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IMMUNOGENICITY OF SLOWLY SEDIMENTING ANTIGEN OF JAPANESE ENCEPHALITIS VIRUS ENVELOPE GLYCOPROTEIN ISOLATED FROM INFECTED CULTURE FLUIDS

GOLAM MASUD MOHAMMAD SHAMEEM

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Abstract: Slowly sedimenting antigen (SE) and the rapidly sedimenting antigen (RE), associated with envelope glycoprotein E were prepared from the concentrated infected culture fluids of Japanese encephalitis (JE) virus. Mice were immunized by each antigen before and after inactivation of virus infectivity by ultraviolet (UV) irradiation. The immunogenicity of the antigens determined by the plaque reduction neutralization test (PRNT), indirect ELISA and hemagglutination-inhibition (HI) test, indicated that SE was almost equally immunogenic as RE, and that the PRNT epitopes on both antigens were more immunogenic before UV-inactivation than after the inactivation.

INTRODUCTION

JE is an acute viral encephalitis and is a serious public health problem in many Asian countries (Umenai *et al.*, 1985). The etiologic agent, JE virus, belongs to the Flaviviridae family (Westaway *et al.*, 1985) comprising about 70 viruses (Calisher *et al.*, 1989), of which many are pathogens for humans and domestic animals (Monath, 1986). Current JE vaccine has been developed as formalin-inactivated and highly purified virion from infected mouse brains and has successfully been used for JE control in Japan, Republic of Korea, and some other countries (Hammon *et al.*, 1971; Huang, 1982; Oya, 1988; Igarashi, 1988). Besides rapidly sedimenting virus antigens associated with complete virion, slowly sedimenting hemagglutinin or soluble complement-fixing antigen (SCF) of flaviviruses have been described in the infected mouse brain homogenates or in culture fluids by sucrose gradient sedimentation (Igarashi *et al.*, 1963; Kitaoka and Nishimura, 1965; Smith *et al.*, 1970; Shameem *et al.*, 1989). However the immunogenicity of SE has not well been documented. In order to clarify the immunogenicity of SE and RE, we examined anti-JE titer of the sera from mice immunized with SE or RE before and after UV-inactivation.

MATERIALS AND METHODS

Cells and virus

Aedes albopictus, clone C6/36, cells (Igarashi, 1978) were grown at 28°C and BHK21 cells

Department of Virology, Institute of Tropical Medicine, Nagasaki University, 12-4 Sakamoto-machi, Nagasaki 852, Japan

at 37°C in Roux bottles with 750 cm^3 volume. The cell growth medium was Eagle's minimum essential medium supplemented with 0.2 mmol/l each of 7 nonessential amino acids (Eagle, 1959) and 10% heat-inactivated fetal calf serum (FCS). The origin of a wild strain JE virus, JaOArS982, was described by Hori *et al.* (1986), and seed virus was prepared in C6/36 cells at 28°C.

Preparation of slowly sedimenting and rapidly sedimenting antigens of JE virus

The SE and RE were isolated by sucrose gradient sedimentation of concentrated infected culture fluids as described in our previous communication (Shameem *et al.*, 1989). In this experiment specimens were harvested 36 hr after infection for both cell lines, but incubated at 28°C for C6/36 and 37°C for BHK21 cells, respectively.

Inactivation of the infective virus by UV-irradiation

Immunogens (2.5 ml) were spread on a petri dish (60 mm) and exposed to 2 UV bulbs (20 W) