the cirrhosis of the liver (LC) and hepatocellular carcinoma (HCC) will develop in 30% to 40% and 15% to 19%, respectively. Our previous figure of the development of HCC from LC was 9% to 13%. Below, some facts on cases with chronic hepatitis C in Jakarta will be presented. Four cases out of 96 chronic hepatitis C cases developed HCC after a period of 2 to 11 years of follow up.

The predictive factors for the progressivity of the

disease are:

1. Viral related factors (viral dose, genotype and quasispecies).
2. Host factors (age of the time of infection and race, gender geographic location); and
3. Extraneous influences

The management of hepatitis C is mostly by administration of interferon α . The first therapy standard is 3 MU, SC injection, 3 times weekly, 6 months. The result is not satisfactory, the biochemical end treatment response (ETR) is around 40%—50%. While sustained response (SR) is 15%—20%. The virological response of ETR: 30%—40% and SR: 10%—20%. Looking at the “unsatisfied result”, the consensus is to increase the duration of treatment to 12 months, increase the frequency and administration to daily to give combination treatment with Ribavirin. A small experience from our

department reveals an encouraging result probably because of very selective cases. Complete response was observed in 52%, partial response in 22% and non-response in 22%. Relapse after 6 months was 22%, and 41% remained sustained responders. It seems that the

important factors associated with a favorable response to treatment include HCV genotype 2 or 3, and low serum HCV RNA level (less than 1 million copies/ml and absence of cirrhosis).

General presentation

1 PURIFICATION AND MOLECULAR CHARACTERIZATION OF A NOVEL NEUTROPHIL CHEMOTACTIC FACTOR FROM *TRITRICHOMONAS FOETUS* ORGANISMS

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A neutrophil chemotactic factor (TfNCF) was isolated from the crude extract of *Tritrichomonas foetus* organisms by a combination of anion exchange chromatography on DE52 and gel filtration on Sephacryl S200. TfNCF showed homogeneity by both polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE. The molecular weight of TfNCF was estimated to be 22KDa and 24KDa, by Sephacryl S200 gel chromatography and by SDS-PAGE under reducing conditions. Immunization of TfNCF caused almost complete protection against *T. foetus* infection in mice. Western-blot analysis probed with anti-TfNCF antibody showed that the epitopes on TfNCF were not commonly shared on the other components of *T. foetus* organisms nor other helminthous parasite-derived components. Furthermore, preincubation of neutrophils with antigens of other helminthous parasites or fMLP did not affect the neutrophil chemotactic activity for TfNCF. These results suggest

that TfNCF is a novel NCF consisted with unique epitopes for both antigenicity and neutrophil chemotactic activity. To clarify the molecular structure of TfNCF, cloning of cDNA encoding TfNCF was performed. Antibodies to a TfNCF were used to screen a *T. foetus* cDNA expression library in lambda gt11. Thirteen positive clones were isolated, and the screening was repeated twice. Sequence analysis revealed that all positive clones were homologs of iron-containing superoxide dismutase (SOD). Native gel electrophoresis showed that the antibodies indeed recognized *T. foetus* antigens with SOD activity. Histidine-tagged forms of *T. foetus* SOD was expressed in *E. coli*, and, after purification, found to have neutrophil chemotactic activity similar to the non-recombinant factor purified from *T. foetus*. Identification of this neutrophil chemotactic factor as SOD provides additional insight into the host-parasite interaction.

2 *TRYPANOSOMA CRUZI*: ROLE OF GLYCOLIGAND-BINDING (LECTIN-LIKE) SITES IN PARASITE-HOST CELL INTERACTION

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Trypanosoma cruzi is an intracellular protozoan parasite represented by many strains, each one harboring common and distinct surface molecules involved in host cell interaction. Since also several cell types from various hosts can be infected by trypomastigote forms, the delineation of both parasite ligands and host cell receptors which could act during this interaction is a

demanding task. Since the binding of *T. cruzi* infective forms to host cells involves glycan-lectin recognition, carrier immobilized carbohydrate structures can probe their contribution to interfere with such a recognitive citoplay. Here we employed neoglycoproteins (NGs), derived from p-aminophenyl- α - and β -N-acetyl-D-galactosaminide (GalNAc), p-aminophenyl- β -N-

acetyl-D-glucosaminide (GlcNAc) and p-aminophenyl- α -D-mannopyranoside (Man) covalently linked to bovine serum albumin (BSA) as carrier, to detect lectin-like sites on *T. cruzi* trypomastigote forms and LLC-MK₂ non-infected host cells, cultured in microplates. The NGs were biotinlabeled and employed in binding assays to determine, by Scatchard analysis, the mean value of bound neoglycoprotein molecules per parasite and host cell, as well as the affinity constants (K_d) of the molecules association. The bindings were partially reversible and saturable. The NGs were also labeled with FITC and employed in a direct fluorescence assay (IF) with trypomastigote collected at different times after *in vitro* infection. The IF analysis revealed

that expression of glycoligand-binding (lectin-like) molecules on trypomastigotes increases according the period of the time elapsed after cell infection. Infected cells were also highly fluorescent, whereas exposure of noninfected host cells to the probes led only to faint IF patterns. Parasite sub-agglutinating concentrations of NGs were tested in order to verify whether the lectin-like sites are involved in trypomastigote-host cell interaction. Inhibition of trypomastigote interiorization to LLC-MK₂ *in vitro* cultured host-cells was observed with the three NGs, the maximum value being 77% when GalNAc was added to the system. These results strongly support the notion for an involvement of lectin-like molecules in *T. cruzi*-host cell interiorization.

3 AN EXPERIMENTAL STUDY OF THE EFFECT OF LPS DERIVATIVE AGAINST LEISHMANIAL MOUSE

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Antimoniate is frequently used for the treatment of cutaneous leishmaniasis [CL]. It is well known that antimoniate causes severe liver and renal disfunction, so other drugs having less adverse reaction than antimoniate should be developed. Our previous data showed that Lipopolysaccharide (LPS) derivative induces TNF- α release from macrophage depending on LPS derivative dosage. This indicates that LPS derivative may activate the function of macrophages. Activation of parasitized macrophage is expected to inhibit *Leishmania* parasites proliferation because *Leishmania* amastigotes proliferate by binary fusion in the cytoplasm of host macrophage.

Total 30 of BALB/c mice, *Leishmania amazonensis* parasites-inoculated to their back skin were examined. The mice were divided into four groups as follows: A; LPS free solution, topical injection, B; LPS free solution, peritoneal injection, C; LPS derivative, topical injection and D; LPS derivative, peritoneal injection. LPS derivative 30 mg/kg twice a week for 8 weeks was injected topically or peritoneally. The size of lesion was measured before and 8 weeks after the LPS derivative administration. Enlargement ratio (ER) was found as

follows: ER (%) = area of 8 weeks after the administration/area before the administration. Skin samples were biopsied from the lesion 8 weeks after the administration, and then investigated pathologically.

The ER rate of Group A (7 mice) was 423.0%, that of Group B (8 mice) was 927.3%, that of Group C (6 mice) was 130.8%, and that of Group D (6 mice) was 254.0%. The ER rate of Group C was definitely decreased, compared with that of Group A, and the ER rate of Group D was relatively decreased, compared with that of Group B. Enlargement of the lesion of the LPS derivative-topically injected group was significantly inhibited than LPS free solution-topically injected group. Enlargement of the lesion of LPS derivative-peritoneally injected group was significantly inhibited than LPS free solution-peritoneally injected group. *Leishmania* parasites were pathologically observed in the skin biopsied sample taken from all groups. Ultrastructural study of the skin samples taken from visibly healed mice in LPS derivative-topically injected group showed no *Leishmania* parasites outside of macrophages.

In contrast to this, many *Leishmania* parasites were observed inside and outside of macrophage of the speci-

mens taken from the other group. LPS derivative is thought to act as leishmaniastatic.

4 CRYPTOSPORIDIOSIS IN HIV-SEROPOSITIVE AND SERONEGATIVE SUBJECTS IN SOUTHERN THAILAND

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The prevalence of *Cryptosporidium* infection in 61 HIV-seropositive and 61 HIV-seronegative subjects (aged less than one to 67 year) in Songkla City, Southern Thailand was studied by centrifugal floatation method using sucrose solution. Most of the HIV-seropositive subjects (72%) were at their twenties and thirties. *Cryptosporidium* oocysts were detected in 10% (6/61) of HIV-seropositive and in 2% (1/61) of HIV-seronegative subjects. Infection rates in these two groups were not statistically significant ($P > 0.05$). The

number of *Cryptosporidium* oocysts range between one to over 12,000 per 20 microscopic field. Among the seven *Cryptosporidium*-positive patients, six were adults (aged 18–42 years) and one was three years old child. All of the seven *Cryptosporidium* infected subjects were male, and two of them were passing formed (normal) feces. Biochemical findings revealed dishepatica in five of six *Cryptosporidium* infected HIV-seropositive subjects.

5 GROWTH INHIBITION OF *ENTAMOEBIA HISTOLYTICA* BY APHIDICOLIN

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We have previously demonstrated that DNA polymerase activity of *Entamoeba histolytica* is inhibited by aphidicolin which is a specific inhibitor of eukaryotic nuclear replicative DNA polymerases. The present study was aimed to evaluate the effect of aphidicolin on the growth and DNA synthesis by this parasite. Aphidicolin blocked the growth of axenic *E. histolytica* strain HM-1: IMSS. DNA synthesis was also inhibited by aphidicolin when assayed by incorporation of ³H-thymidine into the DNA. As it has been reported that aphidicolin blocks eukaryotic cells in "S" phase by

inhibiting the replicative DNA polymerase and allows G2, M and G1 cells to accumulate at the G1/S border and also it does not reduce cell viability and its action is reversible, it was determined whether these action of aphidicolin was also observed in *E. histolytica*. The inhibitory effect of aphidicolin on the growth of *E. histolytica* was abrogated by removal of the drug, and exposure to 3 $\mu\text{g/ml}$ of the drug for at least 48 hr had little effect on the viability. Synchronous growth was observed in the recovery phase after removal of aphidicolin.

**6 ANALYSIS OF CYSTEINE BIOSYNTHETIC PATHWAY IN
ENTAMOEBA HISTOLYTICA: CLONING AND CHARACTERIZATION
OF ADENOSINE TRIPHOSPHATE SULFURYLASE
(SULFATE ADENYLTRANSFERASE) cDNA**

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The enteric protozoan parasite *Entamoeba histolytica*, a causative agent of amebiasis, has been shown to possess cysteine biosynthetic pathway, which was supposed to exist solely in bacteria and plants. Cysteine is a major thiol in this glutathione-deficient organism, and, thus, is considered to be responsible for the oxygen defense mechanism in amoeba. Hence, cysteine biosynthetic pathway is essential for amoeba biology. As an initial process of cysteine biosynthesis, extracellular sulfate is transported into amoeba, and adenosine 5-phosphosulfate is formed from ATP and sulfate, catalyzed by ATP sulfurylase (sulfate adenytransferase). In order to understand biochemical and physiological function of the initial process of cysteine biosynthesis, ATP sulfurylase cDNA was obtained from *E. histolytica* as follows. A part of ATP sulfurylase gene was amplified by PCR using genomic DNA and degenerate oligonucleotide primers corresponding highly conserved regions

(AFQL/TRNP and AP/HDRGV/II) of the protein sequences. ATP sulfurylase cDNA clones were obtained by screening of amoeba Lambda cDNA library with the PCR-amplified probe. An open reading frame of the ATP sulfurylase cDNA encoded a 423-amino acid protein. The deduced amino acid sequence of the amoebic ATP sulfurylase cDNA revealed a highest identity with *Synechocystis species* and *Bacillus subtilis* (45 and 41%, respectively). In contrast, the amoebic enzyme diverged significantly from other eukaryotes including yeasts, fungi, plants, and mammal. However, most of the positively charged residues (arginine and histidine) in the regions where PCR primers were designed were conserved among all the organisms. The enzymatic activity was detected in the amoebic lysate using molybdolysis, suggesting the cloned and characterized cDNA was likely to encode ATP sulfurylase.

**7 ON THE ANTIBODIES IN INDIVIDUALS CHRONICALLY
INFECTED WITH *BLASTOCYSTIS HOMINIS***

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Blastocystis hominis is a protozoan parasite of the human intestinal tract. Many reports suggest that the organism is involved in human gastrointestinal disease. However, another school of thought believes the question of pathogenicity is unsettled. In a recent survey, we found *B. hominis* in a clinically healthy population. The *B. hominis*-positive individuals were symptom-free and colonoscopy failed to reveal evidence of intestinal lesions. Follow-up studies revealed long-term persistence of organism in infected individuals. Therefore, we

think the pathogenicity of *B. hominis* remains unresolved. In the present study, we detected anti-parasite antibodies by enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody (IFA) methods in asymptomatic individuals infected with *B. hominis*. The great majority of stool-negative individuals were negative by serology as well, 14 of 15 by ELISA and 13 of 15 by IFA. In contrast, only about 30% of the stool-positive individuals tested positive either by ELISA or IFA, an indication that it may be difficult to

elicit serum antibodies in symptomfree individuals. We also searched for the antigen(s) recognized by the serum antibodies using immunoblotting methods. The serum of one chronically infected individual specifically reacted with a 12kDa protein of a *B. hominis*-derived antigen. The protein recognized by the serum was detected on the surface of the organism by ELISA staining and immuno-electronmicroscopic observations. It appears that the antibody(s) against the 12kDa protein are produced only in long-term infected individuals. We compared the characteristics of organisms isolated

from the same individual at different times during his long-term infection. The protein and riboprint patterns of the recovered organisms did not change during the course of the infection. The unchanged patterns of the parasite would seem to indicate that this individual was not being constantly re-infected, but had a persistent infection. On the basis of these results, it appears that the production of antibody against the 12kDa protein of *B. hominis* may be dependant on a long-term persistent exposure of the host to the parasite.

8 PURIFICATION AND CHARACTERIZATION OF AN *ENTAMOEB*A *HISTOLYTICA* 150kDa SURFACE ANTIGEN RECOGNIZED BY A MONOCLONAL ANTIBODY, EH3015

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Adherence of *Entamoeba histolytica* trophozoites to host cell is a prerequisite for cytotoxicity. A monoclonal antibody, EH3015 which reacts with a 150kDa protein of *E. histolytica* on Western immunoblot under nonreducing conditions, inhibits adherence and cytotoxicity of the ameba to mammalian cells *in vitro*. In addition, 50% of the passively immunized hamsters by 1 mg of EH3015 were also found to be protected from the formation of liver abscess. Affinity purification of solubilized trophozoites using the monoclonal antibody and electrophoresis, yielded three glycoproteins with molecular masses of 150, 170, and 260kDa, suggesting the existence either of a common epitope or the close association of these proteins. The 260kDa fraction was identified as the previously reported galactose (Gal)- and *N*-acetyl-D-galactosamine (GalNAc)-inhibitable

lectin. The 150 and 170kDa fractions seemed to exist as part of a 380kDa native protein with an isoelectric point of pH 6.9. The *N*-terminal amino acid sequence of the 150kDa protein was unique, indicating that the protein was not a degraded product of the 260kDa lectin. By gel filtration, the 260kDa lectin and the 150/170kDa protein could be separated. When Chinese hamster ovary cells were pretreated with the fraction consisting of the 150/170kDa protein, adherence of trophozoites to Chinese hamster ovary cells were competitively inhibited, equivalent to the 260kDa lectin. The inhibitory effect was lost in the presence of Gal and GalNAc, but was not influenced by the presence of glucose. These results demonstrate that the 150/170kDa protein is a Gal/GalNAc-inhibitable lectin.

9 BACTERIAL EXPRESSION OF HUMAN MONOCLONAL ANTIBODY Fab FRAGMENTS SPECIFIC FOR *ENTAMOEB*A *HISTOLYTICA*

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Genes coding human antibody Fab fragments to *Entamoeba histolytica* were cloned and expressed in *Escherichia coli*. Lymphocytes were separated from 10

ml peripheral blood of a patient with amebic liver abscess. Poly(A)⁺RNA was isolated from the lymphocytes and then genes coding the light chain and

Fd region of the heavy chain were amplified by reverse transcriptase-polymerase chain reaction. Amplified DNA fragments were ligated with a plasmid vector pFab1-His2 and introduced into *E. coli* JM109. One thousand colonies were screened for the production of antibodies to *E. histolytica* by an indirect fluorescence antibody test using trophozoites of the HM-1:IMSS strain. *E. coli* lysates from three clones showed strong fluorescence. When the reactivity of 9 other strains of

E. histolytica was examined, all proved reactive to the antibodies expressed by the 3 lysates. In contrast, these Fab fragments did not react with *E. dispar* SAW1734RclAR. Western immunoblot analysis revealed that the molecular mass of the antigen(s) recognized by the three Fab fragments was 260kDa. These results indicate that the bacterial expression system reported here is effective for the production of human monoclonal antibodies specific for *E. histolytica*.

10 PREVALENCE AND ULTRASONOGRAPHIC FINDINGS IN THE LIVER OF *OPISTHORCHIS* INFECTION IN KHAMMOUANE PROVINCE, LAO P. D. R.

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We reported the distribution of liver fluke within villager residents (Sisomsung, Pavang and Thakhek Neua) in Khammouane Province, Lao P. D. R. as determined by pretreatment egg counts in the stools, and also presented hepatobiliary findings observed by ultrasonography in apparently healthy residents in Thakhek Neua Village and patients attending Provincial Hospital clinic. The overall prevalence of infection in the community was 375 of 670 people (56.1%). There were no significant sex-associated differences in the prevalence or intensity of infection. The prevalence was higher in people over 20 years old, 215 of 294 people (73.1%), and it was different in the three villages (64.9%, 75.2% and

80.0%, respectively). The frequency of heavy infection (>6,000 eggs per gram of faeces) was 3.5% in adults. In ultrasonographic study about the hepatobiliary disorder, dilated intrahepatic or common bile duct and parenchymal disorder such as increased echoes throughout liver were observed frequently in patients who excreted eggs, but there was a significant difference only in the finding of dilated bile duct between people positive and negative egg output. A correlative tendency was observed between the severity of biliary disorder or some symptoms and the intensity of egg excretion, but there was no significance. No cases of cholangiocarcinoma were found in this study.

11 MAGNETIC RESONANCE IMAGING OF SCHISTOSOMIASIS MANSONI IN MICE

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Recently several authors have reported the radiological manifestation of hepatic schistosomiasis as

demonstrated by ultrasound, computerized axial tomograph and magnetic resonance imaging (MRI). MRI is

expected to be a more sensitive indication of disease progression as opposed to the other imaging modalities which are less sensitive to subtle inflammatory changes (Patel *et al.*, 1993). Recently MRI for small animals has become available. In the present study we report the murine hepatic schistosomiasis *mansoni* demonstrated by MRI. Mice (BALB/C) were infected subcutaneously with 150 cercaria of *Schistosoma mansoni*. At 4, 5, 6, 7, 8, 10 and 13 weeks post-infection, the animals were examined by MRI and necropsied for histo-pathological examination. MRI of the abdomen of animals was performed with the use of spin echo. With 1 mm slice thickness, T1-weighted (1,000 TR/19 TE) and T2-weighted (1,800 TR/40 TE) axial images were obtained. No detectable change was obtained in animals examined

at 4 and 5 weeks post-infection by MRI, while a few periportal inflammatory lesions were observed at 5 weeks post-infection by histological examination. In an animal examined at 6 weeks post-infection, scattered lesions with increased signals were obtained on both the T1-weighted and T2-weighted sequences. This pattern is consistent with the inflammation seen early in the disease process of the histological sections. At 7 weeks and afterward, markedly increased signals were obtained along the portal vein on the T1- and T2-weighted sequences. These are consistent with the advanced fibrosis of the histological sections. MRI is a sensitive method to examine the progression or stage of schistosomiasis in murine model.

12 RELATION BETWEEN ULTRASONOGRAPHIC SIGNS AND HAEMATEMESIS OF THE PATIENTS WITH SCHISTOSOMIASIS MANSONI IN NORTHEAST BRAZIL

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Eighty-three Brazilian patients with hepatosplenic schistosomiasis were examined with ultrasonography and endoscopy to assess the major ultrasonographic predictors of upper gastrointestinal bleeding or the grade of esophageal varices. Sixty-eight (82%) out of these patients had suffered at least one attack of upper gastrointestinal bleeding. Fifty-seven (69%), 25 (30%) and 57 (69%) had been treated with chemotherapy, abdominal surgery with splenectomy and endoscopic sclerotherapy, respectively. Abdominal ultrasonography demonstrated that all patients have a hyperechoic periportal fibrosis worse than grade I, liver fibrosis stage more than grade I (WHO, 1991) and Abdel-Wahab score more than grade-2 (Abdel-Wahab *et al.*, 1993). By endoscopy, esophageal varices were demonstrated in 70 patients (84%) of whom 51 patients (61%) had esophageal varices worse than grade II. In the present study, all ultrasonographic and endoscopic data were statistically analyzed using multiple logistic regression (SAS program, SAS Institute, USA). Crude

odds ratio and appropriate confidence interval were estimated for each variable. Abdel-Wahab score was highly associated with a significant risk of variceal bleeding. However, age, sex, liver size, liver surface morphology, PPF grade, liver fibrosis stage, spleen size, the diameter of portal vein and splenic vein, and collateral score were not associated significantly with the risk of bleeding after adjustment for potential confounding variables. In contrast, PPF grade and collateral score were independently associated with the grade of esophageal varices. Other ultrasonographic signs were not associated significantly with the grade of esophageal varices. Our present analysis suggests that Abdel-Wahab score, the degree of PPF and collateral score are good predictors to identify the patients with the esophageal varices or the risk of upper gastrointestinal bleeding. Therefore, these factors could be helpful in the prophylactic management of the patients with complicated schistosomiasis.

13 ULTRASONOGRAPHY FOR ASSESSING MORBIDITY OF SCHISTOSOMIASIS JAPONICA IN ORIENTAL MINDORO, THE PHILIPPINES

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Ultrasonographic and serologic examinations were performed at Malabo (a village where schistosomiasis japonica is highly endemic), San Narciso and San Pedro (moderately endemic) and Poblacion III (low endemic) in Victoria and Naujan, Oriental Mindoro, Philippines, in July and August, 1997. Examined were 241 inhabitants of the above 4 villages whose ELISA OD values indicated positivity for schistosomiasis one year prior to the current study. Studied were 67 females ranging in age from 5 to 76 years and 174 males ranging in age from 7 to 80 years. Ultrasonography, serological tests for hepatitis B and C and liver and gallbladder function, the ELISA test for schistosomiasis, and physical examination were performed. However, examination for hepatitis B antibody revealed positivity rates of 41.1% (Malabo), 38.3% (San Narciso), 39.6% (San Pedro) and 37.9% (Poblacion III), whereas the corresponding rates for hepatitis C were 0.9%, 0%, 1.9% and 3.4%, respectively. The percentages of individuals showing an elevated serum GOT level were 5.4%, 2.1%, 13.2% and 13.8%, and those for serum GPT were 1.8%, 0%, 1.9% and 0%, respectively. Therefore, it was concluded that

there were few cases of active hepatitis B and/or C among the inhabitants examined. In the village with the low endemic rate (Poblacion III), there were no Type 2 (tubular pattern) and Type 3 (network pattern) ultrasonographic liver patterns in individuals under 40 years old, whereas in the village with the high endemic rate (Malabo), even teenagers and subjects in their 20s showed the Type 2 pattern and 5% of subjects in their 30s had the Type 3 echogenic liver pattern. The echogenic liver pattern in moderately endemic villages was intermediate between those in the villages with high and low endemic rates. An overview of the relationship between changes in the liver echogenic pattern and ages of the patients among the three areas showing high, moderate and low endemic rates for schistosomiasis indicated that as the patients grew older, the proportion of typical changes in the liver echogenic pattern due to schistosomiasis increased, regardless of endemicity. It is concluded that ultrasonography is practical for assessing the morbidity of schistosomiasis japonica in areas of different endemicity.

14 SEROLOGICAL STUDY ON *SCHISTOSOMA MANSONI* INFECTION CONDUCTED ON THE INHABITANTS OF TULASHICHAUDA VILLAGE, SOUTHERN NEPAL

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In April 1995, the authors detected *Schistosoma mansoni* like eggs in a stool sample of an inhabitant of

Tulashicahuda village, Dhanusha district, Southern Nepal. In order to clarify the suspected existence of *S.*

mansoni in this area, serological study was conducted. The study was conducted in 4 sites (A—D) of the village. A total of 518 serum samples collected from the inhabitants were examined by micro-ELISA. The antigen was prepared by the extract of *S. mansoni* eggs, and ABTS was used as substrate of peroxidase. When absorbance (OD_{415}) exceeded 0.3, the case was determined to be positive. Out of 518 serum samples 94 were positive (positive rate 18.1%). The highest absorbance was 1.189. Significant difference in positive rate was recognized in the 4 study sites (A: 13.1%, B: 0.99%, C: 18.8%, D: 42.7%).

S. sinensium eggs, rodent's schistosome eggs which has a lateral spine, resemble *S. mansoni* eggs and need to be differentiated. However the eggs detected in the above inhabitant are strongly suspected to be *S. mansoni* eggs due to their size, shape, and the results of serological examination (in *S. sinensium*, human infection is very rare). The possibility of the existence of *S. mansoni* in Southern Nepal is considered to be extremely high. Further epidemiological studies, including studies on intermediate hosts and sero-positive cases, are urgently needed to offer more clarification.

15 DETECTION OF ANTIBODIES TO SOLUBLE EGG ANTIGENS IN URINE SAMPLES FROM PATIENTS WITH SCHISTOSOMIASIS JAPONICA

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Although detection of eggs in stool samples is the most reliable diagnostic method for schistosomiasis japonica, the eggs are not always detected. Therefore, detection of the parasite antigen specific antibodies in serum samples is commonly used. Collections of the serum samples, however, are not necessarily easy. In this research, we intended to use urine samples instead of serum for diagnosis of the parasitosis. Paired serum and urine samples were collected from residents of a village, where schistosomiasis japonica is endemic, located nearby Tung-t'ing Lake in Funan province, China. The sera were kept at -80°C and urine samples were added with NaN_3 and kept at 4°C . Antibodies to soluble egg antigens (SEA) of *Schistosoma japonicum* in both samples were measured by ELISA. Averages of IgG to SEA in ten paired serum and urine samples from

patients were $160\ \mu\text{g/ml}$ and $35\ \text{ng/ml}$, and IgA were $1,887\ \text{ng/ml}$ and $4\ \text{ng/ml}$, respectively. The unbalanced amounts of SEA specific immunoglobulins, that is 1/4,500 of serum IgG and 1/450 of serum IgA in urine, suggests the different mechanisms of their leaks or secretions into urine. Existence of secretory components in anti-SEA IgA in urine suggests its active secretion into urine. Using peroxidase conjugated anti human IgG+M+A as a second antibodies, antibodies to SEA were detected in 20 out of 23 urine samples of which paired sera were positive. No false positive case was observed among urine samples from healthy subjects in China. These results suggest that instead of serum urine can be used for the immunodiagnosis of schistosomiasis japonica with enough sensitivity.

16 APPLICATION OF SPOT IMAGE TO THE STUDY OF GEOGRAPHICAL DISTRIBUTION OF VECTOR SNAIL OF SCHISTOSOMIASIS ON MINDORO ISLAND, PHILIPPINES

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Control of the intermediate host snail, *Oncomelania quadrasi*, is one of the effective measures to prevent schistosomiasis japonica on Mindoro Island, Philippines, because the parasite infects domestic and wild animals besides humans. In the present study, we examined geographical characters such as longitude and latitude, topography, land use and chemical conditions of the soil and water, of 48 spots where the snail colonies had been found, and made maps of the exact locations of their habitats based on these data. We, furthermore, investigated whether the application of satellite images would give us more appropriate information for analysis and clarification of the geographical character. This new technique had been roughly applied for a wide endemic area of schistosomiasis mansoni. However it has not been examined whether the techniques could be used in the geographical environments of the endemic areas where the schistosomiasis japonica is unevenly distributed. The longitude and latitude of the habitats were measured by pocket GPS. Each snail habitat was marked on the maps on a scale of 1: 50,000 published by NMRIA, and on a topographical division map which we drew as a result of field survey. Soil color was measured with a standard soil chart, soil texture by the

finger method, and chemical components by the rapid soil nutrient tester. The pH of soil and water were measured by inserting a glass electrode of a pH meter at the habitat. The COD values and CaO contents of water were obtained by simplified methods. Satellite images derived from a ratio of red and red-infrared Spot HRV were modified geometrically to fit a topographical map on a scale of 1:50,000 and were analyzed with 3 bands of RGB by a personal computer. Based on the analysis on topography and conditions of soil and water, we conclude that favorable environmental conditions for the colonization of *Oncomelania* snails are divided into seven types. All these areas have the same conditions where the alluvial lowland was in the shade of tropical trees and the soils contained CaO and a small amount of Mn and Al, and their pH was neutral. It is easy to make out the topography, vegetation and land use by Spot image. Remote sensing was applicable not only to the place of aerial photographs but also to locating the habitat and their conditions, and to predict the possible location of the habitat. It is suggested that remote sensing is useful for the control program of schistosomiasis japonica on Mindoro Island of the Philippines.

17 TELEPARASITOLOGY: A PROPOSAL FOR TELEMEDICINE ON PARASITE DISEASES

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There are growing interests in telemedicine over the world. National Cancer Center has introduced and applied many telemedicine systems in the recent several years. And in the last year, we developed a new concept telemedicine system named NCC-image. It enables to establish teleconference with interactive remote collaboration among more than two users over the internet. Most important characteristics of NCC-image is (1)

low initial cost, (2) low communication cost, and (3) high compatibility. Doctors can use it by using only a personal computer with a popular internet browser. The communication cost is kept very low because it uses the internet as the transmission linkage. Additionally it can be used by most computers and browsers because it is implemented by java programming language. NCC-image was designed mainly for pathological purposes,

and good results are obtained in telepathology of tissue and cytological samples. Additionally, we are developing a new telepathology system named World Wide Microscope (WWM). WWM is an automatic microscope system that enables to be remote-controlled via the Internet. We think the systems are probably useful to not only telepathology but also to remote conference and remote diagnosis of parasite diseases, especially malaria. In Japan, there is few specialist who can diagnose this disease correctly, even though we have more than a hundred patients every year. Contrary, there are many doctors who are used to treat various malarian cases in tropical countries. It seems beneficial for Japanese doctors, and probably for doctors of such nations, to make discussions on the diseases of remote patients with correspondently displaying microscopic blood images or other medical images on their personal computers. This is the basic concept of teleparasitology that we will propose in this study. To practice the international teleparasitology, to obtain a communica-

tion line with sufficiently broad band width is essential. NCC-image doesn't require very broad communication linkage, however we recommend at least ISDN line (64 Kbps) to use it smoothly. We wonder whether enough ISDN lines for the Internet are constructed or not in many tropical countries. However, this problem may be resolved sooner than we have expected, especially in Asia/Pacific region, because Asia-Pacific Advanced Network Consortium (APAN), an international consortium to promote research and development in advanced networking application and services in this region, was established in this year. Also the Japan Consortium (APAN-JP) will be established as a national organization for APAN. APAN-JP will provide high-speed network for the Asia/Pacific region to encourage regional cooperation and collaboration. So we proposed a telemedicine plan to APAN-JP in the last July. If the plan is accepted, we wish to try international teleparasitology experiment among the region by using our systems.

18 SURVEY OF PREVALENCE OF HUMAN OPISTHORCHIASIS, CERCARIAE IN SNAILS AND METACERCARIAE IN FISH OF NORTHEAST THAILAND

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The opisthorchiasis caused by the liver fluke, *Opisthorchis viverrini* (OV), is an important parasitic disease in Thailand because it can lead to cholangiocarcinoma. We surveyed to determine the recent status of opisthorchiasis and other parasitic diseases by Grants-in-Aid of International Scientific Research Program, Ministry of Education, Science and Culture. One hundred and seventy nine stool samples (78%) were examined in Dondoo Village (population 230) in Khon Kaen Province in 1993. Eighty (45%) were infected with 1 to 3 kinds of parasites. The prevalence of OV was highest (61 samples-34%), followed in order by hookworm, *G. lamblia*, minute intestinal fluke and Echinostome. Fourteen were infected with more than 2 kinds of species. Re-examination was carried out in 1994, 9 months after treatment. One hundred and forty four stool samples (63%) were examined and preva-

lence of OV was 26 (18%). While, 158 stool samples (52%) were examined in Khokklang Village (population 306) in Khon Kaen Province in 1993. Sixty eight (43%) were infected with 1 to 3 kinds of parasites. The prevalence of OV was highest (49 samples-31%), followed in order by hookworm, *S. stercoralis* and *G. lamblia*. Twenty one persons were infected with more than 2 kinds of species. Re-examination was carried out in 1994, 9 months after treatment. One hundred and twenty five stool samples were examined and prevalence of OV was 29 (23%). In addition, infection rates of OV cercariae in snails and OV metacercariae in fish were studied in the pond which is located between two villages for 2 years. No cercariae of OV were detected from 15,955 snails but 1,276 metacercariae of OV were detected in 7,855 cyprinoid fish.

19 IS A JAPANESE STRAIN OF *SCHISTOSOMA JAPONICUM* SAME ONE IN KURUME DISTRICT AND IN YAMANASHI PREFECTURE?

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Schistosomiasis japonica has been perfectly eradicated from Japan since an announcement of the Governor in Yamanashi Prefecture, which was the last endemic area of schistosomiasis japonica in Japan, that there is free from danger of infection. Now the life cycle of the Japanese strain of *Schistosoma japonicum* is maintaining only in the laboratories. There are two Japanese strains, in which one is originally from Kurume (Kurume type; maintained in Department of Parasitology, Kurume University School of Medicine) and another is from Yamanashi (Yamanashi type; maintained in Department of Parasitology, Yokohama City University School of Medicine). In this preliminary works we investigated these two types of a Japanese strain on the morphological and genetical findings. Those two types of a Japanese strain showed the divergence in some points. (1) The length of male worms of Kurume type is longer than that of Yamanashi type, but the length of female worms of Yamanashi type is longer than that of Kurume type. The worms were recovered from C57BL/6 mice infected percutaneously with 20 cercariae at 8 weeks of infection. (2) The size of matured eggs of Kurume type is statistically bigger than that of Yamanashi type (Kurume; length 77.95 ± 4.97 mm, width 63.1 ± 6.32 mm; Yamanashi; length 64.25 ± 3.69 mm, width 52.55 ± 3.69 mm). The eggs were produced by pairs of worms, which were recovered from ddy mice at 8 weeks of infection and cultured in RPMI 1640 medium including 10% FCS and 1 mM of D-glutamine *in vitro* for 9 days after production. The spine of egg is clearly visible in Kurume type, but not in Yamanashi type. (3) The numbers of produced eggs

were compared between two types. As we examined twice, the results were different. In one experiment, the number of produced eggs was almost the same (Yamanashi; $1,037 \pm 368$, Kurume; $1,049 \pm 495$), but in another experiment female worm of Yamanashi type produced almost twice as many as those of Kurume type (Yamanashi; $2,451 \pm 823$, Kurume; $1,168 \pm 417$). A pair of worms was incubated in a well of 24 well culture plate with RPMI medium for 36 hr and more than 20 wells were investigated from each type in each experiment. In addition to these morphological findings, these two types of a Japanese strain and a Chinese strain (Guichi) were examined at the molecular level for genetic divergence. The nucleotide sequence of the internal transcribed spacer regions of the rRNA gene (ITS1 and ITS2) was analyzed. As sequencing primers, ETTS1 and ETTS2 (Kane *et al.*, 1994) were used for ITS1 and ITS2 respectively. The nucleotide sequences of part of the ITS1 region of Yamanashi and Kurume had 85 and 72% of homology to that of a Chinese strain. And the sequences of part of ITS2 region of Yamanashi and Kurume had 86 and 72% of homology to a Chinese strain. The phylogenetic tree of ITS1 and ITS2 region based on UPGMA method suggests that Yamanashi type is closer to a Chinese strain than to Kurume type phylogenetically. Although we need to investigate the morphology and the DNA sequence more in detail to distinguish two types as subspecies, our data suggest that a Japanese strain of *Schistosoma japonicum* should be expressed as a Japanese strain (Yamanashi) or a Japanese strain (Kurume).

20 INFECTIOUS DISEASES IN THE JICA TRAINEES FROM DEVELOPING COUNTRIES —PARASITES, HEPATITIS VIRUSES, ETC.—

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A growing number of foreigners including those from developing countries visit Japan, necessitating us to practice medicine for those people. Therefore, it is imperative to investigate the disease profiles in those countries which could be different from that of our country. As a part of this theme, we studied the infectious diseases among JICA trainees, mainly focusing on parasites and hepatitis viruses they carry. A total of 2,382 JICA trainees who visited Japan between August 1991 and July 1997 were recruited. The numbers varied by country; the largest is 276 from Indonesia, followed by 217 from China and 120 from Thailand. After their arrival, informed consent was obtained for the examination of their blood and urine. Especially for HIV testing, the consent was obtained in a private atmosphere. With regard to parasites, whipworm ova were found most frequently, i. e. in 4.9% of the whole trainees. The positive rate was high in those from Vietnam at 36% (9/25), Bangladesh at 13% (6/45) and Nepal at 12% (5/43). Next, ascaris ova were found in 2.5% of the total, in 40% (10/25) of those from Vietnam and in 8.9% (4/45) of those from Bangladesh. Hookworm ova were

found in 1.3% of the total, in 29% (5/17) of those from Papua New Guinea, and in 16% (4/25) of those from Paraguay. With regard to protozoa, giardia was found in 1.3% and *Entamoeba histolytica* in 0.8% of the whole trainees, with no preferential country distribution. HBs-Ag was detected in 4.6% of the total and distributed preferentially in southeast Asian and African countries. HCV-Ab was found in 0.8% of the whole trainees, and this agent was the most prominent that showed preferential country distribution, i. e. with increased rates in those from Cambodia at 21% (3/14), Mongolia at 20% (7/35) and Egypt at 15% (10/69). HIV-Ab was detected in 0.8% of the whole trainees most of which are confined to those from African countries. The positive rates described above for each agents may not be representative of individual countries. One of the reasons is that the number of cases per country tested was sometimes too small. Another is that ages and the male/female ratios were not matched. Nevertheless, such country profiles of infectious diseases would greatly help us in performing medical practice for foreigners.

21 PREVALENCE OF INTESTINAL PARASITES AMONG JAPANESE RESIDENTS IN DEVELOPING COUNTRIES

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We examined fecal specimens of Japanese residents in developing countries in order to know the prevalence of intestinal parasites in the group. One fecal specimen was collected from each 981 (in 1995) and 1,276 (in 1996) Japanese living in Asia, Middle East, East Europe, Africa and Latin America. The specimens were fixed with 10% formalin in each area, and were examined in Japan by concentration method (formalin-ether sedimentation) to find protozoan cysts or helminth eggs. Cysts or ova of intestinal parasites were found in 29 (in 1995) and 31 (in 1996) Japanese, indicating that the

infection rate of intestinal parasites was 3.0% in 1995 and 2.4% in 1996. The rate was high in Africa (5.7% : 1995, 4.7% : 1996) and Asia (3.8% : 1995, 3.2% : 1996). Among all Japanese examined, the rate was higher in adult (3.7%) than in children (1.4%), and was elevated paralleled with their length of stay. Regarding to the species of the parasites, *Giardia lamblia* (17 cases), *Trichuris trichiura* (14) and *Ascaris lumbricoides* (11) were detected frequently. Additionally, 7 cases of *Heterophyes heterophyes* infection were found in Asia and Middle East.

The character of 57 Japanese infected with intestinal parasites was further analyzed. The average age was 29.8yo and average length of stay was 20.2 months. Symptoms such as diarrhea and abdominal pain were observed in 37.5% of them. Especially, the Japanese infected with *Giardia lamblia* frequently had the symptoms. It is also noteworthy that 29.1% of the infected Japanese had the history of gastric diseases such as gastric ulcer. Among all Japanese examined, the infec-

tion rate was statistically significantly higher in the group with gastric diseases than that in the group without gastric diseases ($p < 0.005$). Although the infection rate of intestinal parasites among Japanese residents in developing countries dropped for the last decade, the rate is still higher than that in Japanese living in home country. It is necessary to continue preventive measures such as health education in order to eradicate intestinal parasitic infections from this group.

22 THE KAP STUDY ON INFECTIOUS DISEASE AND ITS PREVENTION WITH JAPANESE OVERSEA TRAVELERS

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【Purpose】 KAP (Knowledge, Attitude and Practice) of Japanese oversea travelers and IEC (Information, communication - Education and communication) activity by travel agents to their guests were investigated to develop educational program to Japanese oversea travelers. **【Methods】** Investigation was carried out by questionnaires from August 1996 to January 1997. The questionnaires were delivered to travel agents and the persons who visited Yokohama quarantine station to be vaccinated. The questions (to the travelers) are travel history, means of obtainment of information, whether vaccinated or not, knowledge on preventive effectiveness of vaccination and condom. the questions to travel agents were raised on whether information on such diseases is collected or not, how to collect it and also whether such information is offered to their guests or not. **【Results】** *Overseas travelers:* The total 262 questionnaires were collected including 144 males and 118 females. The mean frequency of travel to overseas was 3.6 times and the main travel countries were 60 to developing Asian countries, 89 to Africa, 26 to Central and South America, 11 to world round travel, 38 to North America and Europe and so on. 218 persons equivalent to 83% had taken care of or concerned about infectious disease prevention, 228 (87%) had not believed the effectiveness of vaccination and 88 (34%) had believed the effectiveness of condom use in the prevention of HIV or HBV infection. 119 (45%) had

collected information before traveling, 90 (34%) had vaccination against cholera but only 19 (7%) had taken anti-malaria drug. 142 (54%) had hoped collecting information about diarrheal diseases in future but only 51 (19%) about arthropod borne diseases. 140 (53%) of the persons had expected travel agents to collect such health information in future, then followed by 70 (27%) of passport centers. *Travel Agents:* 19 (63%) of 30 travel agents answered that they were collecting health information. Only 9 of them answered they had offered such information to their guests. But 21 (70%) of 30 travel agents answered they would like to or should provide their guests with such information in future. 14 (47%) of the agents had expected quarantine stations with broad network to provide the health information with travelers. 9 (30%) agents expected tour conductor and 8 (27%) agents expected video display in the aircraft as measures of health education for travelers. **【Discussion】** Many travelers had been vaccinated against cholera even though they did not believe the effectiveness of vaccine generally. Also more than one third of travelers did not believe the effectiveness of condom use for the prevention of STD. It should be noted that less than one third of travel agents did not provide health information to their guests. In view of the above, the mass-education campaign on the health of overseas travelers should be promoted.

**23 AN EVALUATION OF IMMUNE RESPONSE TO MULTIPLE
AND SIMULTANEOUS VACCINATION AMONG THE PERSONNEL
OF JAPANESE SELF DEFENSE FORCES
DETACHED TO PKO FIELDS**

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The immune response and persistence of antibodies to simultaneous immunization against tetanus, Japanese encephalitis B, poliomyelitis, rabies, hepatitis B, cholera, hepatitis A and yellow fever in Japanese UN (United Nations) Peace-keepers were studied. 169 male personnel aged 19-52 who received full planned former 6 vaccines and human immune serum globulin (HISG) within 7 months before or after the second detachment to PKO (Peace keeping operation) in Cambodia (Group A), 14 personnel who received a yellow fever vaccine before the second detachment to PKO in Mozambique (Group B) and 31 personnel who received inactivated hepatitis A vaccine before the second detachment to PKO in Golan-highland (Group C) as well as several kinds of vaccine as mentioned above were investigated. The results of this study are summarized as follows :

① tetanus: Because all to the personnel had already been taken the tetanus toxoid vaccine when they enlisted in the forces, seroconversion rate showed almost 100% determined by passive haemoagglutination (PHA) technique prior to the vaccination in this plan. It has been keeping 100% consistently through 43 months after the vaccination in Group A. ② Japanese encephalitis B: Seroconversion rate determined by complement fixation (CF) method was lower than by haemoagglutination inhibition (HI) method. And seroconversion rate by HI was as much as 84% at the point of 42 months after the vaccination in this plan among the additional immunized group, whereas only 40% among the initial immunized Group A. ③ poliomyelitis: Seroconversion rate determined by CF method was lower than by neutralization test (NT), which has been keeping 100% even at 40

months by NT method in Group A. ④ rabies: Seroconversion rate reached almost 100% after the second or the third injection determined by enzyme immunosorbent assay (EIA) method, but nearly half of subjects turned to seronegative at 10 months after the third injection. ⑤ hepatitis B: Seroconversion rate turned to almost 100% after the third injection in Group A and kept more than 70% at 22 months after the third injection determined by radioimmunoassay (RIA) method, which resulted as we expected. ⑥ cholera: Immune response against cholera vaccine determined by agglutination test was very poor, especially against Ogawa type (seroconversion rate was less than 10% even at the best point of 6 months), and almost all subjects were seronegative at 15 months in Group A. ⑦ hepatitis A: The effective duration of the passive immunity afforded by HISG was less than 6 months in Group A, whereas almost 100% of seroconversion rate was keeping more than 6 months in immunized troop by inactivated hepatitis A vaccine in Group C. Both were determined by RIA method. ⑧ yellow fever: 80% of seroconversion rate by HI and 100% by NT method were obtained, whereas less than 60% by IgM-captured enzyme linked immunosorbent assay (ELISA) at 6 months in Group B. These data were investigated at Fundacao Oswaldo Cruz in Brazil. Overall, it can be concluded that simultaneous and multiple immunization against tetanus, Japanese encephalitis B, poliomyelitis, rabies, hepatitis B, hepatitis A and yellow fever in adults would be effective as much as being expected respectively by immunized separately.

24 STUDY ON THE MORBIDITY OF INFECTIOUS DISEASES AND THE PROPHYLACTIC ATTITUDE AMONG ADULT JAPANESE WHO HAD LIVED IN DEVELOPING COUNTRIES

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As a result of globalization and development in transportation, the number of Japanese who travel to foreign countries has been increasing. The travelers who are suffering from infectious diseases during travel is suspected also increasing. It is well known that immunization is the practical way for preventing people against vaccine preventable diseases. In addition to immunization to babies like EPI, attention should be drawn to the importance of immunization to adults with vaccine such as hepatitis A, hepatitis B, Japanese encephalitis, rabies and yellow fever, particularly in those disease endemic countries. This study was conducted to know the situation of immunization and the morbidity of infectious diseases among adult Japanese who had lived in disease endemic developing countries. Questionnaires on receiving vaccination and morbidity of infectious diseases were distributed to 637 persons of over 20 years old who have lived in developing countries to work as bilateral collaboration project member or as their family member from 1991 until 1995. All the 637 persons responded to the questionnaires. Among total 637 persons who had lived in developing countries, 307 (49%) lived in Asia/Oceania, 149 (23%) in Latin America and 179 (28%) in Africa. The highest morbidity among overseas Japanese are common cold and diarrheal diseases, followed by dental problems. There were also a few cases suffering from the diseases such as malaria, parasite diseases and hepatitis. Although hepatitis A and Hepatitis B have been endemic in Asia, Oceania, Latin America and Africa, less than 20% of

them received hepatitis A vaccine, gamma-globulin or hepatitis B vaccine. With regard to Japanese encephalitis which is endemic in Asia, only 5% of people lived in Asia/Oceania received Japanese encephalitis (JE) vaccine while 24% of people lived in Latin America where Japanese encephalitis is not endemic received JE vaccine. Over 50% of people lived in Africa received Cholera vaccine which is not recommended by WHO and less than 20% of people lived in either Asia/Oceania, Africa, or Latin America received polio vaccine. These results showed that immunization rate of vaccines necessary for prevention of infectious diseases is generally low among Japanese adults who lived in Asia/Oceania, Africa and Latin America. Several explanation can be cited for the observation. One is shortage of knowledge and concern about infectious diseases. This was partly supported by the results of other study of us about knowledge, attitude and practice on infectious diseases with Japanese overseas travelers. The other is the problem of access to the immunization service. Interview of the studied groups showed that number of health facilities providing immunization to overseas travelers is limited in Japan and many health facilities are not familiarized with providing immunization. In view of the increase of overseas travelers in Japan and increasing chance of exposing infectious diseases, more efforts should be taken to make Japanese people aware of the importance of prevention against infectious diseases and encourage them to receive necessary immunization.

25 MAMUSHI (*AGKISTRODON BLOMHOFFII*) BITES IN GUNMA PREFECTURE

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Mamushi (*Agkistrodon blomhoffii*) bites are estimated less than 2,000 per year in Japan. This report concerns mamushi bite patients who were treated in 20

hospitals and dispensaries in Gunma Prefecture during three years from 1993 to 1995. The results indicated that the bites occurred in warmer season from May to

September. 75 percent of the patients were concentrated in ages between fifties to seventies, in which bites in male were 3 times as frequent as in females. Thirty-six or 73% of the bites occurred in agricultural field, residences, and on road which indicated that the bites are closely related with human life. Although the

mamushi is nocturnal, 34 or 69% of the bites occurred in daylight hours, whereas 13 or 26.5% of bites occurred in dark hours. Those figures of mamushi bites were compared with those of habu-bites on the Amami islands.

26 STUDY OF THE PREPARATION OF THE ANTI-HABU GOAT SERUM

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Okinawa prefecture, situated at the southern tip of Japan is home to the 3 species of the habu family, *Trimeresurus flavoviridis*, *T. elegans* and *Ovophis okinavensis*. Although there are around 150 cases of people being bitten by these species annually, due to the easy access to antivenom and well developed medical facilities, fatal cases are rare. Even cases of severe permanent damage are a rarity; only around 5% of the victims are left with minor permanent disabilities. Although the antivenom is an efficient form of treatment, some people suffer from serum sickness due to the administration of heterologous protein. Since it is produced from immunized equine blood. The number of serum sickness gradually increases when the antivenom produced from the same types of animal is used repeatedly, and the symptoms become more serious. In

order to produce the antivenom used for patients suffering from serum sickness due to the administration of anti-habu horse serum, we have attempted to produce the anti-habu goat serum. Goat seems to have a lower susceptibility than horse and so the potency only rose to about a third or half that of the horse. The goats with high potency were chosen for bleeding and 500 ml was used repeatedly. Although the goats were bled at 1-2 weeks interval, the potency level never fell dramatically. The potency of the goat serum was only half that of the horse serum, but the potency rose to a similar level salting-out and refining by ion exchange on DEAE-cellulose. There were no allergic reactions when the anti-habu goat serum was injected into a rabbit susceptible by injection of anti-habu horse serum.

27 A CASE OF FEBRILE DISEASE PROBABLY INFECTED AT SOUTHEAST ASIA

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A 60-year-old male surgeon was admitted to our hospital because of the second attack of remittent fever up to 40.0°C. He had a history of a travel to Thailand and Burma about one month before. And he had an episode of remittent fever with liver enzyme elevation of 7 days duration starting from 8 days after arrival to Narita. On admission, there was no lymphadenopathy or hepatosplenomegaly. There were insect bite like skin rashes on the back site of the both thighs, which were noticed by his family next day arriving to Narita

and were not worsened during his entire clinical course. Laboratory studies revealed slight elevation of liver enzyme, serum fibrinogen, and FDP. Platelets count was also slightly decreased. WBC was 7,700. Proteinuria and microhematuria were observed. The bacteriological studies of blood, urine, and faces were all negative. Weil-Felix and Brucella reaction were also negative. The both of IgG and IgM antibody to Tsutsugamushi were slightly increased, but these values were unchanged during his hospitalization period. Malaria

protozoa was not identified in thick blood films. After the ineffective fosfomycin therapy, tetracycline was administered, and general condition improved markedly,

the body temperature became normal within 4 days. Although there were no direct evidences, rickettsial disease was suspected.

28 ARE FLUOROQUINOLONES USEFUL IN PARATYPHOID FEVER A IN A PERIOD OF INCUBATION?

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It is well known that oral administration of fluoroquinolones for 14 days is effective against acute phase paratyphoid fever A. But to our knowledge, there is no report whether fluoroquinolones are useful in paratyphoid fever A in a period of incubation. We treated a 26-year-old Japanese female with bacillary dysentery, who returned from India and Nepal, with oral administration of daily doses of 600 mg of norfloxacin from

February 24 to March 1. Fever was appeared on March 17 and she paid a visit to our hospital on March 19 because of fever, and her blood culture revealed *Salmonella* serovar Paratyphi A. This fact indicates that 5 days administration of fluoroquinolones on incubation period of paratyphoid fever A are not useful to prevent the patient from onset of this bacterial infectious disease.

29 A CASE OF SEVERE DENGUE FEVER WITH HYPOXEMIA IN A JAPANESE TRAVELLER

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A 37 year-old male patient was admitted to our department with chillness, fever, general malaise with myalgia, headache, orbital and retrobulbar pain, back pain and anorexia. He visited Manila city, Philippines from May 11 to May 17, 1997. On May 22, he abruptly developed the symptoms described above. He was diagnosed as dengue fever by polymerase chain reaction (PCR) of serum, isolation of dengue virus and seropositive for anti-IgM antibody to dengue virus (type 2 and 3) by IgM antibody-capture ELISA method. A respiratory rate of 20/min and hypoxemia (PaO₂ 66.7 Torr) were observed. During the course, he was treated

symptomatically by acetaminophen and fluid transfusion. Biphasic high fever, transient thrombocytopenia (minimum: 100,000/ μ l), erythematous skin rash, purpura, minor manifestations caused by increased permeability of blood vessels such as nasal and gingival bleeding, and positive occult blood of stool were also observed. He was discharged on June 3, 1997 after the improvement of the above symptoms, signs and restoration of normal laboratory tests. Interestingly, elevation of interferon- γ was observed just after the transient elevation of interleukin (IL)-12 in this patient.

30 A CASE OF SUBCUTANEOUS DIROFILARIASIS : THIRTEENTH CASE IN JAPAN

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The thirteenth case of subcutaneous dirofilariasis in Japan is reported. A 53-year-old female, native of Hyogo Prefecture complained of subacute progressive swelling of her left lower abdomen. On the basis of pathohistological findings in the transverse sections biopsied and specific antibodies of her paired sera examined by ELISA revealed *Dirofilaria immitis* infection in a elastic hard nodule associated mainly with eosinophils, lymphocytes, and histiocytes. To date,

about 148 cases of human dirofilariasis including pulmonary (123 cases) and extra-pulmonary (22 cases) has been reported (5 cases are not clarified yet in their parasitic sites). In the time of pet-boom, the treatment of parasitoses for companion animals are not only necessary but also the protection of mosquito bites for them are indispensable strategy against the emerging disease such as dirofilariasis and other zoonoses.

31 A CASE REPORT OF SPOTTED FEVER CONTRACTED IN ZIMBABWE

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Diagnosis of rickettsial diseases is often missed, because the symptoms are not characteristic and the routine laboratory findings are of only marginal help. However, rickettsiosis continue to constitute major problems in many parts of the world, especially in tropical or temperate areas, and the number of travelers at risk is likely to increase as more people visit the endemic areas. Spotted fever group rickettsiosis (SFGR) that are prevalent from southern Europe to southern Africa are recently classified into two groups, i.e. Mediterranean spotted fever (MSF) caused by *Rickettsia conorii* and African tick-bite fever (ATBF) caused by *R. africae*. Here we focus mainly on a case of SFGR contracted in Zimbabwe and also briefly mention another case experienced very recently. Case Report: A 40-year-old Japanese male presented with a low-grade fever and an eschar of the left lumbar part 13-days after returning from a trip in Zimbabwe. A physical examination demonstrated scarce maculopapular rash on the

face, trunk, and both legs, and mild hepatomegaly but no lymphadenopathy. Laboratory studies yielded the following values : WBC, $4.9 \times 10^9/l$ (neutrophils 34.5%, lymphocytes 50.5%, atypical lymphocytes 4.0%, monocytes 6.5%, eosinophils 3.0%, basophils 1.0%); hemoglobin, 14.8 g/dl; platelets, $235 \times 10^9/l$; alanine aminotransferase, 27 IU/l; aspartate aminotransferase, 17 IU/l; lactate dehydrogenase, 572 IU/l; and erythrocyte sedimentation rate, 35 mm/hr. Weil-Felix test were all negative. Antibodies against *Rickettsia conorii* on the 17th day after onset of symptoms were negative (IgM < 1 : 10, IgG < 1 : 10), with a significant rise in these titers occurring over a period of 7 days (IgM 1 : 160, IgG 1 : 160). The patient was treated with minocycline 200 mg/day orally for 4 weeks, and made an uneventful recovery. Additional case: A 34-year-old man presented with fever while he was staying in the Republic of South Africa. A few days later, he noticed a rash affecting his entire body and an eschar on the left lum-

bar part. 11-days after returning to Japan, he made an uneventful recovery without any medicine except for rash. Antibody examination against *Rickettsia conorii* done on the 18th day after the onset of the symptoms was positive (IgM 1 : 160, IgG 1 : 320). In both cases the diagnosis of SFGR was confirmed serologically by in-

direct immunofluorescence assay. However, because the causative agents were not isolated, differentiation between MSF and ATBF was not possible. With increasing international travel, a need for the recognition of rickettsial diseases by physicians is becoming more important.

32 TWO CASES OF AMOEBIC LIVER ABSCESS

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Case 1 : 58 years old man was admitted who complained high fever, upper abdominal pain and appetite loss. He had been performed aortic valvular replacement because of severe aortic regurgitation 5 years ago. Abdominal CT showed a space occupying lesion suspecting liver abscess in left lobe 8 cm in diameter. As he had been given anti-coagulant, the abscess was drained by surgery. Chocolate colored pus was evacuated, however, any kind of bacteria was not detected by cultivation. Abscess cavity was irrigated continuously, nevertheless, he was getting worse and three new hepatic lesions were appeared after 10 days of the previous operation. As he developed into diffuse peritonitis 12 days after drainage, emergency laparotomy was performed which revealed perforated liver abscess. The patient died the next day after surgery because of septicemia. Autopsy revealed invasive amoebiasis in the large bowel and the liver. Case 2 : 66 years old homosexual man was admitted because of fever and diarrhea. As high fever was continued in spite of treatment of antibi-

otics, he was referred to our hospital. CT showed a solitary space occupying lesion 7 cm in diameter in the right liver lobe. Catheter abscess drainage was performed under ultrasonic guidance. Having an appearance like coffee cream, the discharge was urgently examined microscopically to prove *E. histolytica*. The patient went quite well immediately after administration of metronidazole. *E. histolytica* in homosexual men is a commensal organism, and all available information indicated that enteric protozoal infection are several times more frequent in homosexuals than in heterosexuals. The time to clearance of the parasite was not significantly different between patients who did and did not have antibody to HIV. In these points, the reason why the prevalence of amoebic liver abscess in homosexuals is the high rate of protozoal infection. It also might be emphasized that amoebic liver abscess should be considered in case of ineffectiveness of antibiotic treatment.

33 THREE CASES OF AMEBIASIS

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We reported three cases of amebiasis, which was clinically not correctly diagnosed and resulted in death.

Case 1: A 72-year-old man manifested bloody stool and anemia. He had been in Myanmar at World War II. A diagnosis of acute hypoplastic undifferentiated leuke-

mia was made at seven month before his death. Three months before his death he complained of fever and abdominal pain and abdominal CT scanning showed liver abscess. He died from peritonitis and paralytic ileus. Autopsy revealed yellowish white fragile tumors

up to 12 cm in diameter in the right lobe of the liver. Histological examination revealed amebas in and near the necrotic tissue. In addition amebas were disseminated in the peritoneal cavity, bone marrow, skin and tonsils. We cannot find amebas in the rectal ulcer with surrounding fibrosis. Case 2: A 58-year-old man complained of fever and abdominal pain. He and his family had not been abroad. He had received aortic valve replacement three years before. Abdominal CT scanning showed liver abscess and drainage operation and administration of antibiotics was performed with some improvement. Recurrent fever, right lower abdominal pain and multiple liver abscess appeared and he died from cachexia. Autopsy revealed multiple yellowish white fragile tumors, up to 8 cm in diameter in the liver and many ulcers on cecum and ascending colon. Histological examination showed many amebas in the liver

and colon. Case 3: A 72-year-old man displayed signs of common cold and received ampicillin at a clinic. He and his family had not been abroad. After three days, he had high fever and bloody stool and admitted to the hospital and received supportive therapy with no improvement of the symptoms. On administration of prednisolone, peritonitis was evident and colectomy was made. The resected colon showed yellowish white necrotic material all over the mucosa and many flask-shaped ulcers. Histological examination showed numerous amebas beneath the ulcers. He died from peritonitis. Recently, the number of patients of amebiasis is increasing. Only one third of the patients has been abroad. Amebiasis is easy to cure if a correct diagnosis is made. Thorough search for amebas is essential in colonic biopsy if a patient manifests fever and diarrhea, and antiameba antibody is useful if liver abscesses are evident.

34 A CASE OF VIVAX MALARIA

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We reported a case of relapsed vivax malaria, of which parasitemia reached to one present on admission. The case was an Iranian man, 29 years old. He had suffered from vivax malaria in India in 1994 and 1995. He was medicated with chloroquine for three days in each time. He moved to Japan in August 1996, and settled in a city in Ibaraki Prefecture. On November 2 1996, he felt fever, headache and fatigue. The fever repeated about every 48 hours. He visited to a clinic and was medicated with a pain killer. Since the symptoms had continued and gotten strong, he visited to Shioya General Hospital on November 10. He was suspected as malaria because of the symptoms and a labo datum, the number of platelets was $30 \times 10^3/\text{mm}^3$, and was sent to Jichi Medical School Hospital as an emergent case. His

body temperature was 39.5°C on the admission. His blood was sent to the Department of Medical Zoology, and was immediately made diagnosis as vivax malaria with acrydine orange staining method. Parasitemia was 1.0%. He was treated with chloroquine, 600 mg per day, for three days. By the third admission day, body temperature recovered and general fatigue was alleviated. Malaria parasites vanished from the 4th day. After checking of his glucose-6 phosphate dehydrogenase level in his RBC, we started to give him primaquine, 30 mg per day for seven days, for the purpose of killing hypnozoite of vivax malaria in the liver. The Duffy blood group was Fy (a+, b-), which was sensitive for vivax malaria merozoite to attach and invade into the RBC. After 10 days' admission, he discharged.

35 STUDIES OF EFFECTOR CELLS AND MHC CLASS II IN MOUSE INFECTED WITH ERYTHROCYTIC STAGE OF *PLASMODIUM YOELII*

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It has been suggested that pathogenesis and prognosis are associated with MHC molecule expressed in patients in malaria infection. The relationship between MHC class I and pathogenesis were reported in a few cases, however, the studies of pathogenesis association with MHC class II in malaria infection were not well known. From these findings, the studies of the relationship between MHC and malaria are necessary to develop vaccines involvement of antigenic epitope against malaria infection. In the present study, we explored the relationship between MHC and pathogenesis, effector cells and antigenic epitope recognized by these effector cells in mouse infected with *Plasmodium yoelii* (*P. yoelii*). H-2b, d, or k and these F1 mice were employed in this study; C57BL/10 congenic mouse (H-2k, a, d, s) and BALB/C congenic mouse were also used. *Plasmodium yoelii yoelii* 17 \times lethal (L) strain or non-lethal (NL) strain of erythrocytic stage were used as infectious malaria. Parasitemia of mouse

inoculated with NL strain were slowly increased and all mice were survived after infection. Mice infected with L strain showed rapid and high parasitemia, and all mice were died except H-2b or H-2bF1 mouse. Spleen cells of surviving H-2b/d mice against L or NL *P. yoelii* infection were transferred into SCID mice of the same strain and these SCID mice were subsequently inoculated with either L or NL *P. yoelii*. The recipient SCID mice were resistant to both L and NL strain infection; however, CD4⁺ cells eliminate SCID mouse were died after both strain of *P. yoelii* challenge. Proliferative responses of spleen cells in H-2b/d mouse against MSP-1 peptide were observed after infection of *P. yoelii*. These study suggested that pathogenesis of malaria infection of erythrocytic stage were associated with MHC class II molecule, and L and NL strain had same antigenic epitope. Moreover, CD4⁺ cells were critical component of protective immunity against erythrocytic stage malaria infection.

36 KINETICS OF LYMPHOCYTE SUBSETS FROM PERIPHERAL BLOOD IN A PRIMATE MODEL OF SEVERE HUMAN MALARIA: *PLASMODIUM COATNEYI*-INFECTED *MACACA FUSCATA*

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During the acute phase of falciparum malaria, dramatic modifications of immunocompetent blood cell populations may be detected, including a decreased number of lymphocytes, especially T cells. Recently, we reported that a Japanese macaque-*Plasmodium coatneyi* infection system provided a model for the study of severe human malaria. In this system, pathological changes and manifestations similar to those seen in severe human malaria were observed. In the present study, variations of lymphocyte subsets in peripheral blood were followed longitudinally in two infected

monkeys. Two Japanese macaques (*Macaca fuscata*), nine and ten months of age, respectively, were used in this experiment. Both monkeys were inoculated intravenously with 1×10^7 *P. coatneyi*-infected erythrocytes. Between ten and thirteen days after inoculation, the animals became moribund. Samples of heparinized, peripheral blood were obtained from the infected monkeys before inoculation with the infected cells, and subsequently on days 3, 6, 10, and 13. Twenty microliters of MoAb (anti-human CD4, CD8, CD16, CD20, and CD14) was added to 100 μ l of whole blood and

incubated for 15 min at room temperature. The cells were washed twice, resuspended in phosphate-buffered saline, and analyzed using a Becton Dickinson FAC scan. The population levels of CD4⁺ T cell showed little change, while the CD8⁺ T cell population decreased

when the animals became moribund. In addition, CD20 and CD14-positive cells gradually increased in the population during the course of infection. These results suggest that a strong Th2 response early in infection is associated with a lethal outcome of malaria.

37 ROLES OF IL-12 IN BLOOD-STAGE MALARIA INFECTION

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We have been investigating the mechanism to develop the protective immunity to blood-stage malaria infection and also that to cause the pathogenesis by comparing immune responses induced by a lethal strain *Plasmodium (P.) berghei* NK65 and its irradiation-induced self-limiting variant *P. berghei* XAT. In the present study, we analyzed the involvement of IL-12 in the protective immunity to blood-stage *P. berghei* XAT infection and its effector molecules. Immune competent mice spontaneously clear *P. berghei* XAT parasites and recover from the infection after two peaks of parasitemia. The infection enhanced mRNA expression of IL-12 p40 and also of IFN- γ , IL-4, IL-10 and cytokine-inducible nitric oxide synthase (iNOS) and augmented natural killer (NK) cell lytic activity in spleen during early course of the infection. Increased production of IL-12 p40, IFN- γ , IL-10 and NO₂⁻ was also evident in culture supernatant of spleen cells from the infected mice. Treatment of these mice with neutralizing monoclonal antibody against IL-12 or IFN- γ led to the

progression of parasitemia and fatal outcome. Anti-IL-12 treatment of the infected mice strikingly reduced the secretion and mRNA expression of IFN- γ but minimally affected the increase in IL-4 and IL-10 mRNA expression. In addition, the augmentation of iNOS mRNA expression and NK cell lytic activity was greatly diminished by the treatment. Consistent with the important role of endogenously produced IL-12, rIL-12 administration delayed the onset of parasitemia due to the enhanced IFN- γ production. However, experiments using anti-NK1.1 to deplete NK cells in vivo and those using iNOS^{-/-} mice revealed that NK cells and NO production appear not to be critically involved in the protective immunity. Taken together, these results suggest that blood-stage *P. berghei* XAT infection induces IL-12 production in spleen which is important for the development of protective immunity via IFN- γ production. We are currently investigating other effector molecule(s) involved in the protective immunity.

38 INDUCTION MECHANISM OF *P. BERGHEI* INFECTION INDUCED HEPATITIS

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We can induce liver injury by sequential treatment of the mice with *Propionibacterium acnes* and LPS. We found that, inoculation of the mice with *P. berghei*

infected red blood cells induce a shock-like state at the third week. We also found such infected mice suffered from severe liver injury with high serum transaminases

level and IL-18 at the second week after infection. IL-18 is a new cytokine capable of inducing IFN- γ production from Th1 cells and NK cells. Further this cytokine induces FasL on Th1 cells and some type of NK cells. Liver is a unique organ, containing CD3^{int}, IL-2R β ^{pos} T cells, NK cells and Kupffer cells. We found that, CD4^{pos} T cells in CD3^{int}, IL-2R β ^{pos} T cells completely

disappeared at 8 days after infection. Such liver lymphocytes have shown activity to produce perforin and to kill the YAC-1 cells, suggesting that this cytotoxicity of these infected mice were mediated by extrathymic T cells and NK cells. We need further study to reveal the pathological roles of these cells in the liver injury.

39 PLASMODIUM FALCIPARUM PRODUCES PROSTAGLANDINS

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Plasmodium falciparum causes the most severe form of human malaria which kills about 1.5 to 2.7 million people every year. Malaria symptoms are general inflammatory responses, including periodic fever with shivering, headache, body pains, sleepiness, and loss of appetite, and are commonly accompanied by suppression of both T and B cell-mediated immune responses. However, the molecular mechanisms underlying the clinical symptoms and the host-parasite interaction remain unclear. We show here that *P. falciparum* produces prostaglandins (PGs) D₂, E₂, and F₂. After incubation with 1 mM arachidonic acid (AA), cell homogenates of *P. falciparum* produced PGs as determined by enzyme immunoassay and gas chromatography-mass spectrometry. PG production in the parasite homogenate was not affected by nonsteroidal anti-inflammatory drugs, aspirin or indomethacin, and was

partially heat resistant, whereas PG biosynthesis by mammalian cyclooxygenase was completely inhibited by these chemicals and by heat treatment. Addition of AA to the parasite cell culture markedly increased the ability of the parasite cell homogenate to produce PGs and of parasite-containing cells to accumulate PGs in the culture medium. PGD₂ and PGE₂ accumulated in the culture medium at the stages of trophozoites and schizonts more actively than at the ring stage. Since PGD₂ and PGE₂ are well known somnogenic, pyrogenic, and immunosuppressive substances in mammals, they may, in part, be involved in the clinical manifestation of malaria. These findings are the first evidence of the direct involvement of a malaria parasite in the generation of substances that are pyrogenic and injurious to the host defenses.

40 CLONING OF 100kDa MEROZOITE RHOPTRY PROTEIN (Rhop 100) GENE FROM *PLASMODIUM YOELII*

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All invasive forms of Apicomplexa possess a complex of organelles located at the anterior end of the parasite which comprise rhoptries, dense granules and

miconemes. The rhoptries are pear-shaped, electron-dense, membrane-bound organelles and are considered to have roles in host cell attachment, invasion and

formation of parasitophorous vacuole membrane. We have developed monoclonal antibodies (mAb) against rhoptry proteins of the rodent malaria *P. yoelii* merozoites. These mAbs specifically bind to the antigens of 140/125kDa or 100kDa as assessed by immunoblots of Western transfers of SDS-PAGE gels. mAb # 25 reacted with 140/125kDa antigens and mAb # 32 reacted with 100kDa antigen respectively run under reducing conditions. These mAbs also reacted with *P. berghei* rhoptry proteins. mAb # 25 reacted strongly with a 135kDa antigen of *P. berghei* merozoites run in SDS-PAGE under reducing conditions. Subcellular localization of the antigens was confirmed by IFA and immunoelectron microscopy. We affinity purified 100 and 140kDa *P. yoelii* rhoptry proteins by using the affinity column conjugated with mAb # 25. After partial purification,

we analyzed the partial amino acid sequences of the *P. yoelii* rhoptry proteins. The amino acid sequences of five peptides obtained from 100kDa rhoptry protein are decided. Based on these partial amino acid sequences, we synthesized several sets of degenerate PCR oligonucleotides. Using these primers in a PCR reaction, a gene was amplified from the *P. yoelii* cDNA library. Analysis of the amino acid sequence deduced from the 648 bp single open reading frame of this gene revealed that this protein is the homologue of RhopH3 of *P. falciparum*. This partial amino acid sequence of *P. yoelii* 100kDa rhoptry protein has 35% similarities with that of *P. falciparum* RhopH3 exon 3 to 6. We also amplified the gene of the N-terminus of the *P. yoelii* 100kDa rhoptry protein from genomic DNA library and determined the nucleotide sequence.

41 A NOVEL OOKINETE SURFACE PROTEIN FROM *PLASMODIUM VIVAX* IS A TRANSMISSION-BLOCKING VACCINE CANDIDATE

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Vaccination of humans to prevent transmission of the malaria parasite to the mosquito vector is one of the control strategies currently being developed. Targets of transmission-blocking immunity are proteins expressed by the sexual stages of *Plasmodium* species. In *P. falciparum*, the prime vaccine candidates are Pfs25 and Pfs28, ookinete surface antigens of 25- and 28-kDa, respectively. The analogous proteins, Pgs25 and Pgs28, from *P. gallinaceum*, and Pys25 and Pys21, from *P. yoelii*, and Pbs25 and Pbs21, from *P. berghei*, have been described previously. However, many researchers have tried unsuccessfully to isolate the analogous genes from *P. vivax*. To search for a gene encoding the homologue of Pfs25 and Pfs28 from *P. vivax*, the gene sequences of the eight known proteins were aligned and one set of degenerate PCR oligonucleotides were synthesized.

Using these primers in a PCR reaction, a unique gene was amplified from the *P. vivax* genomic DNA. The complete sequence of this gene was determined from DNA amplified from the splinkerette DNA library template by using pairs of gene-specific and splinkerette-specific primers. Analysis of the amino acid sequence deduced from the single open reading frame of this gene revealed a presumptive secretory signal sequence, followed by four EGF-like domains with a total of 20 cysteines, and a short hydrophobic region at the carboxy-terminus. The presence of four cysteines in the fourth EGF-like domain suggested that this gene is the homologue of Pfs28, Pgs28, Pys21 and Pbs21; therefore we refer to this gene as Pvs28 based on its primary structure.

42 ANTIMALARIAL MECHANISM OF CYCLOPRODIGIOSIN

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Cycloprodigiosin hydrochloride (cPrG · HCl), a member of the prodigiosin family, is a red pigment obtained from the marine bacterium *Pseudoalteromonas denitrificans*. In recent study we found that cPrG inhibits the H⁺ translocation by vacuolar type ATPase (V-ATPase) in mammalian and plant cells. Here we report that cPrG has high antimalarial activity and unique properties. cPrG showed antimalarial activity *in vitro* against cultured *Plasmodium falciparum* (FCR-3 strain). As a result, 50% growth inhibitory concentration (IC₅₀) was 10 nM, showing high antimalarial activity compared with the IC₅₀ value of chloroquine (18 nM). cPrG was also examined for *in vivo* antimalarial activity against *P. berghei* (25 mg/kg, consecutive 3 days, i.p. injection). Macrobiotic effect was observed in

infected mice, but they were not cured. We evaluated the IC₅₀ value of chloroquine when using together with cPrG. It has been thought that weak base chloroquine shows the antimalarial activity by accumulation in acidic vacuole. As a result from the experiment in which chloroquine was used together with cPrG, there was no change in the IC₅₀ value of chloroquine. The result suggests that antimalarial activity of cPrG has another mechanism in addition to inhibition of V-ATPase. Using the property that cPrG fluoresces itself, we confirmed cPrG was uptaken in malaria parasites with fluorescence microscopy. We are going to elucidate the relation between food vacuole function and V-ATPase by using cPrG.

43 EVALUATION OF ANAEROPACK CAMPYLO® : CAN ANAEROPACK® SERIES BE A NOVEL TOOL TO CULTURE AND DETERMINE DRUG SENSITIVITY OF *PLASMODIUM FALCIPARUM*?

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Infestation of drug resistant strains of *Plasmodium falciparum* (*P. falciparum*) is an immense obstacle for prevention and treatment of falciparum malaria. It is important to monitor drug sensitivity to document the extent and distribution of resistance not only against chloroquine but other anti-malarials, such as mefloquine, quinine and so on. The drug sensitivity assays are usually carried out using candle jar technique to determine drug sensitivity in the field. The assay system is required to be easy to carry out, because of unsatisfied facilities in the field. The candle jar, heavy and taking labour intensive to manage, occupies the huge space in incubator. Therefore, standardised method will be anticipated to compare the result obtained from fields with laboratory. AnaeroPack Campylo* system has

been developed by Mitsubishi Gas Company (MGC), Tokyo, Japan, to culture the microaerophilic bacteria, such as *Campylobacter* spp. and *Helicobacter* spp. The system maintains the gas phase in a specific jar (AnaeroPack Kakugata jar*) at 10% of CO₂ and O₂ respectively. Jar itself is very light and easy to handle. We evaluated three methods : 1 Gas blowing 2 candle jar 3 AnaeroPack Campylo*, in terms of growth efficacy and drug sensitivities. The growth curve of among *P. falciparum* K1 and HB3 strains showed no difference in all methods. The results of IC₅₀ both strains showed no remarkable difference in between original gas condition and AnaeroPack Campylo* System. We expect AnaeroPack* as a novel tool to monitor drug sensitivity of *P. falciparum* in the field. In addition, it would be feasible

to use this system for the transportation of wild strains from rural field to well equipped urban laboratory for studies. Further modification should be required to

evaluate the potential of this system for field application.

44 A VARIANT OF *PLASMODIUM OVALE*: ANALYSIS IN THE SEQUENCE OF THE 18S RIBOSOMAL RNA GENE

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We report here a variant of human malaria parasite, *Plasmodium ovale*, which was found by diagnostic results from a new DNA diagnostic method (microtiter plate-hybridization, MPH) and traditional microscopic examination. Six cases of infection of *P. ovale*-variant were found by us; one case was in epidemiological research in Vietnam and five cases were in clinical diagnosis of imported malaria in Japan. Although parasites in these cases were morphologically indistinguishable to *P. ovale* in microscopy, they were diagnosed as negative by previous MPH, because these parasites had different in 18S rRNA gene sequence of reported *P. ovale*. We suppose that the parasite may be a kind of new plasmodial species. We performed sequence analysis of 2.1 kilobase of 18S rRNA gene of *P. ovale*-variant, which was obtained from the blood of a patient who had

been infected in Mali, western Africa. Sequencing procedure was as follows. Two DNA fragments which have either upper half (5'-side) or lower half (3'-side) of total length of 18S rRNA gene were amplified by PCR. PCR products were subcloned into plasmid pCR 2.1 vector by TA cloning method, and transfected to *Escherichia coli* INV α F' strain. Cloned DNA was extracted from transfected cells and nucleotide sequence was determined by autosequencer. As a result, three types of sequence were revealed. These sequences from *P. ovale*-variant were compared with previously published sequences of *P. ovale*, i.e. clone 9 and clone 26. They differs 3.5-3.7% in sequence from *P. ovale* clone 9, and 3.6-3.8% from *P. ovale* clone 26. They were closely related to published sequence of *P. ovale* 18S rRNA gene and far apart from that of any other plasmodial species.

45 MALARIA GENOME PROJECT IN JAPAN

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Malaria genome project, an effort to determine all the nucleotide sequences of plasmodium has started by several groups in the world. With a modest genome size, i.e., 3×10^7 base pairs divided in 14 chromosomes, *Plasmodium falciparum* is a good target because of its medical importance. Since 1990 we have studied the

structure of the chromosome # 4 on which DHFR-TS (dihydroforate reductase-thymidine synthase) enzyme is located and established the high resolution physical map. Extending this we have started Malaria Genome Project in Japan. 1) For genomic DNA sequencing, we are preparing genomic libraries constructed with P1

phage. It is a single copy phage which can maintain a 100kb insert in *E. coli*. With the genome notoriously rich in AT, high frequency of recombination has hampered previous efforts. Lack of intermolecular recombination between phage DNAs is one of the advantages. 2) Genomic DNA data should be supplemented with information about the expression of the genes. Using the method we have established to clone a full length mRNA, a cDNA library was produced from the erythrocytic stage *P. falciparum*. Sequencing of five

hundred clones revealed that most contained AT rich malaria sequences, two third of which were previously unknown ones. Moreover, a full length mRNA library of murine malaria should enable the screening of vaccine candidates as DNA vaccines in mouse model.

Reference: Establishing a physical map of chromosome No. 4 of *Plasmodium falciparum*. Junichi Watanabe and Joseph Inselburg, Mol. Biochem. Parasitol., 65, 189-200, 1994.

46 SEQUENCE DIVERSITY OF THE *PLASMODIUM FALCIPARUM* SERINE REPEAT ANTIGEN GENE EXON II IN THE WORLDWIDELY COLLECTED WILD ISOLATES

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Serine repeat antigen (SERA) of *Plasmodium falciparum*, a protein inducing antisera that inhibit the growth of the parasites *in vitro*, was one of the five antigens identified by the WHO as malaria vaccine candidates of high interest for their introduction to human vaccination trials. Protective effects in monkeys immunized with recombinant SERA protein were also reported. But, malarial parasites may escape from the attacks of host immunosystem by the antigen variation based on the genetic recombination and mutations and thus such mechanism becomes a barrier to the malaria vaccine development. For the design of a vaccine containing SERA protein, it is essential to understand in what extent the genetic diversity of this gene is present among the natural isolates of *P. falciparum* from different endemic areas in the world. The upto date reported SERA gene diversity was from the laboratory cultured strains. In this study, two high variable areas of the exon II within the immunogenic 47kDa peptide encoding region of the SERA gene were sequenced for the analysis of diversity among the wild isolates worldwide collected. The two areas of 68 wild isolates were amplified by PCR and then directly sequenced. Structurally the two variable areas can be named as anterior area and posterior area, with the repeat sequence I and

II in former and the 13 amino acid insert/deletion part, the serine repeat part and the 12 point mutation cluster part in the later one. From the culture strain derived data, SERA gene were identified as HB3/SL3/FCBR type, Camp/T9-102/K1/PA-7 type and Honduras-1/3D7/T9-96 type. The sequence data of 68 wild isolates showed that in the posterior area only the number of serine repeat was highly variable in all of three types except 4 isolates also having other point mutation. But the 68 samples showed the much higher diversity in forms of sub-repeat insert/deletion and frequent point mutations within the anterior area. Totally, in addition to the normal types, 2 mutants of HB3 type and 12 mutants of Camp/T9-102 type were identified while for Honduras-1 type only several isolates were found to be its mutants. Geographic distribution showed that: 1. Among 32 isolates from Southeast Asia, 18 were Camp/T9-102 type and their mutants, 9 were HB3 type and its mutants, and 5 were the mutants of Honduras-1 type. 2. In 5 isolates from Solomon Islands, 4 and 1 isolates were the HB3 type and the T9-102 type, respectively. 3. Most of the 20 isolates from Tanzania and west Africa countries were the Camp type and its mutant beside 2 isolates were the mutants of Honduras-1 type. 4. 11 isolates from Brazil were the identical mutants of HB3

type, characterized by the longest 54 repeats of serine codes. Generally, the Camp/T9-102 type and their mutants are widely present in the endemic areas of the Old World. HB3 type and its mutants are likely

restricted in southeast Asia and Solomon Islands while an unique mutant with the longest serine repeats is detected only in Brazil, an New World country.

47 CLONING OF A GENE ENCODING VARIABLE DOMAIN OF ANTI-Pbs21 MONOCLONAL ANTIBODY

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Malaria control strategies include the prevention of transmission of the parasite from the host to the female *Anopheles* mosquito vector. The targets of the transmission-blocking are the *Plasmodium* sexual stages. Pbs21, a 21kDa protein expressed on the plasmalemma of the macrogamete, zygote, ookinete and oocyst of *Plasmodium berghei*, has been shown to induce an effective transmission-blocking immunity. A monoclonal antibody (Mab), called 13.1, directed against Pbs21 molecule has been reported to inhibit the development of oocysts in mosquitoes. In this study, we cloned and sequenced the variable region genes of the heavy and light chains (V_H and V_L) of Mab 13.1 by RT PCR. The V_H and V_L genes were connected to construct a single-chain variable fragment (sc-Fv) by $(Gly_4Ser_1)_3$ linker. The assembled 13.1sc-Fv gene was cloned into a phagemid for display of the sc-Fv as a fusion protein with the

M13 gene 3 coat protein. The resulting phages were found to express the sc-Fv gene by ELISA using plates coated with recombinant Pbs21 synthesized in baculovirus. Binding of the phage to the native Pbs21 of *P. berghei* ookinete was also shown by indirect immunofluorescence microscopy. In an attempt to obtain large amount of 13.1 sc-Fv, the 13.1 sc-Fv gene was cloned into pET expression vector, and produced in the periplasmic space of *E. coli* following IPTG induction. Western blotting was performed to determine specific binding of 13.1 sc-Fv purified from periplasmic fractions. Like Mab 13.1, 13.1 sc-Fv reacted with a 21kDa band of ookinete lysate, which corresponds to the molecular weight of the native Pbs21 molecule. The results presented here indicate that the 13.1 sc-Fv gene will be useful in studying the interaction between 13.1 sc-Fv and ookinetes in mosquito midgut.

48 EXPRESSION OF IRON-SULFUR CLUSTER SUBUNIT OF MITOCHONDRIAL COMPLEX II FROM *PLASMODIUM FALCIPARUM* IN *ESCHERICHIA COLI*

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The favorable effect of low O_2 level on the *in vitro* cultivation of *Plasmodium falciparum* suggests that energy metabolism of the parasite is different from that of human host. The preliminary study in recent years suggested that the parasite mitochondria, as well as glycolysis, could function in energy metabolism in asexual intraerythrocytic stages. For elucidating the role of

this organelle, analysis of enzyme complex II is critical because it is exclusively mitochondrial enzyme and plays unique role as a direct link between the TCA cycle and the electron transport chain. Complex II is generally composed of nuclear-encoded four subunits including two catalytic ones (flavoprotein subunit : Fp and iron-sulfur subunit : Ip), and functions as succinate

dehydrogenase (SDH) in aerobic mammalian-type mitochondria or fumarate reductase (FRD) in the mitochondria of parasitic nematode under anaerobic condition. In the previous study, we have characterized the genes for two catalytic subunits of *P. falciparum*: these are single copy genes on different chromosomes and no evidence was obtained for the presence of isoforms. The expression at erythrocytic stage was also shown by Northern blot analysis. The physiological catalytic function (SDH/FRD) of this enzyme is the critical question, but preparation of workable quantities of functional mitochondria from parasites are difficult at present. So, we planned alternative strategy using cloned genes: production of recombinant proteins and getting functional complex II by co-expression of these subunits. As the initial step toward this goal, in the present study we ligated DNA fragment corresponding

to the putative mature region of the Ip subunit into expression vector. IPTG-induction of transformed *E. coli* produced the recombinant Ip, which was confirmed by the amino terminal sequence analysis. The purified protein was then used to raise antiserum in rabbit. The antiserum showed weak crossreaction with mammalian (*Bos taurus*; bovine heart) Ip and no reaction with the counterparts from parasitic nematode (*Ascaris suum*) or bacterium (*E. coli*), which indicates that the epitope structure of plasmodial Ip is similar to that of mammals and the prepared antibody is adequately specific to *Plasmodium*. Immunofluorescence assay on *P. falciparum* and *P. yoelii* parasitized-erythrocytes demonstrated the expression of complex II, confirming the result of Northern analysis and encourages further effort toward the overall goal of this study.

49 ONSET OF CLINICAL SYMPTOMS AND ANTIBODY RESPONSES TO MSP1 IN INDIVIDUALS INFECTED WITH *P. FALCIPARUM* IN GUADALCANAL, THE SOLOMON ISLANDS

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Transmission of malaria is holo-endemic in Guadalcanal, the Solomon Islands. Among the Solomon's people infected with *Plasmodium falciparum*, most inhabitants are healthy carriers. In order to analyze biological function of host immune response in clinical malaria, we compared IgG production to MSP1 between symptomatic and asymptomatic individuals infected with of *P. falciparum*. Of 67 subjects tested, 23 were symptomatic patients with falciparum malaria showing high fever (>38°C), chill, head ache, and/or vomiting, and 44 were symptom-free individuals. There was no significant difference in sex ratio and the mean age between the two groups, but mean parasitic density in the symptomatic group was higher than that in

asymptomatic group. Twenty healthy Japanese donors were tested as controls. Symptomatic group showed a lower IgG level to MSP1 than that in asymptomatic group ($p < 0.001$), whereas there was no difference in antibody levels to polio virus. Moreover, we observed that the impaired IgG response was more apparent for the dimorphic block 6 compared with that for block 3, suggesting the presence of non-random immunoregulation. These results suggest that onset of clinical symptoms of falciparum malaria is somehow associated with impaired antibody response to malaria antigens, and the immunoregulation seems to be effective not for random epitopes, but for selective ones.

50 EXPRESSION AND PURIFICATION OF THREE ALLELIC FORMS FOR BLOCK 2 OF *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE PROTEIN 1

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Merozoite surface protein 1 (MSP1) of *P. falciparum*, a candidate molecule for malaria vaccine, contains 17 blocks. To date, there have been few studies on human immune responses to variable blocks and their roles in protection from the infection and/or the disease. Although variation in variable blocks is principally dimorphic, the exception is block 2, which is polymorphic but represented typically by either of three allelic forms according to the presence or absence of tripeptide repeats and the type of repeating units; K1-, MAD20-, and RO33-types. Our recent analysis of MSP1 allelic in field isolates revealed that the prevalence of MSP1 allelic types in block 2 differs in endemic areas. Therefore, it is of interest to see if human immune responses

to block 2 might influence the prevalence of MSP1 allelic forms in natural populations. To conduct seroepidemiology of MSP1, three forms of block 2 were expressed in *E. coli* as a fusion protein with glutathione S-transferase (GST) using pGEX-2T vector and purified with glutathione column. Antibodies to the fusion proteins were made in rabbits and showed high titers in ELISA. The antibody against K1-type did not cross-react with antigens of either MAD20- or RO33-type, and vice versa. From these results, the expressed antigens can be used for seroepidemiological study of MSP1. We are analysing human serums from patients in malaria endemic areas in Vietnam by using these antigens, comparing with DNA typing of block 2 of the patients.

51 EVALUATION OF THE *PARASIGHT F* TEST IN IMPORTED MALARIA

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The dipstick test, under the trade name of *Para-Sight F*, detects the histidine rich protein II which is specifically synthesized by *Plasmodium falciparum*. Previous studies done in field conditions reported excellent sensitivity and specificity of the test although the figures never reached 100%. Here we took advantage of applying the test to imported malaria in which the details of the patients can clearly be defined. Blood samples were obtained from febrile patients who visited the Institute of Medical Science, University of Tokyo, Ebara Metropolitan Hospital or Jikei University School of Medicine. The results of the dipstick test were compared with those of microscopy of Giemsa-stained

blood films, and also with those of the species-specific PCR assay which amplify a part of the 18S rRNA gene and can confirm the microscopical diagnosis of malaria and its species identification. In some cases, the above tests were performed serially after the start of antimalarial treatment. All of 19 cases of falciparum malaria showed a positive dipstick test. Most of the cases were contracted in sub-Saharan Africa, several in Oceania and the least in southeast Asia. Three (17.6%) of 17 cases of vivax malaria were also positive, although the reaction was weak. Only one case of ovale malaria was tested and yielded a negative result. A total of 40 non-malaria cases were all negative for the dipstick

test. The urine from 3 out of 9 patients with falciparum malaria were tested positive. Although serial assays after the start of treatment were not performed systematically, a positive dipstick test was observed in some cases 1-2 weeks after microscopy has become negative, and even 4 weeks in a rare instance. This led us to the opinion that caution is required when applying the

dipstick test to the evaluation of treatment, e.x. for predicting recrudescence of falciparum malaria. Although the number of patients tested is small and more cases contracted in various countries should be included, we conclude that the dipstick test could be a very useful supplementary tool in clinical practice of malaria.

52 DEVELOPMENT OF AUTOMATIC ANALYZING SYSTEM FOR MALARIA PARASITES USING FLOW CYTOMETRY

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Malaria is one of the most serious disease in the world because of the lack of effective vaccines and the rapidly spread of drug resistance of malaria. Thus, the basic studies purposing the malaria control are becoming more and more important. The effects of newly developed vaccines and antimalarial drugs should be evaluated in the laboratory as well as in the field, *in vitro* and *in vivo*. Moreover, it is necessary to analyze a plenty of samples for diagnosis in order to control malaria. In these circumstances, the biggest problem is that microscopic examination of stained smears to detect and count parasites as gold standard is really time consuming and that the data often seems to be strongly influenced by the technical ability or human errors, especially when a lot of samples should be analyzed at the same time such as in the laboratory studies. The objective and reproducible and, if possible, automatic method to detect and analyze parasites is desired. The method by flow cytometry is one of the most promising ways to satisfy these requirements. The method using DNA specific fluorescence dye such as Hoechst 33258 or 33342 has been proved to be sensitive and stable, although flow cytometry with ultraviolet

laser, which is expensive and rarely equipped even in the central hospitals and laboratories of developed countries, is required. On the other hand, the method by flow cytometry with argon laser using DNA and RNA fluorescence dye such as acridine orange has not been able to obtain the satisfactory sensitivity because of the difficulty to distinguish the infected red blood cells from the reticulocytes. We report here a newly developed method to detect the parasites by flow cytometry using acridine orange, aiming at the point that the influence of reticulocytes can be omitted if lysis is caused before analysis. Because the parasite could not be analyzed probably due to the damage of membranes of erythrocytes and parasites when we lysed red blood cells using hypotonic solution and/or saponin, first we developed the new lysing solution. In this system, *P. falciparum* in cultured samples could be detected at the similar level of microscopic examination. *P. falciparum* could be also differentiated from white blood cells and platelets. Moreover, stage-specific parasites (merozoites, ring forms, trophozoites and schizonts) could be clearly distinguished in this system. This system seems to contribute especially for the laboratory study.

53 AN IMPROVED SINGLE-STEP SCREENING METHOD FOR GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) DEFICIENCY

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One of the complications in the malaria chemotherapy is drug-induced hemolytic anemia in G6PD-deficient subjects particularly by primaquine. It is thus quite important to screen G6PD-deficient subjects before starting the chemotherapeutic control. We report here an improved single-step colorimetric method suitable for rapid screening for G6PD-deficient subjects in the field. The principle of our method is the formation of blue formazan with NADPH produced by G6PD absorbed on a DEAE-Sephadex anion exchanger. All the reactions are carried out in a 1.5 ml microcentrifuge tube at room temperature. A tube containing 200 μ l each of DEAE-Sephadex A-50 equilibrated in 0.1 M Tris-HCl, 10 mM MgCl₂, pH 6.5, the substrate mix with 5 mM G6P, 0.4 mM NADP⁺ and 0.2% Saponin in H₂O, and the MTT-PMS mix with 0.025% each of MTT and phenazine methosulphate in H₂O is prepared. The reaction is started by adding 5 μ l of whole blood to the tube and mixing by shaking several times. A gel bed forms

immediately by natural sedimentation and is clearly separated from the upper reddish aqueous layer. After 20 minutes incubation, the development of blue color on the gel with patient's blood is compared with that with control blood. With normal control samples, color development is apparent after 20 minutes incubation and the intensity reaches a maximum after 40 minutes. Samples with less than 30% residual activities show very slow color development. In our method, the whole single-step procedure can be completed in less than 30 minutes without any special equipment other than micropipettes. In comparison with other procedures, our method has the great advantage of its rapidity and simplicity with similar reliability. These features make our method particularly suitable for field detection of G6PD-deficient subjects prior to administration of primaquine *in situ* as well as for ordinary laboratory tests.

54 CLINICAL EVALUATION OF THE NEW METHOD FOR QUICK DETECTION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY

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To eliminate exo-erythrocytic parasite and gametocyte which transmit malaria to mosquito, primaquine and related drugs are required. However, primaquine may cause acute hemolytic attack if the patient lacks glucose-6-phosphate dehydrogenase (G6PD) activity. We checked the G6PD activity of a patient who had clinical malaria caused by *P. vivax*, to start early treatment against gametocyte and exo-erythrocytic stage malaria parasite using new methods; formazan-ring method and single-step screening method, then, could treat him earlier. We also used these methods in the field trying to adapt them for field study. Method: G6PD is the first enzyme of pentose-phosphate

cycle. Both of two methods uses production of blue-colored formazan from MTT. The difference of them is; formazan-ring method (Fujii *et al.*, 1984) requires agar and cation exchange paper (Whatman P81), single-step screening method (Hirono *et al.*, 1997) requires anion exchange resin (DEAE-Sephadex A-50). We prepared agar plate and test tubes, then, compared G6PD activity of a malaria patient to a male adult who had normal G6PD activity. Results: 1) The patient was 29 years old male, who had worked as a volunteer at a pariative care center in Calcutta, India. He stayed there since had fever. Patient's blood showed same G6PD activity compared with a normal control, we could start

primaquine treatment against exo-erythrocytic stage parasite within the same day on admission. The patient was cured from clinical malaria within 3 days. 2) We used these two methods for field screening, and found that we needed some device to use them. The test tube was quite sensitive against sunlight, the color was changed within 2 minutes when the tubes were exposed under sunlight. We put test tubes with blood sample into dark and cold place to keep them inactive, until some samples were collected. We could not use formazan-ring method in the field where incubator of 37°C was not available. Conclusion: Both of formazan-ring method and single-

step screening method are quite useful for quick diagnosis of G6PD deficiency for early treatment and field survey. Formazan-ring method seems to be more efficient in laboratory testing, because it is more qualitative than single-step screening method. Single-step screening method is more suitable for field survey because it does not require incubator and it is time saving. However, we can use both methods in laboratory testing and field survey. It is a big problem to measure reagents correctly when we try to introduce the method to developing countries. If we can get a kit, using the principle, we can check the G6PD deficiency easier.

55 FIELD SURVEYS ON G6PD DEFICIENCY IN LAOS, THAILAND AND INDONESIA

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Recently Hirono *et al.* (1997) have developed a novel and rapid, single-step method for detection of glucose-6-phosphate dehydrogenase (G6PD) deficiency by using a small aliquot of blood (5 μ l). We applied this rapid test at fields for the first time at the Pakson district, the Chanpasack Province, southern Laos, and Halmahera island, the Maluku Province, eastern Indonesia, in combination with malaria survey by a rapid diagnosis using the acridine orange (AO) staining method. Blood samples were collected by a finger pricks from volunteers. First, 5 μ l of blood for G6PD test was taken and quickly mixed with a reaction mixture in a 1.5-ml microcentrifuge tube on ice. Then, two drops of blood were collected on a Whatmann P-81 paper for further confirmation of the rapid test of G6PD deficiency by the formazan-ring method in laboratory. Finally, a thin and thick smear was made for the rapid diagnosis of malaria infection by the AO method. At two camps for the Karen refugees near Mae Hong Song,

northern Thailand, 2-3 drops of blood samples were collected by finger pricks, and brought back to Japan. Then, they were examined in a laboratory. In Laos, a total of 677 volunteers were examined, and 66 (10.8%) were G6PD deficient. In the Halmahera island, a total of 407 volunteers were examined, and 40 (9.8%) were found to be G6PD deficient. On the other hand, blood samples obtained from the blood bank at Surabaya, eastern Java island, were also examined by two methods, the rapid test and the formazan method. In a total of 378 tested, G6PD deficiency was detected in only 7 (1.9%). In Karen peoples, G6PD deficiency rate was 6.8% (11/151). All of the results on G6PD deficiency obtained by the rapid test were informed to patients on-site, and these results were later confirmed by the formazan method in laboratories, indicating that the rapid test for the detection of G6PD deficiency is useful method at fields.

56 INHIBITORY EFFECTS ON THE *PLASMODIUM FALCIPARUM* GROWTH IN ERYTHROCYTES WITH SEVERE GLUCOSE-6 -PHOSPHATE DEHYDROGENASE DEFICIENCY

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It is well known that G6PD deficiency is inhibitory to the growth of *Plasmodium falciparum*, but its inhibition mechanism is still unknown. In addition, most of the previous studies have been focused mainly to the growth rates in the G6PD deficient erythrocytes, and there has not been reported in details on the morphological aspects of the parasites. The aim of this study is to investigate the development of *P. falciparum* (strain G 2300 isolated from Irian Jaya) in erythrocytes with severe deficiency of glucose-6-phosphate dehydrogenase (G6PD) and the effect on the morphology of the parasites during *in vitro* culture. Blood samples were obtained from Indonesian six males, 15-28 y.o. with G6PD activities ranging from 6-16 mU/10⁹ erythrocytes. For statistical comparison, the normal erythrocytes were also obtained from five males, 20-28 y.o. with normal G6PD activities ranging from 100-140 mU/10⁹ erythrocytes. All these blood samples were the same blood type of A or O, and they had normal hemoglobin level. The parasite growth was compared in normal and G6PD deficient erythrocytes in *in vitro* culture in

RPMI-1640 with 10% human serum. Each day 5,000 erythrocytes taken and stained with Giemsa were evaluated for the parasite growth and their morphology until sixth day of culture. The results obtained were analyzed by t-test. Inhibition of the parasite growth in G6PD deficient erythrocytes could be detected by the third day. All stages of parasites were seen in G6PD deficient erythrocytes until the sixth day, but numbers of ring forms and schizonts were much decreased in G6PD deficient erythrocytes when compared with those grown in the normal erythrocytes. Ring forms and schizonts appeared degenerate and showed abnormality in morphology, especially after the fourth day. In G6PD deficient erythrocytes, decreases in NADPH production and then glutathione production have been proposed to explain the inhibitory mechanism for the parasite growth. Effects of the chemicals which activate or inactivate the glutathione metabolism on the parasite growth in the G6PD deficient erythrocytes is now in progress.

57 MALARIA SURVEYS BY A MOBILE MALARIA CLINIC SYSTEM USING A RAPID DIAGNOSIS

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In many malaria endemic countries, the rainy season brings large malaria outbreaks and also creates bad road conditions that prevent access to malaria-infected areas. Poor transportation, lack of electricity, and the absence of facilities to test for malaria make for inferior health care in the areas where have the largest numbers of malaria patients. An external halogen illuminator equipped with an interference filter and driven by an automobile battery has recently been developed for use

of the acridine orange (AO) staining method under field conditions. This has inspired us to develop a mobile unit using the rapid diagnosis method of malaria with the AO method. 'Mobile malaria clinic' system using this illuminator and a light microscope has been implemented in 4WD vehicles and used for rapid diagnosis in small villages where there is no electricity in South East Asia and Brazil. From October 1996 to October 1997, we surveyed malaria in Thailand (Mae Hong Son), Myan-

mar (Taninthavyi State), Vietnam (Giarai Province), Laos (Chanpasack Province), China (Suchian Province), Indonesia (Halmahera island, Maluku Province) and Brazil (Rondonia State). In Halmahera island, we used a speed boat instead of vehicles since there is no road to access villages. Except in China, there was no electricity during our surveys, and we used a car battery (50AH) directly connecting with the halogen light source. Frequent switching on-off of the illuminator provided long-time operation of battery (about 5 hours) without engine idling, and the maximum number of examined slides was 220 for 8 hours in a day. Total

numbers of malaria patients detected were 51/332 in Mae Hong Son, 59/402 in Taninthavyi, 69/220 in Giarai, 77/677 in Chanpasack, 27/826 in Suchuan, 119/407 in Halmahera, and 169/1570 in Brazil. These results may indicate that a mobile unit can make the rounds of villages despite poor road conditions, facilitating early diagnosis and prompt treatment, and thus it will be useful in improving local health care. This study was supported by grants from the Toyota Foundation (96B3-011) and from the Japanese Ministry of Education, Science, Culture and Sports (07041159 & 09041179).

58 PROMOTION OF RAPID MICROSCOPIC DIAGNOSIS OF MALARIA USING ACRIDINE ORANGE STAIN AND HALOGEN LIGHT IN TANZANIA

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In order to improve the speed and accuracy of malaria diagnosis in Tanzania, a microscopic method using acridine orange stain and halogen light (AO method) was introduced nation-wide through JICA's small scale cooperation schemes. Total 123 laboratory technicians have been exposed to the in-country training courses since 1994. Total 52 microscopes equipped with according light sources have been donated and distributed to the four consultant hospitals, 20 regional laboratories, 10 district hospitals and 3 health centres. The overall ratio of blood smears examined with AO method in 20 regional hospitals in 1996/97 was roughly 50%, ranging between 0 and 100% at individual region. The acceptance of the method depended basically on (1) the initiative of the laboratory technicians and (2) the acknowledgment of the medical officers on rapidness and accuracy of the method. Some technicians were reluctant to use the new equipment believing that the blue filtered light was harmful. Many doctors were

confident on their clinical diagnosis more than the laboratory results. Collaboration between laboratory technicians and nurses was crucial for the application of the method to the ward patients. In order to secure the sustainability, two workshops were held with the laboratory technicians to discuss on the supply of the consumable materials such as reagents, glasses, bulbs and fuses. Although the total number of the spare parts donated by Japan was sufficient for five years' activity nation wide, their distribution from the centre to particular laboratories was not smoothly done. However, most materials were locally available, and some regions have started to utilize the users' fee to purchase them. The equipment should be improved to resist heat, dust and voltage fluctuation. The whole practice showed that even a simple technology like AO method could not be transferred without proper institutionalization and system approach.

59 CONGENITAL MALARIA CASES IN DAR ES SALAAM, TANZANIA —RATHER LOW PREVALANCE—

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Many cases of malarial vertical infection have been reported in hyper-endemic area, especially in Africa. It was recognized that the placenta worked as a barrier against the fetal infection of malaria. However, the frequency of these reported cases were recently increasing. We investigated the prevalence of congenital malaria in the neonatal ward of Muhimbili Medical Center (MMC), by way of PCR method by which we might expect higher sensitivity than the ordinary Giemsa staining method. We underwent this research just after the rainy season when the malaria prevalence becomes at peak. 303 neonates who were admitted to the neonatal care unit of MMC were randomly selected and investigated by heel prick method after the informed consent through the MMC ethical committee. 24% of the neonates were suspected to have infections on admission, but there was only one among their mothers, who had malaria parasites in her peripheral blood. For PCR, a few drops of the blood was absorbed in a filter paper and was fixed with 100% methanol. DNA templates were extracted from these filter papers

by boiling with Chelex 100. We, then, did PCR with 18s ribosomal DNA primers. PCR revealed two positive cases and both were *P. falciparum* by nested PCR. Giemsa staining also showed two positive cases. Totally, we got three suspected malaria patients, one of which was positive by both Giemsa and PCR. Other two were not congenital malaria because one of them turned out to be negative after checking the same thick smear again. The other case was out of the definition because he had blood transfusion. As a conclusion, there was only one vertical malaria infection among 303 neonates and there was no significant difference on Birth Body Weight, Gestational age, Mother's age, Parity, Hematocrit and Total bilirubin between the infected neonate and non-infected ones. However, the frequency of congenital malaria from our result is much lower than those of recent reports in Africa, we can not point out any reasonable factors which lead us to the low frequent ratio of the vertical infection including technical problems. The congenital malaria prevalence of 0.33% seems to be real at Dar es Salaam in Tanzania.

60 EPIDEMIOLOGICAL STUDY ON MALARIA IN SOUTHERN CHINA —LONGITUDINAL STUDY OF SERO-EPIDEMIOLOGICAL STUDY—

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To elucidate the influence of malaria spreading due to global warming, we have been conducting sero-epidemiological study in three villages of southern China, Yunnan Province. Sero-epidemiological study has been carried out during from August 1993 until now.

Blood samples were collected on filter papers and were tested for *Plasmodium falciparum* and *P. vivax* using IFA test. The prevalence rate of IFA positive (>16) case showed clear seasonal fluctuation and we could find some evidence of recent malaria epidemics in those

villages for both *P. falciparum* and *P. vivax* from not only positive rate but also frequency distribution of IFA titer.

61 MALARIA EPIDEMIOLOGY AT SUMBAWA ISLAND, NTB, INDONESIA

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The epidemiological survey of malaria at Sumbawa Island has begun since August, 1996. In the preliminary survey three villages, Desa Penyaring, Desa Medang and Desa Stowe Berang, were selected according to the data from local health centers. Spleen examination of school-children in the first and second grade of elementary schools, and simple entomological examination were carried out to check applicability of the regular malaria survey to the above villages. Desa Penyaring showed only 0.6% spleen rate, consequently was selected as a control village. Desa Medang and Desa Stowe Berang showed 42.3% and 54.8%, respectively and were selected as proper areas for the next regular survey. In December, 1996 during the rainy season the first regular survey was done. Desa Penyaring showed 8 cases of slide positive although the spleen rate was 0% and no vector mosquito was caught. Since ages of all cases were between 25 and 52, suggesting they were imported cases,

we concluded that Desa Penyaring has no malaria endemic or extremely low endemic as we expected before. Desa Medang was replaced by Desa Labangka IV because of difficulty and danger of transportation. Desa Labangka IV showed low slide positive rate and spleen rate, indicating low endemicity. Curiously entomological examination showed only *Anopheles subpictus* was caught by human baits but no larva of *An. subpictus* was found but those of *An. minimus* and *An. barbirostris*. Desa Stowe Berang showed relatively high slide-positive rate even in the rainy season when low transmission was inferred from the behavior of *An. subpictus*. The entomological survey revealed the presence of *An. subpictus* by night collection and many breeding places such as lagoons and fish-ponds and speculated that active transmission would take place at some periods in the dry season.

62 LIMITATIONS OF CLINICAL DIAGNOSIS OF MALARIA AND STREPTOCOCCAL INFECTION AMONG FEBRILE CASES IN SOLOMON ISLANDS

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We investigated the usefulness and limitations of the clinical diagnosis of malaria in primary health care (PHC) activities in malaria-endemic area on Guadalcanal in Solomon Islands. In some clinics in Guadalcanal, the validity of the clinical diagnosis based on symptoms such as fever and chill, was evaluated by its

sensitivity and specificity. As PHC activities, the number of severe malaria cases has decreased in Honiara, capital city of Solomon Islands. In some clinics of Honiara, as slide positive rate of malaria parasite among febrile cases was reduced from 60% to 20% for recent 5 years, sensitivity of clinical diagnosis was

reduced from 65% to 25% and its specificity was maintained at a high level more than 80%. In a clinic of rural mountain area, slide positive rates of the parasite among malaria suspected febrile cases have been about 20 to 30% for a recent few years. And only based on results of the clinical diagnosis, most of the febrile cases were treated by chroloquine and quinine. In some clinics of Honiara and rural mountain area, malaria suspected febrile cases were also examined by Strep ID test to detect A, β -haemolytic streptococcal infections. In about

half number of non-malaria febrile cases, we could find A, β -haemolytic streptococcal infections. Out of them, about 20% cases had treatment with quinine, and only a few cases had treatment with penicillin in a rural health clinic. As progress of PHC activities, sensitivity of the clinical diagnosis of malaria decreased. Among some false-positive cases, we could find A, β -haemolytic streptococcal infections. But most of them had no treatment with penicillin and some of them had treatment with chroloquine and quinine.

63 A NEW MATHEMATICAL MODEL OF MALARIA TRANSMISSION FOCUSED ON HUMAN BEHAVIOR

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Since the Ross-Macdonald model, enormous improvements have been added to the mathematical models on malaria transmission dynamics. However, as Sattenspiel (1990) pointed out, human behavioral factors are essential for malaria transmission but have rarely been involved in these mathematical models. The present study aimed to combine stochastic fluctuation with the Susceptible-Exposed-Infective-Recovered (SEIR) model because human behavior has a nature of random fluctuation. Therefore expected numbers of those who do protective behavior and who do not in 'Susceptible' and 'Infective' population were calculated by random function with binomial distribution. The other major assumptions were, 1) total numbers of humans and mosquitoes were fixed, 2) mosquitoes can never recover if infected by parasites, 3) every bites to 'Susceptible' humans by 'Infected' mosquitoes lead to transition of those humans to 'Exposed' status. Examined conditions were expected proportion of protection (12% as baseline, and 70% to 95% according to the extent of compliance) and the efficiency of protective methods (highly effective and ineffective). These mechanisms were implemented as the computer pro-

gram written in C language and the 2 years transmissions were simulated 50 times for each condition. Major results were, 1) ineffective protection even of 95% compliance only achieved about 4% reduction of mean parasite rate, 2) highly effective protection of 70% compliance achieved about 13% reduction of mean parasite rate, and 3) highly effective protection of 95% compliance achieved eradication in all simulation runs. The necessity of more than 90% compliance to eradicate malaria showed good agreement with the recent models (Saul, 1993; Gupta and Snow, 1996). The results strongly suggested the necessity of not only alternative protection to bed net distribution but also of health education.

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64 ESTABLISHMENT OF ANTI-MALARIA CONTROL PROGRAM IN KHAMMOUANE, LAO PDR

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Scientific data on malaria prevalence, vector mosquito and evaluation of effect of preventive action from malaria infection were carried out as establishment of effective anti-malaria control program under JICA PHC Project in Khammouane from late 1995, because recently scientific information on malaria situation in Lao PDR has not published. In mass surveys on villagers conducted in three villages, malaria infection was demonstrated in 5.3-10.5% of the villagers examined throughout year in 2 villages locate in mountain forest, but malaria infection was rarely observed in the other village locates in plain along Mekong River. More than 90% of the positive villagers were occupied under 14 years old. Etiologic species of malaria parasite was almost *Plasmodium falciparum* in these villages. *Anopheles minimus*, *A. maculatus* and *A. dirus* were considered to be possible vector for malaria transmission in the areas, however, vector for malaria is still not concluded. In srveys for malaria knowledge on villagers conducted in 400 household selected randomly from 40

villages in Khammouane, 23% of villagers were not know relationship between mosquito bite and malaria, however, there were bed net in 90% of household and more than 90% of villagers slept in bed net. According to the above results, two villages in forest were selected as pilot area. The control program was planned to target mainly children and to implicate in rainy season. After education on villagers for effectiveness of impregnated bed net, bed net were supplied to the pilot area. In 6 month after setting impregnated bed net, malaria infection was demonstrated in only 2%, while positive rate in control villages located only 4 km far from model village were 7%. The net revolving system was operated for further delivering the impregnated bed net, and collection fund were carried out within 6 month. More than 90% of fund could collected in one village, while in other village where race of villagers were minority, more than 50% of fund could not collected. Necessity of socio-economic survey and training of supervisor were suggested for expanding this system.

65 MALARIA CONTROL IN THE JICA/CHSU PROJECT IN MALAWI: PRE-INTERVENTION SURVEY IN SALIMA DISTRICT

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Malawi is located in the Central of the tropical savanna region of Africa. This is the perimeter of the most malarious region. It is about 1/3 of the size of Japan, but 1/5 of which is the water mass of lake Malawi. It has a population of 12 million people. There

are three seasons, dry, wet, and cool. Malaria continues to be the most significant public health problem to the country's population. Malaria transmission seasonally fluctuates with the highest peak during the wet season from November to March. The prevalent parasites are

Plasmodium falciparum (Pf, 90%) and *P. malariae* (Pm, 10%). Responding to the declaration of malaria summit in 1992, malaria control has been chosen as a vertical program in JICA-Community Health Sciences Unit (CHSU) Project in Malawi. Malariometric survey was conducted to obtain baseline data in the rainy season before the intervention to control malaria in Salima District (a model area for the project). In January 1997, the surveys were carried out in Maonga village and surrounding primary schools in Salima District. The survey included mass blood screening for the community and spleen examination mainly for school children. All febrile cases were treated by antimalarials on the spot. Parasitological diagnosis of malaria was made microscopically at CHSU. Thick and thin blood smears were stained with 10% Giemsa solution for 10 minutes and examined by the investigators. A total of 608 villagers were examined and 252 (41%) were malaria positive, which consisted of 223 Pf cases and 16 Pm cases and 13 mixed infection. Nine (3.8%) of 236 Pf/mixed cases had Pf gametocytes. A total of 349 children were examined and 217 (62%) had splenomegaly. According to aging, parasite rate increased with the highest peak (86%) in 2 to 5 years old, and then gradually decreased to 23% in >26 years of age. This tendency was clear in

Pf rate. Pm rate decreased more markedly: Pm rate in 0 to 5 years are about 10 fold higher than that in >5 years of age. The similar tendency was observed in age specific spleen rate.

The results of this survey showed a typical picture of holoendemic malaria in Malawi. Age-specific malaria prevalence reflected high immunity in ≥ 5 years old.

The intervention by improvement of malaria case management at the periphery, distribution of impregnated bednets and installations of drug revolving funds have been initiated at Maonga in Salima District. However there are many constraints to conduct the project successfully as follows: insufficient drug supply, difficulties in transportation, poor compliance of drug intake, lack of supervision, no monitoring of efficacy and side effect of drugs, lack of training of village volunteers in malaria diagnosis and drug administration, difficulties for villagers to pay for bednets though subsidized to 350 yen per net, and lack of knowledge by villagers about malaria control. It is for this reason that JICA expertise is of vital role to collaborate in putting the program to a sound footing as we eagerly look forward to reducing malaria problems as much and as soon as it is possible.

66 ISLAND MALARIA CONTROL BY MASS DRUG ADMINISTRATION (MDA) AND IMPREGNATED BED NETS IN VANUATU: FIVE YEARS AFTER THE INTERVENTION

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Vanuatu is located at the southeast perimeter of the malarious band extending from Southeast Asia to eastern Melanesia. Aneytyum is the southernmost inhabited island of Vanuatu with a population of about 700 in 3 major villages. Malaria in the South is usually less prevalent than in other parts of Vanuatu. Suitable intervention measures were more likely to lead to sustainable lowering or even elimination of the parasite reservoir. The 9-week course of MDA and distribution of impregnated bed nets was carried out between September and November 1991, just before the start of the transmission season. MDA was well supported by the

community (compliance rate of 88.3%) and resulted in a marked reduction in the prevalence of malaria. We reported that the situation in Aneytyum was well maintained at least until February 1993. Since that time, the bednet program has been well progressed by Malaria Section with a coverage of about 60% of total population at the end of 1995. In 1996, five years after MDA, we conducted follow-up survey about malaria situation in Aneytyum and some selected islands in relation to malaria control activities. We conducted surveys at Aneytyum, Tanna, Tongoa and Malakula from February to April 1996 in collaboration with the District

Malaria Supervisor in each district. In Aneytyum we examined all 639 villagers and found only 3 Pv cases (PR=0.5%). High parasite rates were found in the Southwest of Malakula (PR=12.9%), where bednets were not yet distributed. The moderate parasite rates (2-7%) were found in most islands with only impregnated bednets. In Aneytyum we examined 288 and found spleen rate (SR) 2.1%. High spleen rates were found in the Southwest of Malakula (SR=43.2%). In Aneytyum, the malaria situation has been well suppressed since the first intervention in 1991. This finding confirms that time-limited MDA, in addition to impregnated bed nets, can eliminate malaria from small

island communities. It is remarkable that the community microscopist, who was trained in microscopy in 1992, is well maintaining his capacity of malaria case detection. To successfully conduct MDA in Aneytyum, we consider the following factors were important:

1. Aneytyum is an isolated island with a small population.
2. Malaria shows a clear seasonal fluctuation.
3. Good community participation & high compliance with MDA.
4. Population movement is limited.
5. Degree of Pf drug resistance is still low.
6. G6PD deficiency is not prevalent.

67 HLA-B*4601 INCREASED IN THE ADULT PATIENTS WITH SEVERE MALARIA AT MAE SOD HOSPITAL IN THAILAND

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To investigate the host genetic factors affecting the clinical course of falciparum malaria, HLA-B gene polymorphism was 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 were examined for their complete blood cell counts (CBC), hemoglobin concentration (Hb), hematocrit (Ht), and the percentage of the infected RBC in the blood smear. 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 13 severe malaria patients at Mae Sod National Hospital. The nationality of the patients was Myanmar (115 from Karen, 105 from other Burmese). The average ages of the outpatients and the hospitalized patients with severe malaria were

27.6 ± 9.2 and 23.3 ± 7.15 the sex ratios were 187:33 and 9:4 respectively. 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 (Corrected P value < 0.02). In the present study, we focused on the adult patients who would be expected to be resistant against malaria by the acquired immunity. Therefore, the clinical symptoms are relatively milder than small children but still it was possible to categorize their severity by using several criteria. Within the criteria, especially CNS symptoms and hemoglobin concentration could successfully identify susceptible HLA-B alleles, HLA-B*4601 and 1301 respectively. Pathogenesis of cerebral malaria and anemia must be different each other according to the previous studies. Further immunological analysis of those susceptible alleles should be done to explain the mechanisms involved in the pathogenesis.

68 RESISTANCE TO POST-SCHISTOSOMAL LIVER FIBROSIS ASSOCIATED WITH HLA-DRB1*1101 IN CHINA

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In 1995, over 100,000 patients have been estimated to be infected with schistosomiasis japonica in Jiangxi province along Yangzhe River and Poyang lake. One of the most serious complications is post-schistosomal liver disease that develop within several years after the infection. To identify the host genetic factors affecting the prognosis after infection, we have typed 232 of unrelated patients who are living in the endemic village named Beishan, Yushan County, Jiangxi, including 45 patients with no fibrotic change in the liver (Grade O) and 187 patients with typical fibrotic change (Grade I through Grade III) for their HLA-DRB1, DRB3, DRB5, DQA, DQB, DPA, DPB and B by PCR method. The schistosomal fibrotic changes in the liver were observed

by using ultrasound and the grades were determined by the WHO standard (Cairo1991). HLA-DRB1*1101-DQB1*0301 haplotype significantly decreased in the fibrotic change group (Grade I \leq) indicating that this haplotype is resistant against fibrosis* (Pcorrected<0.001, RR=0.25). On the contrary, HLA-DRB1*1501-DRB5*0101 haplotype was significantly increased in the fibrosis group indicating that this haplotype is susceptible against fibrosis (Pcorrected<0.02, RR=8.7). DPA1*0202-DPB1*0201 haplotype significantly decreased in the advanced fibrotic group (Grade 2 \leq) indicating that the individuals who had these haplotypes were resistant against advanced fibrosis (Pcorrected<0.0008, RR=0.2, Pcorrected<0.02 RR=0.3).

69 PROGUANIL EFFICACY IN MALARIA PATIENTS FROM VANUATU WITH HIGH FREQUENCY OF CYP2C19 MUTATHINS

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(Background) The cytochrome P450 isoenzyme CYP2C19 mutations have been found to be associated with poor metabolism of the antimalarial drug proguanil (PG) to its active form, cycloguanil (CG). We have studied the therapeutic efficacy, adverse effects and the pharmacokinetics of PG in malaria patients from Vanuatu, where high frequency of CYP2C19 mutations have been recently found. (Methods) In rural communities on Malakula Island, uncomplicated malaria

patients received PG for 3 days in daily doses of 5 mg/kg body weight and were followed up for 28 days. Finger prick blood samples were collected on filter paper for determinations of pharmacogenetic profiles by PCR and drug levels by HPLC. (Findings) The selected 95 patients were genotyped to 33 extensive metabolisers (EMs) and 62 poor metabolisers (PMs). Capillary blood concentrations of PG were similar in the two groups whereas significant CG concentrations were only

found in the EMs. The PG treatment was effective in about 70% of *P. falciparum* and 90% of *P. vivax* patients and this efficacy was similar in PMs and EMs. Mild adverse effects were reported in 45% of the treated patients, also similarly in the two group. However adverse effects were positively correlated to PG concen-

trations, and there was a similar tendency with efficacy. (Interpretation) PG represents an important antimalarial drug also in populations with high frequencies of PM phenotype. Mechanisms other than dihydrofolate reductase inhibition by CG may be responsible for the antimalarial activity.

70 THE POTENTIAL TO THE SCIENTIFIC UTILIZATION OF ASIAN TRADITIONAL MEDICINAL PLANTS FOR THE CONTROL OF PARASITIC DISEASES (MINIREVIEW)

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There are a large number of obstinate parasitic diseases still nowadays in Asian and other countries on the earth. Though several excellent drugs have been synthesized for their treatment and control, there still remain matters to be considered (Zhang *et al.*, 1997, *Jpn. J. Trop. Med. Hyg.*, 25, 209-213). Excellent synthesized drugs are not readily available in local villages because of their high cost and inconveniences in transportation. This fact might be rather a minor one. However, there will be an important problem. Some strains (isolates) of protozoal and helminthic parasites have been found to be insensitive to excellent synthesized drugs (Boreham, 1995, *Int. J. Parasit.*, 25, 1009-1022; Geerts *et al.*, 1997, *Parasitology Today* 13, 149-151). For instance, according to Boreham (1995), some lines of *Giardia* resistant to quinacrine and albendazole have been isolated. The situation with these facts is thought to be where the possible development of new drugs would hopefully come in, based on scientific exploitation of traditional medicinal plants like a global strategy for the control of obstinate parasitic diseases (Maki *et al.*, 1996, Abstracts for XIV Congress for Tropical Medicine and Malaria, 355). This is, at least in part, in accordance with the famous fact that chloroquine-resistant malaria is treated nowadays with quinghaosu, originally a traditional Chinese drug from the plant, *Artemisia annua*. In thought of this and the facts above mentioned, the present authors have been working experimentally for these years to find the possible efficacy of their extracts on a number of obstinate protozoal and helminthic para-

sites. They have been testing efficacy of extract from medicinal plants on various kinds of parasites (Maki *et al.*, 1997, *Parasit. Int.*, 46 (suppl.), 65). These work were partly reviewed and/or presented by Maki *et al.* (1997: Program and Abstracts for the 38th Annual Meeting of Japanese Society of Tropical Medicine, p.115) with special emphasis on the laboratory studies on efficacy of extracts from the Chinese plants, *Artemisia annua*, *Cnidium monnieri*, *Phellodendron amurense*, *Pulsatilla chinensis*, and *Sophora flavescens* against *Trichomonas vaginalis* (Zhang *et al.*, *ibid.*) and on that from Nepalese *Embelia ribes* against *Trichuris muris* (Maki and Ito, unpublished). The preliminary study showed that the aqueous decoction from the plants, *A. annua*, *P. amurense* and *P. chinensis* had the activity to reduce the number of *T. vaginalis* cultured for 1-5 hrs in our laboratory. The extract from *E. ribes* was not found to be effective against adult *T. muris* (S-isolate) in B10.BR mice despite the demonstration of the efficacy of pure powdered mebendazole as a positive control. Based on the data so far obtained, it might be concluded that our studies would be beneficial for the fundamental research leading to the development of new anti-parasitic drugs from the wealth of traditional medicinal plants. Unfortunately, however the local traditional customs and information on medicinal plants are dying out in many areas in Asia. We have to continue making efforts for the collection of the information on medicinal plants, which are believed and used without scientific evidences for the treatment of parasitic diseases in Asia.

71 SPATIAL ANALYSIS BETWEEN MALARIA TRANSMISSION AND VECTOR BREEDING SITE IN LOMBOK ISLAND, INDONESIA

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Conventional methods of monitoring mosquito populations and malaria prevalence require extensive field work and manual sampling. Because they are time-consuming and costly as well as impractical at regional large scales, an application of new approaches to malaria surveillance and control is expected. To introduce new technology into Malaria investigation, we tried to conduct spatial analysis between malaria transmission density and the anopheline vector abundance using a newly devised program. Three villages in Lombok Island were designated for this spatial analysis. GPS (global positioning system) accurately measured the locations of residence and mosquito breeding site in order to combine into a GIS (geographical information system). The study areas of three villages were divided into 258×258 meshes (one mesh is about 10m square in size), and the sample values (number of anopheline larvae and malaria positive case) were accumulated in individual mesh. The unknown sample values were

estimated interpolatively using Weighted Averages Using Inverse Distance Methods. Then, computer aided mapping of distribution pattern of malaria prevalence and anopheline larvae was made to perceive the difference visually. *An. sondaicus* and *An. subpictus* considered as a main vector of malaria in Lombok were similarly distributed in seaside regions. These distributional pattern definitely corresponded to that of vivax malaria, not to that of falciparum malaria distinctly. To clarify this discrepancy between falciparum and vector distribution, a new line study of environmental analysis is required. Field study and remote sensing (RS) findings of environmental elements can be integrated into GIS to facilitate characterization of the landscape in terms of transmission risk factors. This spatial analysis combined with landscape analysis would enable us to define predictors of malaria transmission and vector breeding site.

72 EVALUATION AND METHODOLOGY OF *B.T.I.* AGAINST *ANOPHELES* LARVAE IN HONDURAS, CENTRAL AMERICA

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We have evaluated in the laboratories and in some natural conditions the *B.t.i.* products, which were the microbiological insecticides produced by *Bacillus thuringiensis israelensis*, and were donated by USAID and PAHO to Honduras, Central America. The Investigations were carried out in the division of vector-borne diseases, the ministry of public health, Honduras, Central America. In the laboratories, we recognized an influence of water contamination to effects of the *B.t.i.*

granule, so that a rate of mortality decreased depending on a proportion of contaminated water. It also indicated that a relationship of the contamination and PH of water, therefore, from 5 to 9 in the range of PH, the effect of *B.t.i.* was corresponded with the PH value. The additional laboratory investigations suggested that the liquid type of *B.t.i.* has more effective in rapid action than the granule type one, however, in the light of the stability the granule maintained more durable efficiency

than another one. From a practical situation, we have chosen the granule type for the investigation in the field. According to the application of the granule in the canals of rice field, we have investigated better method to apply it in the field. We would tried to develop an application system which was put in the upper stream of canals and had an efficiency to the lower reaches. Moreover, on basis of a behavioral character of *Anopheles* larvae, the granules would be maintained in the surface of water. We have investigated materials and forms of containers kept on the water surface. Some results indicated that sufficient space was demanded in the container to prompt water currency and to adjust to the granule expansion, which was occurred in wet condi-

tion. Some materials like cotton cloth or gauze, were not acceptable for the container. We have selected the plastic materials for the container that have some holes covering with the iron flames and a plastic float of the same size. The effect of *B.t.i.* granule with the plastic container was that: 1 lbs (approximately 450 g) granule have kept more than 80% mortality until 4weeks, to 100 m distant of lower reaches in a canal. From these results, this plastic container system was only applied in the canal with water currency. We have developed the system and have increased the holes and the float of used plastic containers to accept for a water system without currency.

73 MICROSTRUCTURES AND OPTICAL PROPERTIES OF SCALES OF TROPICAL BUTTERFLY WINGS

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All butterfly scales share the same basic architecture, but various elements of this architecture are particularly complex in these scales that exhibit structural colors. The beautiful colors of butterfly wings have attracted our attention and much work has been carried out to clarify the origins of the beautiful and brilliant hues on butterflies. This paper describes the mechanism generating the beautiful wing colors of various male tropical *Morpho* butterflies and the relationship between the wing material and the color appearance. The microstructure of the scales covering the upper surface of the wings was analyzed with the aid of a scanning electron microscope. Reflection spectra of these wings are measured at various incidence and viewing angles, they are characterized by strong anisotropy. The basic

mechanism of color generation of structurally colored scales is determined for the first time in accordance with the theory of optical interference in thin film layers using a model of wing scales. Optical properties were found in relation to the three-dimensional spectral reflectance of the samples, and differences were observed between the brightness perceived in subjective evaluations and calculated values based on the reflective spectra of the structurally colored wings. The results of this study suggest that the microroughness of the upper wing surface may influence the perceived gloss of structurally colored wings. In the course of studies on the color mechanism and optical properties of *Morpho* butterflies' wings, we found that these butterflies contain blue fluorescent compounds in their wings.

74 BIONOMICS OF DENGUE VECTOR MOSQUITOES IN HOUSES AND THEIR CONTROL IN AN ENDEMIC AREA

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Dengue fever/dengue hemorrhagic fever is one of the most important infectious diseases for reasons of medical and public health problems in endemic and epidemic areas, and also is counted among re-emerging diseases. Understanding of the relationship between mosquito infection rates with dengue virus and mosquito bionomics, especially inside houses, may provide us important information when and how to plan and implement vector control strategies for dengue. For these purposes, we carried out mosquito surveillance to understand their bionomics as well as evaluation on the effectiveness of Olysetnet against mosquitoes in selected houses in Banhom village, Nakhon Phanom Province, Thailand during February and June 1997. About 1,000 mosquitoes were collected by human-baited collection and resting mosquito collection in 8 houses for 5 days in the village. Most of the mosquitoes were identified as *Aedes aegypti*, however very few *Ae. albopictus* were collected inside the houses. The number of *Ae. aegypti* mosquitoes collected in June was slightly higher than in February, 1997. Although mosquito larvae were found inside several water jars which were placed around the houses, no adult mosquitoes could be collected by human-baited collection. After 2 days incubation at room temperature, all collected female *Ae. aegypti* were tested for the presence of dengue virus genome by using RT-PCR in Nakhon Phanom Hospital, Thailand, and in Japan. The results, however, were all negative. Since no clinical dengue patient was reported during the survey periods in the village, we suppose that dengue virus infection rate of the mosquitoes was extremely low in the village. On the other hand, parallel experiments on patients' serum specimens collected in Nakhon Phanom Provincial Hospital, could detect genomes of dengue

virus type 1, 2 and 3 by RT-PCR accompanied by virus isolation from dengue patients who lived in different villages in June. Abdominal tergal scale pattern values of *Ae. aegypti* were scored to see the relationship between the pattern variations of the females and the virus infection rates in the village. The mosquitoes predominantly consisted of the dark type form (# 1 to 3 as scoring values). However we could not clarify whether the values and behavior of the vector mosquitoes will change by dengue virus infection, because virus genome was not detected in the collected mosquitoes. It was found that *Ae. aegypti* adult mosquitoes came flying every day and were hiding inside the houses before/after feeding human blood during daytime, especially in the village. Because of these mosquito behavior, we tried to reduce mosquito-biting chance by setting the Olysetnet (Sumitomo Chemical Co., Ltd. and Sumika Life-Tech Co. Ltd., Japan) mainly around mosquitoes which could be hiding inside of 4 houses. The Olysetnet is wide mesh net woven of polyethylene thread which had been incorporated with pyrethroid. Twenty square meters of the Olysetnet were distributed in each house, and used to cover the clothes in boxes and/or hangers inside of houses, as well as curtains on windows and doors. We observed that the number of mosquitoes in houses was reduced, and also almost no biting activity of the mosquitoes were recognized by villagers in houses. These data may encourage us to control dengue vector by a combination of the Olysetnet and larvicides in the village. This field research was supported by the Grant-in-Aid for International Scientific Research (Principal Investigator: Prof. Akira Igarashi) from the Ministry of Education, Science, Sports and Culture of Japan, in the fiscal year 1996-1997.

75 ESTIMATION OF FLIGHT RANGE AND DAILY SURVIVAL RATE OF MALARIA VECTORS: RESULTS FROM A MARK-RELEASE-RECAPTURE STUDY IN NORTHERN THAILAND

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Mark-release-recapture studies of anopheline mosquitoes were conducted in northern Thailand in October–November, 1995. To examine the flight range of anopheline mosquitoes, 3,744 (12 species) were collected from the field and were released after sprayed a fluorescent dye. The mosquitoes were recaptured for 4 days at 5 places of different distance from the release point (60, 400, 1,000, 2,000, and 4,000 m) by human and a water buffalo bait collection. The recapture rate of *A. aconitus* was 0.924% and 3 marked *A. aconitus* were recaptured at 4,000 m away from the release point. Total of 9,374 (11 sp) anopheline mosquitoes were sprayed and released to estimate the daily survival rate and confirm their flight range. The following 3 methods were used for the estimation of the survival rate: (1)

modified triple catch method, (2) log-regression method, (3) renewal equation method. To apply these estimation methods, unfed mosquitoes were marked with 2 different fluorescent dyes and released in 2 successive days, and 874 engorged females were also marked and released. Recapture was made for 7 days at 5 places of different distance from the release point. The survival rate of *A. aconitus* was estimated as 0.618 and 0.624/day by the 1st and 2nd methods, respectively. The duration of the feeding cycle and the survival rate per feeding cycle were estimated as 3.56 days and 0.80, respectively using the 3rd method. The recapture rate of *A. aconitus* was 1.46% and 1 marked *A. aconitus* were recaptured at 4,000 m away from the release point.

76 PREPARATION OF *PLASMODIUM VIVAX* INFECTIVE MOSQUITOES BY MEMBRANE FEEDING

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We have studied transmission blocking vaccine using a rodent malaria, *Plasmodium berghei*, as a model of human malaria. Now we are going to study transmission blocking vaccine of *P. falciparum*. In the beginning, technic for preparing infected mosquitoes with human malaria and facility for maintaining the infected mosquitoes are required. We prepared a maintenance room with triple-doors for avoiding the infected mosquitoes to escape from the laboratory. To infect Anopheline mosquitoes with human malaria, we prepared membrane feeding method. Under the bottom of a plastic bottle filled with 37°C water, we put a parafilm membrane extended enough for mosquitoes to put in their proboscis. In the small chamber with the bottom of the plastic bottle and the parafilm membrane, malaria infected blood was injected with a syringe. Mosquitoes were allowed to feed the blood under the parafilm

membrane via mosquito cage. Fully fed mosquitoes were collected in a small plastic pot, and maintained in the maintenance room. We had made practice of the membrane feeding with infected mice blood for several times. One day in March 1997, we had a malaria patient who infected with vivax malaria. It was relapse of vivax malaria after 8 months of the first infection in India, because he had taken chloroquine but not primaquine. We received 2ml of his blood for confirming diagnosis. With one drop of the blood, we made a thin-smear slide and stained with acridine orange. Within 10 minute after receiving the blood, we observed trophozoites and gametocytes of vivax malaria in the slide. We reported the result and gave information of treatment of vivax malaria to the doctor for the patient. After that we gave the rest of the patient's blood to Anopheline mosquitoes with the membrane feeder.

Mosquitoes could start feeding the blood within 30 minutes after being taken from the patient. Fully fed mosquitoes were isolated in the maintenance room, and rest of the mosquitoes were killed in the cage by heating in 70°C incubator. Twelve days later, the isolated mosquitoes were anesthetized with CO₂, removed their legs and wings on ice, and gotten out from the maintenance room. The mosquitoes were dissected, and the midguts and the salivary glands were observed under light microscope. Ten mosquitoes were dissected.

Seven mosquitoes had oocysts on the midgut. Eight mosquitoes had sporozoites in the salivary gland. We confirmed that we could infect Anopheline mosquitoes with human blood containing malaria parasites using membrane feeding method, and could maintain the infected mosquitoes in a isolate place without accidents. Now we are culturing gametocytes of *P. falciparum* for preparing *P. falciparum* infected mosquitoes in order to evaluate the effect of transmission blocking vaccine when we can produce the vaccine.

77 PURIFICATION OF THROMBIN (FACTOR IIa) INHIBITOR FROM THE SALIVARY GLANDS OF *ANOPHELES STEPHENSI*

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The salivary glands of *Anopheles stephensi*, an important malaria vector, contain an anticoagulant. In the presence of salivary gland extract prothrombin time (PT) and activated partial thromboplastin time (APTT) were markedly prolonged. Based on the inhibition of thrombin-directed cleavage of synthetic chromogenic substrate, the anticoagulant has been shown to be a thrombin inhibitor, but not factor Xa inhibitor. By means of thrombin-sepharose affinity

chromatography and anion exchange chromatography the anticoagulant was purified. SDS polyacrylamide gel electrophoresis indicated the inhibitor had a molecular weight of 45kDa. It has a non-covalent and reversible interaction with thrombin. Like other blood feeding arthropod, mosquitoes have evolved a highly potent inhibitor capable of interfering with the host homeostasis facilitating blood feeding and subsequently parasite transmission.

78 CHIKUNGUNYA VIRUS IN MIDGUT OF *Aedes albopictus* (OAHU STRAIN): AN ELECTRON MICROSCOPIC STUDY

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Aedes albopictus (Oahu strain) mosquitoes were infected with chikungunya virus from a blood meal. The distribution of virus particles in midgut epithelial cells on 3 days and 14 days after infection with electron microscopy was observed. On 3 days after infection, virus particles were observed in basal labyrinth. The basal labyrinth was branched channels formed by numerous infolding of the basal plasma membrane.

Virus particles budding from a cytoplasm were observed in the basal labyrinth. In different area in the cell, the particles like virus nucleoids were seen near the basal labyrinth of midgut cell. On 14 days after infection, mass of virus particles was seen in the basal lamina consisting of several layers and between the muscle cell. From these observations, disperse of virus particles from midgut was thought.

79 MORPHOGENESIS OF ARBOVIRUSES: (4) DENGUE VIRUS MATURATION SITE IN CULTURED MOSQUITO C6/36 CELLS AND VERO CELLS

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Dengue virus (type 2, New Guinea B strain) maturation was observed by an electron microscopy. The growth of dengue virus was examined in the cultured mosquito C6/36 cells and Vero cells. The titration of dengue virus was performed with the use of PAP (peroxidase-anti-peroxidase) method (Okuno *et al.*, 1977). Highest virus titers in C6/36 and Vero cells were obtained after 6th day incubation at 28°C and 37°C, respectively. These cultured cells were treated and

prepared as described previously in order to observe them electron-microscopically. The results so far obtained showed that dengue type 2 virus matured mainly at the sites of cytoplasmic vesicles and vacuoles despite the exception of a few budding viral particles in the cell surface membrane. There were no apparent differences of its maturation site between C 6/36 cells and Vero cells.

80 TRANSIENT POPULATION BYPASSED BY POLIO VACCINATION PROGRAMS IN YUNNAN PROVINCE, CHINA

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(Background) Efforts to achieve global eradication of poliomyelitis have shown considerable success since the WHO designated the year 2000 as the deadline. Wide distribution of vaccine is crucial to eradication of polio. The number of patients with poliomyelitis in China has decreased dramatically since the implementation of supplemental immunization programs, including National Immunization Days (NIDs). However, immunization programs may have bypassed certain groups of individuals. We have already reported wild poliovirus circulation occurred in residents who lived in Myanmar near the border of China. As well as people who cross the border, there is a domestic transient population in China consisting of market tradespeople, construction workers, domestic workers, and others. Rapid economic growth and advances in transportation systems have led to an increase in this population. (Materials and Methods) We surveyed the parents or custodians of 91 children aged 1 to 3 years in 4 markets in Yunnan Province in southwestern China, where there appear to

be many unregistered children. Children were classified into the following groups: settled population, intra-provincial transient population, extra-provincial transient population and foreign transient population. We collected data on the following immunization indices: routine OPV (oral live polio vaccine) administration, BCG scar, NIDs participation, OPV supplementation other than NIDs. (Results) More than half of the children (54/91) in the surveyed market areas were transients whose families were not registered in those areas. 47 (87 %) of the surveyed transient children were intra- or extra-provincial transients. All indices of immunization participation were significantly lower in the overall transient population, in the intra-provincial transient population, and in the extra-provincial transient population as compared with the settled population. (Conclusion) The present results showed that children in the transient population were much less likely to have received OPV than children in the settled population. Because it is illegal to live outside the area in which

one's family is registered, transients may not participate in immunization programs for fear of being identified by the authorities. Furthermore, they often have more children than allowed by the strict family planning law

of China. Thus, this population may be at high risk of poliomyelitis. Our results suggested that the supplemental immunization programs in China should focus on the transient population.

81 ISOLATION OF DENGUE VIRUS FROM PATIENT BLOOD OF DHF BY USING CELL CULTURES MAINTAINED IN HEPARIN-ADDED MEDIUM: DATA ON A DHF EPIDEMIC IN INDONESIA

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One of the difficult aspects regarding DHF study is that the rates of virus isolation are much lower than those of classic dengue fever. During an outbreak of DHF in Jakarta, Indonesia in 1988, we attempted to apply a new method to isolate dengue virus (DENV) from DHF patients. (Blood specimens) Single blood specimens were collected from each of 98 patients who were clinically diagnosed as having DHF of Grade I to Grade IV at Cipto Mangunkusmo Hospital, Jakarta. (Procedures of virus isolation and typing) *Aedes albopictus* C6/36 cells grown in a 24-well plastic plate were temporarily maintained in a medium containing 30 u/ml heparin and the plates were covered with a cellophane sheet. Immediately after blood was taken by venipuncture using a syringe, the needle was inserted through the cellophane cover. Two drops of blood were introduced into each well. The blood was evenly distributed on the surface of cells by shaking the plate which was then placed in an incubator at 28°C overnight. The next day the fluid phase was replaced by new medium without heparin. Six to seven days thereafter, the medium was taken and inoculated into BHK-21 monolayer cell cultures in 8-chamber slides that were then held in a CO₂ incubator at 35°C. After 5-6 days, the slides were examined with an immunofluorescent antibody test to confirm the presence of DENV. The serotypes of DENV were determined using the type-specific monoclonal antibody. (HI tests) Sera separated from the remaining portion of the blood were titrated for anti

-DEN and anti-JE HI antibodies. (Results) (1) From the 98 individual blood specimens, 17 strains of DENV were isolated (three type 1 viruses, and 14 type-3 viruses). No concurrent infections with multiple types were observed. (2) Successful virus isolation was clearly related to the time of blood sampling (within 3 days after admission of the patients to hospital); no virus was recovered 4 or more days after the time of admission. (3) Apparently the viruses were isolated from patients without significant regard to DHF Grades nor to the concomitant HI titers. (4) The isolated viruses multiplied well in BHK cultures. It was notable that a strain isolated from a patient with Grade IV DHF showed a significantly rapid growth rate. (Discussion and Summary) The procedure reported here was simple and easily done at bedside. When the blood samples were collected during early stages of illness, the virus isolation rates by this method were significantly high compared with those of the similar trials using serum. The success of virus isolation was apparently not related to the DHF Grades of patients nor to the HI antibodies coexisting in the blood. The data may be explained that the DENV located in lymphocytes or macrophages contained in whole blood was not affected by the antibodies, and probably a coculture system was formed between the virus-harboring cells and culture cells. Since the isolation of DENV from DHF patients is not easy, our method may effectively contribute to the DHF works in tropical areas.

**82 COMPARISON OF NUCLEOTIDE AND AMINO ACID SEQUENCES
OF prM AND E PROTEINS OF NEWLY ISOLATED JAPANESE
ENCEPHALITIS VIRUS (JEV) ISHIKAWA STRAIN WITH
OTHER JEV STRAINS: GENETIC RELATEDNESS WITH
THAILAND JEV STRAINS**

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Japanese encephalitis virus (JEV) Ishikawa strain was isolated from the mononuclear cells of swine which seemed to be persistently infected with JEV in Ishikawa prefecture (Takashima *et al.*, 1994). To characterize the biological properties of Ishikawa strain, multiplication and reactivity to monoclonal antibody were examined. Growth rate and yields of Ishikawa strain in Vero cells were slightly lower than those of JaGAR-01 strain. Ishikawa strain was neutralized by the JEV-specific monoclonal antibody 503 and the neutralization titer was similar to those of other JEV strains, JaGAR-01 and Nakayama which were isolated in Japan. The sequencing study, however, clarified that the nucleotide and amino acid sequences of prM region of Ishikawa

strain were different from other JEV strains isolated in Japan (genotype III), but very similar to those of JEV strains isolated in Thailand (genotype I). The sequence of Ishikawa E protein region which is important for the virus infection, was also very similar to Thailand strain. Four amino acids, Met₁₂₉, Ser₂₂₂, Tyr₃₂₇ and Ser₃₆₆ in the Ishikawa E region were substituted from Tyr₁₂₉, Ala₂₂₂, Ser₃₂₇ and Ala₃₆₆ in other isolates in Japan. The substitution of those amino acids are commonly observed in the isolates in Thailand (Ali *et al.*, 1995). These facts are very interesting to discuss the origin of Ishikawa strain, although they are not explained so far. Further biological and genetic analyses are necessary to identify the origin of Ishikawa strain.

**83 COMPARATIVE ANALYSIS OF NTPASE ACTIVITIES OF
NON-STRUCTURAL PROTEIN 3 IN FLAVIVIRUCES**

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Flavivirus genomic RNA is translated into a large polyprotein that is processed into structural and non-structural proteins. The sequence analyses of flavivirus nonstructural protein NS3 suggested that NS3 is a multifunctional protein with sequence motifs characteristic of a serine protease in the N-terminal domain and a NTPase-RNA helicase in the C-terminal region. Recently RNA helicase and NTPase activities in recombinant NS3 proteins of bovine viral diarrhea virus (BVDV) and HCV have been reported, confirming the predicted enzymatic activities. However NTPase-RNA helicase activity of dengue virus (DEN) is still unknown. To assess the NTPase and RNA helicase activity of DEN (1,636-2,091 a.a.), JEV (1,668-2,123 a.a.) and HCV (1,193-1,658 a.a.) NS3 protein, containing the putative NTPase-RNA helicase domain, were ex-

pressed in *Escherichia coli* by a pET expression vector. The recombinant NS3 proteins were purified and analyzed by SDS-PAGE. The requirements of mono- or divalent cation and polynucleotide and pH ranges necessary for optimal ATPase activity of purified NS3 protein of DEN were determined. 1) About 56 kDa of DEN4, JEV and HCV NS3 proteins were genetically engineered and expressed in *E. coli* with a histidine tag at the N terminus (DEN4/NS3hel, JEV/NS3hel, HCV/NS3hel). 2) The purified recombinant proteins containing the C-terminal half of each NS3 protein, possessed polynucleotide stimulating ATPase activity. 3) Yield of DEN4/NS3hel was low compared to the other 2 proteins and easily inactivated within short time after purification. 4) Optimal pH of the ATPase activity of DEN4/NS3hel was nearly pH 6.5. 5) ATPase activity of

DEN4/NS3hel was enhanced by addition of poly(A) or poly(U). 6) MgCl₂ or MnCl₂ were required for ATPase activity of DEN4/NS3hel and the optimal concentra-

tions were 0.625 mM and 1.25 mM respectively. 7) ATPase activity of DEN4/NS3hel was inhibited by Ca²⁺ or high concentrations of monovalent cation.

84 AN ULTRASTRUCTURAL STUDY ON AN ORIGIN OF THE CELLS IN KAPOSI'S SARCOMA

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Since the occurrence of the first case of AIDS, Kaposi's sarcoma (KS) has been considered to be one of its main complications. Presently, KS is classified into several forms, based on historical, epidemiological and virological background. However, its etiology, pathogenesis, histogenesis and disease entity are still obscure. We report the results of an ultrastructural study on an origin of the cells in African endemic form KS. All materials were taken from the western part of Kenya, East Africa. The histological features of African endemic form KS can be divided into four types with its stages: granulation tissue-like, angioma/angiosarcomatous, fibrosarcomatous and anaplastic lesions. We examined five granulation tissue-like, six angiosarcomatous and 16 fibrosarcomatous types of KS, respectively. Ultrastructurally through its stages, we

found there are three types of cells in KS tissues: endothelial cell-like cells which contain Weibel-Palade bodies and show erythrophagocytosis, pericyte-like cells which contain dilated rough endoplasmic reticulum (RER), pinocytotic vesicles (PV), thin filaments (F), collagen, focal densities (FD) and dense patches (DP) and smooth muscle cell-like cells which contain PV, F, FD, DP and thick collagen-like materials in their cytoplasm. Immunohistochemically, pericyte-like cells and smooth muscle cell-like cells were positive for vimentin, laminin, α -smooth muscle actin, collagen-IV and CD-34. These findings suggest that the origin of the cells in African endemic KS are primitive and pluripotent mesenchymal cells which, may grow to be immature endothelial cells, pericytes and smooth muscle cells.

85 MICROBIOLOGICAL DIAGNOSIS AND ANTIBIOTIC THERAPY FOR COMMUNITY-ACQUIRED PNEUMONIA (CAP) IN UGANDA

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(Back ground) It is estimated that 2 million people are infected with HIV in Uganda. In the previous study of CAP in Uganda, we found that high prevalence (76%) of these patients was HIV-1 seropositive and the major causative organisms were *H. influenzae*, *S. pneumoniae*, *M. catarrhalis*. In the present study, we evaluated the clinical usefulness of ampicillin (ABPC) therapy for CAP in HIV-endemic, developing country. (Design) Patients with community-acquired pneumonia, which was confirmed by clinical symptoms and findings of

chest radiographs, were enrolled at Mulago Hospital, Makerere University from November 1996 through July 1997. (Method) Quantitative culture on rabbit blood agars, Gram's staining of sputum, blood culture, pulse oximetry oxygen saturation (SpO₂), CRP, HIV serostatus (PA and WB), CD4 and CD8 lymphocyte count, serological tests for *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and antibiotic susceptibility tests for causative organisms were performed. Treatment with ABPC 1 g twice a day for 3 days followed by amoxicillin 1,500

mg for 4 days were given for patients after enrollment. (Result) Among evaluable 60 patients, 47 (78.3%) were HIV-1 seropositive and 13 (21.7%) were seronegative. There were no differences in proportion of sex, symptoms, median age, extent of pneumonia between HIV-1 seropositive and seronegative patients. HIV-1 seropositive patients had lower CD4 lymphocyte count, complication such as oral candidiasis, pleural effusion, and pulmonary tuberculosis. The causative organism was identified in 29 (61.7%) in 47 HIV-1 seropositive patients and in 6 (46.1%) in 13 HIV-1 seronegative patients respectively. The organisms identified com-

monly was *S. pneumoniae*, *S. aureus*, *M. catarrhalis*, *H. influenzae*. Clinical response rate to antibiotics therapy was 74.5% in total 47 cases, 70% in the HIV-1 seropositive group, and 100% in the HIV-1 seronegative group. Fair and poor responders were associated with low CD4 cell counts, severe underlying disease and superinfection after treatment. Serum CRP level and SpO₂ value were useful markers of the drug treatment of pneumonia. (Conclusion) Treatment with ABPC appears to be useful for patients with CAP. However, careful microbiological diagnosis and treatment are necessary for CAP in HIV-infected patients.

86 TREATMENT OF COMMUNITY ACQUIRED PNEUMONIA IN NORTHERN THAILAND

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We had a study about "Treatment of Acute Respiratory Infections in Thailand" during three years from April, 1994 to March, 1997. In the second year, we studied about the patients with community acquired pneumonia who were admitted in Nakorn Ping Hospital of Chiang Mai. Major organisms of community acquired pneumonia were *H. influenzae*, *S. aureus* and *K. pneumoniae*. MIC of Ciprofloxacin was 0.006 µg/ml to *H. influenzae*, 0.025 µg/ml to *K. pneumoniae* and was sensitive than Gentamicin to major organisms. In the third year, we compared with effect of chemotherapy between Gentamicin and Ciprofloxacin. 31 patients with community acquired pneumonia were enrolled in Nakorn Ping Hospital from January to March in 1997. The sex distribution from the total number of patients were 18 for male and 13 female. Age was from eighteen to seventy six, mean of age was 44.5 years old. Underlying diseases were 3 cases of chronic obstructive pulmonary disease, 3 cases of alcoholism, 2 cases of chronic heart failure and so on. HIV antibody was resistered in 19 of

31 patients, this was positive in 7 patients. We estimated the effect of chemotherapy about 16 cases of community acquired pneumonia except 15 cases dropped out. Major organisms of community acquired pneumonia were *H. influenzae*, *S. pneumoniae* and *K. pneumoniae*. MIC of Ciprofloxacin was under 0.003 µg/ml to *H. influenzae*, between 0.006 µg/ml and 0.013 µg/ml to *K. pneumoniae* and was sensitive than Gentamicin to major organisms. In chemotherapy, Penicillin G was selected for mild pneumonia, Penicillin G pulus Gentamicin or Penicillin G pulus Ciprofloxacin were selected for moderate and severe pneumonia. Mild pneumonia has infiltration shadow within one lobe in chestradiograph. Moderate and severe pneumonia have more wide infiltration shadow than mild pneumonia. Effect of chemotherapy was evaluated by course of clinical symptoms, physical examination, chestradiograph, blood examination and sputum culture. 40% (2/5) of the patients given Penicillin G, 0% (0/2) of the patients given Penicillin G pulus Gentamicin and 78%

(7/9) of the patients given Penicillin G plus Ciprofloxacin were effective in community acquired pneumonia. For a few cases given Penicillin G plus Gentamicin, if we added the cases of pneumonia treated by Penicillin G

plus Gentamicin from January to February in 1996 to it from January to March in 1997, 17% (1/6) was effective. We will recommend Penicillin G plus Ciprofloxacin for therapy to community acquired pneumonia.

87 SERUM LEVEL OF CYTOKINES IN PATIENTS WITH BRUCELLOSIS

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Human brucellosis is a zoonotic disease which often turns into chronic or recurrent stages, is prevalent in many parts of the world. In mouse and macrophage models of brucellosis several cytokines have been shown to have pathophysiological effects in the bacterial cell. However the cytokines associated with this infection in human is not yet been well elucidated. In the present study we measured IL-1 β , TNF- α , IL-4, IL-2, IFN- γ , IL-8 and IL-12 in 46 serum of patients with suspected brucellosis. Tube agglutination titer of $\geq 1:160$ was taken as a cut-off point for the diagnosis of brucellosis. The level of IL-1 β , TNF- α , IL-4 and IL-2 were nega-

tive in all samples. The IFN- γ level was significantly ($p < 0.05$) higher in patients with brucellosis (165.0 ± 46.0 pg/ml; Mean \pm SE) compared with the patients without brucellosis (41.3 ± 13.3 pg/ml). The IL-12 level also significantly ($p < 0.05$) higher in patients with brucellosis (327.5 ± 48.1 pg/ml) than without (130.9 ± 50.0 pg/ml). Regarding the level of IL-8 there was no statistically significant between brucellosis positive (184.7 ± 23.2 pg/ml) and brucellosis negative (138.0 ± 13.6 pg/ml) patients. This result indicates that IFN- γ and IL-12 may have effects on the pathogenesis of human brucellosis.

88 DETECTION OF *ESCHERICHIA COLI* O157:H7 IN MALAYSIA

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Frozen beef imported from an Asian country and retained in Malaysia were examined for the presence of *Escherichia coli* O157:H7. Nine of 25 samples contained this organism. Twelve strains isolated from these samples had established virulence-associated traits; these strains produced Shiga toxin 2 with or without Shiga toxin 1, had the corresponding *stx* genes, *eae* gene and a 60 MDa plasmid. None of the 12 strains appeared to

belong to an identical clone; the antibiograms, plasmid profiles, and the profiles of the arbitrarily primed PCR of the strains were diversified. These results suggest that diversified strains of *E. coli* is widely distributed in Malaysian environment. Distribution of and cases associated with *E. coli* O157:H7 should be investigated not only in the Western world but also in Asian countries.

89 ROLE OF SUPEROXIDE ANIONS AND NITRIC OXIDE IN HOST DEFENSE AGAINST INFECTION WITH *PENICILLIUM MARNEFFEI*

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Penicillium marneffeii is the dimorphic fungus and causes deep-seated systemic fungus disease. The fungus is one of the most important opportunistic pathogen in patients with AIDS in Southeast Asia. Although many epidemiological and clinical studies have been reported, the underlying immune mechanisms responsible for protecting the host against infection with *P. marneffeii* are poorly understood. In the present study, we investigated the role of superoxide anions (O_2^-) and nitric oxide (NO) in the fungicidal activity against *P. marneffeii* of IFN- γ -stimulated murine peritoneal macrophages and chemically generating system. Peritoneal macrophages suppressed the intracellular growth of *P. marneffeii* yeast cells. The number of live yeast cells within macrophages was significantly reduced by activation of macrophages by IFN- γ . IFN- γ -induced macrophage fungicidal activity against yeast cells was mediated by NO and almost completely inhibited by N^G monomethyl-L-arginine (L-NMMA), a competitive inhibitor of NO synthesis, while N^G monomethyl-D-arginine (D-NMMA), an optical isomer of L-NMMA, did not show any influence. Yeast cells were susceptible to the killing

effect of chemically generated NO. These results suggest that NO plays an important role in host defense against infection caused by *P. marneffeii*. On the other hand, oxygen radical scavengers, such as superoxide dismutase (SOD) and catalase, did not suppress, but rather enhanced, the fungicidal activity of IFN- γ -stimulated macrophages against *P. marneffeii* yeast cells, although *P. marneffeii* yeast cells were susceptible to chemically-generated O_2^- . This inconsistency was explained by the release of insufficient concentrations of O_2^- by activated macrophages compared with the amount of O_2^- necessary for the killing of yeast cells which was predicted in the chemical generating system. These oxygen radical scavengers enhanced the production of NO by IFN- γ -activated macrophages and their increased fungicidal activity was significantly inhibited by L-NMMA. Our results suggest that O_2^- is not involved in the fungicidal activity of macrophages against *P. marneffeii*, but rather plays an important role in the regulation of NO-mediated killing system by suppressing NO production.

90 DETECTION OF *BURKHOLDERIA PSEUDOMALLEI* FROM SOIL IN CENTRAL LAO P.D.R.

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Burkholderia pseudomallei causes melioidosis, an endemic disease in tropical areas such as Southeast Asia, northern Australia and Indian subcontinent. There exist overwhelming reports on the detection of *B. pseudomallei* from clinical and environmental samples in Thailand especially in northeastern areas. Laos faces Thailand crossing Mekong river, but no study on the detection of *B. pseudomallei* from environmental samples has been reported in Laos. In the present study, we

have tried to detect *B. pseudomallei* from the soil of central Laos. (Materials and Methods) Soil samples were collected from 20 sites in the dry season and 21 sites in the rainy season in Khammouane province located in central Laos. Soil was taken from the surface (0 cm) and at the depth of 20 and 50 cm mainly in rice fields. Approximately 3 cm³ of soil were placed in a sterile plastic tube. 2 ml of distilled water were added and the tube was shaken vigorously. 0.5 ml of suspen-

sion were transferred to 8 ml of the selective trypticase soy broths, CVC50, which contains crystal violet 5 mg/l and colistin 50 mg/l. After incubation at 42°C for 48 hrs, 10 µl of surface broth was plated on to Ashdown's agar and incubated aerobically at 42°C for 4 days. *B. pseudomallei* was identified by conventional method and confirmed by API ID 32 GN (bio Mérieux sa France). (Results) Only one strain was isolated from the surface in the rainy season. The isolation rate of *B. pseudomallei* from soil was 0.8% (1/123). This strain shows positive arginine dihydrolase, negative lysine decarbox-

ylase and negative ornithine decarboxylase. This strain was also confirmed by API ID 32 GN (bio Mérieux). (Discussion) The results of this study clarified the existence of *B. pseudomallei* in the soil of central Laos. In Laos little is known about this organism among medical staffs. Without their more detailed knowledge on *B. pseudomallei*, the number of the reports on the detection will not be changed in Laos. Further investigation is necessary to realize the situation of the prevalence in Laos.

91 A PRELIMINARY REPORT OF *BORRELIA* SURVEY AROUND WARM TEMPERATE ZONE IN CHINA

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In May, 1997, we conducted a survey for Lyme *Borrelia* in some areas along the Yangtze River as warm temperate zone in China. Collection size of vector ticks and reservoir rodents were small, because natural forests were generally poor in all survey areas. Nine isolates were obtained from BSK-culture on ticks and rodents. *Ixodes ovatus*, as a newly confirmed tick in

China by us, was positive for *Borrelia*. The isolate was a related to *B. japonica*, common in Japan. Some isolates from rodents were closely related to *B. garinii*, a well-known genospecies of Lyme *Borrelia* throughout northern areas of Eurasia. Hereafter these isolates and the vectors should be examined in comparison with Japanese strains.

92 LEPROSY ELIMINATION THROUGH INTERGATED BASIC HEALTH SERVICES IN MYANMAR: ROLE OF MIDWIVES FOR CHALLENGES AND POSSIBILITIES TOWARD POST-ELIMINATION PHASE

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Myanmar, being one of the leprosy endemic countries, is implementing the set target by WHO to reduce the prevalence rate to below one case per 10,000 population at national level by the year 2000 in order to eliminate leprosy as a public health problem. MDT was introduced in Myanmar in phased manner from 1988 as a vertical program which was gradually integrated into the Basic Health Services (BHS) and achieved 100% of MDT coverage of the registered cases in 1995. Both vertical and BHS staff especially the midwives were

trained to implement MDT while performing other Primary Health Care activities. Between 1988 and 1996 reduction of registered prevalence rate by 97% was noted with 4.72 cases per 10,000 population in 1996. In order to analyze the LEP work load of the midwives and their attitude towards LEP activities a survey was conducted by trained interviewers from July to November in 1995 in three of the six divisions where MDT was introduced in 1988-89 and interviewed one hundred and eighty-eight midwives. The average patient load of 2.2

cases per midwife is seen among 188 midwives interviewed. Thus, 2.2 leprosy patients count 0.75% of midwife's total work load for a month. For case finding activities the midwives conduct school survey, contact survey and a mixture of other survey methods. A midwife spent 2.1 days in last six months for the surveys and the figure equals to 1.9% of total number of working days in half a year for each midwife. All of the midwives interviewed perceived that leprosy activities were not an extra load of work as 50% of them could perform it together with other BHS activities and 27.6% presumed that it was a part of their routine activities under BHS since integration. 78% of 188 midwives

interviewed responded that they spend less than ten percent of their monthly working time for LEP activities. MDT is a simplified and effective approach and after the integration the skill was well transferred from the specialized vertical staff to the midwives of the BHS. On the other hand, the midwives consider that LEP activities are simple and is not an extra load of work of them. The commitment generated among the midwives in Myanmar with lessen LEP work load would be an essential component to achieve the elimination goal even at the sub-national level and to make LEP sustainable towards post-elimination phase.

93 DEVELOPMENT OF RAPID DIAGNOSIS METHOD OF ENTERIC *SALMONELLA* INFECTION IN THAI AND JAPAN

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Salmonella is a major causative agent of enteric infections, including food poisoning, both in Japan and Thailand. The genus *salmonella* comprises over 2,000 serotypes and the identification of the pathogenic *salmonella* in foods and clinical samples has been so laborious and so tedious that many of regional laboratories have been unable to identify the species accurately. Nonetheless the accurate and rapid diagnosis are necessary for the epidemiological analysis and for the prevention of the diseases. The most important matter regarding to the diagnosis is whether the isolate is virulent to human. The virulent factors of *salmonella* have not been well characterized as yet, but we focused on the enteric invasion factor and on the enterotoxin of which genes were identified recently and tried to develop rapid diagnosis method by using those genes as the virulence targets. Owing to the development of DNA technology, it became possible to identify the pathogenic *salmonella* gene rather quickly and easily comparing with the conventional method. We have designed certain primers for enterotoxin gene that reported previously (*stn* 101, *stn* 111) and enteroinvasive gene (*sin* 106 and *sin* 112) from the reported sequence for PCR method. The tested standard strains showed reproducible result to identify *salmonella* from other enterobacteriaceae. Then various serovar (46 serovar) of *salmonella* isolates in Thailand were examined by the method. As the control 30 strains

of different bacterial strains including enteric bacteria were also examined. All the *salmonella* strains tested, 301 strains with 46 serovar, showed positive in *stn* and *sin* by PCR method but none of the other strains, 176 strains of 30 different species, showed positive with those primers. The experimental data were consistent with the results and the handling were rather easy because of the stability of DNA. This method took only 3 hr. for the whole procedure, indicating the method could be practically applicable for routine use at regional laboratories where PCR is available. From the other point of view, the examination or confirmation of the gene product is also important because some genes of bacteria seems to be silent under a certain environmental conditions. To confirm the *stn* gene product in *salmonella*, the antibody against *stn* was needed. An artificially synthesized polypeptide was used as the antigen and raised antibody in rabbit, because the cloned *stn* gene in *E. coli* was not expressed enough amount for the purification to homogeneity. The antibody reacted specifically with 6kDa protein of *salmonella* by the Western-blott analysis but the molecular weight was different from the expected *stn* product of 30kDa. It is possible that the *stn* product could be processed during the excretion or the sample processing procedure. This point will be elucidated and proceed to the establishment of ELISA method for the toxin identification.

94 RAPID DETECTION OF CHOLERA CAUSED BY *VIBRIO CHOLERAE* O139

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Hybridomas secreting specific monoclonal antibodies (MAb to *Vibrio cholerae* serogroup O139) were produced. Six monoclones (hybridomas) secreting monoclonal antibodies specific only to *V. cholerae* O139 strains and did not cross-react to 137 strains of other enteric pathogens were obtained. These clones were designated clones 12F5-G11, 12F5-G2, 15F5-H5, 5B9-F8, 14C9-D2 and 6D2-D8. The immunoglobulin heavy chain isotypes secreted by these clones were IgG2b, IgG2b, IgG2b, IgM, IgG2b and IgG3, respectively. The clone 12F5-G11 was selected for mass production of MAb which were used in the development of an immunological assay, i.e. MAb-based dot-blot ELISA (membrane ELISA) for the detection of *V. cholerae* O139 antigen in stools of patients with watery diarrhoea for diagnosis of cholera caused by *V. cholerae* O139. Rectal swab cultures in alkaline-peptone water (APW) were collected from 6,497 patients with watery diarrhoea. All of the rectal swab cultures were sent to the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University where the antigen detection assays were performed using the MAb-based dot-blot ELISA. The bacterial isolation and identifications of the stools were carried out by scientists of Bamrasnaradura Infectious Diseases Hospital, Krung Thon Hospital, Children Hospital, Armed Force Research Institute of Medical Sciences U.S. Com-

ponent (AFRIMS) and National Institute of Cholera and Enteric Diseases (WHO Collaborating Center for Diarrhoeal Diseases Research and Training), Calcutta, India. The results of the bacterial cultures were revealed only after the MAb-based dot-blot ELISA had been completed to avoid bias. It was found that among the 6,497 stool samples, 45 samples were positive while 6,452 samples were negative by the ELISA. The bacterial cultures and identification of the recovered bacteria by agglutination with *V. cholerae* O139 polyvalent antiserum showed that 42 cases were *V. cholerae* O139 positive, while the rest were negative. Statistical analysis of the MAb-based dot-blot ELISA involving the MAb from clone 12F5-G11 in comparison with the bacterial culture method revealed that the ELISA had 100%, 99.95%, 99.26%, 93.33% and 100% of diagnostic sensitivity, diagnostic specificity, efficacy, and positive and negative predictive values, respectively. Moreover, the test was negative when tested on rectal swab cultures of 50 healthy, enteric pathogen free individuals; thus indicated 100% specificity of the assay. The kappa coefficient (K) and kappa probability (Z) value of the MAb-based dot-blot ELISA when compared with the bacterial culture method were 0.965 and 77.85, respectively which indicated excellent degree of agreement the two tests beyond chance and extremely high reliability of the ELISA ($p < 0.0000001$), respectively.

**95 A SURVEY OF INTESTINAL PARASITIC INFECTIONS
AMONG WHOLE RESIDENTS OF PREK RUSSEY COMMUNE,
CAMBODIA; WITH SPECIAL REFERENCE TO THE
RELATION WITH THE DIFFERENCE OF SOCIO-ECONOMICAL
DEVELOPMENT AMONG 3 VILLAGES**

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A survey of intestinal parasitic infections among whole residents of Prek Russey Commune, Cambodia, was performed as part of our international cooperation project for intestinal parasitic diseases control. The main objects are to grasp exact condition of infection and then to apply to the prevention activity against repeated parasites infections. By the way, the agar plate method, which we adopted as one of the examination methods, was developed in 1988 by Arakaki *et al.* has been shown to be very effective in detecting *Strongyloides stercoralis*. We have been reporting that the agar plate method is very efficient, not only for its reliability but also as an appropriate technology for developing countries based on our previous studies in Thailand, Cambodia and Indonesia. The field is Prek Russey Commune, Takmau District, Kandal Province, Cambodia, and involving 3 villages, Kroppeu Ha, Prek Russey and Prek AngChanh. This commune is adjacent to Takmau Town, which is the central town of Kandal Province, and is located 15 km south of phnom Penh. It

is farming villages but comparatively developed area in this country because of good access to the capital. Among 3 villages, Kroppeu Ha is next to the Takmau Town, and most socio-economically developed. Prek Russey Village is the next, and Prek AngChanh is located the opposite side of small river. The positive rate of intestinal parasites seen among 3,533 residents was 42%. The most common parasite was hookworm (22%) followed by *Ascaris lumbricoides* (15%) and *S. stercoralis* (15%). The other parasites were not common (under 5%). The positive rates of these 3 major parasites differ among 3 villages. Kroppeu Ha was lowest, followed by Prek Russey and Prek AngChanh was highest throughout 3 parasites. The differences were statistically tested using logistic analysis. The P value was <0.0001 for hookworm, 0.001 for *Strongyloides* and 0.04 for *Ascaris*, respectively. The value was quite small for hookworm and *Strongyloides*. Therefore, it was suggested that the socio-economical difference influenced the soil transmitted infection of these parasites.

**96 PREDICTORS OF SOIL-TRANSMITTED HELMINTHIASIS
IN A RURAL COMMUNITY IN MALAYSIA**

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Soil-transmitted helminthiasis is found worldwide with the three most common worms, *Ascaris lumbricoides*, hookworm and *Trichuris trichiura* amongst the top ten most common infections afflicting people today. Socio-environmental factors are important in the transmission of soil-transmitted helminthiasis. However, these factors varies from one community to the other and identifying the significant risk factors within communities is pertinent for effective control of soil-transmitted helminthiasis. This prevalence study

was undertaken in the subdistricts of Tawang and Perupok within the district of Bachok, Kelantan, Malaysia between October 1992 to December 1992 to compare significant risk predictors of soil-transmitted helminthiasis in these two subdistricts. A total of 194 children four years and below were randomly selected from a list of children attending the "under 4" government clinics in the two subdistricts. All children in Tawang participated in the study while in Perupok a total of 169 children finally participated in the study.

Demographic as well as socio-environmental factors were collected from the participating children. Stools for ova of soil-transmitted helminthiasis were examined by the formal-ether method. Important risk predictors of soil-transmitted helminthiasis in Tawang following adjustment include large household size, poor household knowledge about soil-transmitted helminthiasis and poor household hygiene. In Perupok, children

from poverty households were at higher risk of getting infected. It can be concluded that in general socio-environmental factors are important in the transmission of soil-transmitted helminthiasis, however such risk factors differed from one community to another and future strategies in the control and prevention of worm infection should be tailored according to the prevailing risk factors in the community.

97 A STUDY ON *WUCHERERIA BANCROFTI* ANTIGENEMIA IN SRI LANKA

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Detecting microfilariae (mf) in the peripheral blood has been the method to confirm filarial infection. In recent years, a new immunological technique to detect circulating filarial antigens became available, and the use of the method revealed the presence of many people who were antigen (Ag) positive but mf negative. We report here the prevalence of antigenemia and microfilaremia in Sri Lanka, where *Wuchereria bancrofti* has been endemic, and analyze the Ag positive but mf negative people. A total of 353 people in Matara area, southern part of Sri Lanka, were examined for filarial infection by three different methods, 60 μ l finger-prick blood smear method (Fp), 1 ml Nuclepore filtration method (Np) and antigen detection method using the ELISA kit based on Og4C3 monoclonal antibody and developed by JCU Tropical Biotechnology Pty Ltd., Australia. The antigenemia detected by the kit is believed to indicate the existence of live adult worms. All blood samples were collected between 22:00 and 01:00 hours. The prevalence rates determined by the three methods were 7.9% (Fp), 11.3% (Np) and 20.7% (antigenemia). Of 313 Np negative persons, 42 (13.4%)

were Ag positive. The levels of antigenemia and Np counts were correlated positively, when Ag positive but mf negative subjects were excluded. The Og4C3 ELISA was found to be effective in finding filarial infections. In addition, the method is said to be applicable even in the daytime. The use of the kit revealed that quite a big portion of Matara residents were Ag positive but mf negative. This category of people included young people who probably had still a 'few' number of adult parasites which were not enough to produce microfilaremia, and older people in whom mf had probably been eliminated as a result of immune reactions against mf. It has been known that most elephantiasis/edema cases are mf negative, and this may be implying that Ag positive but mf negative people constitute a future high-risk group in terms of clinical filariasis. The epidemiological significance of this new entity has to be investigated by conducting a long-term follow-up study. This study was supported in part by the Grant-in Aid for International Scientific Research by the Ministry of Education, Science, Sports and Culture.

**98 BASIC STUDIES ON THE MONGOLIAN GERBIL AS A
SUSCEPTIBLE HOST TO FILARIAL INFECTION (16)
SENSITIVITY TO THE DIABETOGENIC SUBSTANCE, ALLOXAN**

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The Mongolian gerbil (*Meriones unguiculatus*) is world wide used in the laboratory practice for its suitability as a host for filarial infection. A rodent whose natural habitat is the desert, its propensity to develop obesity and mild diabetes in laboratory conditions has been documented. In this study, we compared the sensitivity of the Mongolian gerbil to that of the ddy mouse toward alloxan. Animals were injected intravenously with a single dose of alloxan and their diabetic symptoms were studied using biochemical and immunohistopathological techniques during 3 months. The optimal required dose of alloxan to induce diabetes in Mongolian gerbils was 140 mg/kg BW, 2 fold the dose needed for mice. The peak average blood glucose level

was equally 460 mg/dl in both animals at 3-7 days post injection. Body weight loss was transient in gerbils, whereas that in mice persisted until 10 weeks. The normal HbA_{1c} level in mice was $3.2 \pm 0.2\%$ with a significant increase up to 9%, 4 weeks post injection. Gerbils showed 2.5-3.0% of HbA_{1c} throughout the experiment with no significant changes. Hypoplastic changes of insulin secreting cells and a remarkable hyperplasia of glucagon secreting cells were demonstrated in the endocrine pancreas of both animals by immunohistochemical staining. The Mongolian gerbil may be one of the indicated rodents for the etiopathogenesis of idiopathic cases of diabetes in some tropical areas.

**99 THERMAL INSULATION OF FUR AND CIRCADIAN RHYTHM OF
BODY TEMPERATURE IN PIKA**

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Since 1985, Afghan pikas supplied from the Central Institute for Experimental Animals, Japan were reared and bred in the Animal Research Center for Infectious Tropical Diseases, Institute of Tropical Medicine, Nagasaki University. The autonomic characteristics of the pika are high body temperature, high metabolism and poor heat losing ability. In this study thermal insulation of the skin and hairs and the circadian rhythm of body temperature in pika were observed. (Materials and Methods) Exp.1: Anatomical study of the skin and hairs. Eleven pikas (185-210 g) and six wistar rats (200-230 g) were used in this experiment. The animal

were sacrificed by an overdose of ether inhalation. After cutting the hair with electrical clippers, the skin was cut off from the chest, abdomen, back and waist. The thickness of the skin from the epidermis to the dermis was measured with micrometer under a light microscope. The density of the hairs in the horizontal skin sections was detected under a light microscope. Several strands of hairs were pulled out from each region of the chest, abdomen, back and waist and the length of the hairs from the hair papilla to the tip were measured. Exp.2: Telemetry-recording of the circadian rhythm of body temperature and locomotive activity.

Seven pikas, two rabbits and seven wistar rats were used in this experiment. In 25°C and 60% rh with a 12L:12D Light-dark photoperiod, A battery-operated transmitter was intraperitoneally implanted under anesthesia, 7-14 days before the experiment began. The body temperature and locomotive activity were recorded every one minute using a bio-telemetry system. (Results and Discussion) Exp.1: The skin thickness of pikas was significantly thin compared to that of rats on the all of the 4 determined regions ($p < 0.01$). The hairs on the back and waist were longer in pika than in rats ($p < 0.01$). The hair density on the dorsal surface of the pikas was higher than that of the ventral surface. Exp. 2: The rabbits and rats showed the nocturnal rhythm of the body temperature, but the pikas did not show diurnal nor nocturnal rhythm in body temperature. Subcutane-

ous fat plays a role as an insulative barrier in human beings and hairless animals. In animals with fur and hairs, however, insulation depends on the fur and not on the subcutaneous fat. The hairs in the pikas were longer than the rats and more dense and two times longer on the dorsal region than on the ventral region although the skin was thinner. 20 mm hair length of pika was estimated to provide 3 clo of insulative property. Pika is considered to be adaptive to cold not only ecologically and autonomically but also due to the insulative cold defense mechanisms. Our present finding of the absence of the circadian body temperature rhythm in the pika is quite unique and is encouraging for further research investigations related to body temperature rhythm in pika.

100 A SURVEY OF THE LIVING CONDITIONS OF NEPALESE PORTERS

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The purpose of this survey is to clarify the characteristics of the ways of transportation of goods under undeveloped social conditions. In Nepal, people living not only in the mountain area but also in the downtown area often have to depend on porters when they move things. In Kathmandu, the capital of Nepal, we notice the following four patterns when the porters carry luggage and/or commodities: putting the goods 1) in the baskets of a yoke on their shoulders, 2) on a handcart, 3) on the top of the head, and 4) on the back using a rope. The fourth pattern, putting things on the back, is most frequently observed. In this connection, we designed a survey by investigating living conditions of Nepalese porters using oral interview. For this purpose we selected seven porters, whose ethnicity was Magar

or Tamang. The following results were obtained:

- 1) They could carry luggage of about 60 kg to 200 kg by themselves.
- 2) Six out of the seven porters habitually smoked and drank alcohol. They felt these stimulants helped refresh their body condition.
- 3) They did not complain of severe neck pain but all porters complained of knee and/or back pains.
- 4) Many porters wore cloth tightly twisted around their waist based on their belief that their god gave them power if they did so. Actually the binding helped to prevent lumbago.
- 5) Their income was extremely low and unutterable compared with that of other citizens.

101 THE INFLUENCE OF *ASCARIS LUMBRICOIDES* INFECTION ON
SERUM VITAMIN A AND E LEVELS IN YOUNG WOMEN IN
RURAL AREA OF THE PHILIPPINES

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Vitamin A deficiency is common in developing countries. A study was conducted in rural area in the Philippines in September 1996 to determine the influence of *Ascaris* infection on the levels of vitamin A and E amongst women of childbearing age. A total of 123 female subjects aged 15 to 34 years of age (mean age 19.9 years old) participated in the study. Out of 81 respondents 45 (55.6%) and 51 (63%) were positive for *Ascaris* and *Trichuris* ova respectively. Serum vitamin A ($p > 0.05$) and E ($p < 0.05$) decreased with the intensity of *Ascaris* ova, which was not influenced by age and dietary habits. However, serum vitamin A levels were higher in those with light infection compared to those not infected with *Trichuris*. Further studies would be

required to clarify this occurrence. It was also found that serum vitamin A and E levels of individuals recently infected with *Ascaris* were $77 \pm 38 \mu\text{g/dl}$ and $352 \pm 239 \mu\text{g/dl}$, respectively. The serum vitamin A and E amongst 100 subjects with no *Ascaris* eggs on stools were $96 \pm 38 \mu\text{g/dl}$ and $398 \pm 161 \mu\text{g/dl}$ respectively. Vitamin A and E levels were significantly lower amongst those infected compared to those not infected ($p < 0.05$). It is thereby concluded that *Ascaris* and other soil-transmitted helminthiasis may enhance serious vitamin A and E deficiency in low socio-economic areas and effective health care system for health promotion and voluntary anthelmintics is warranted.

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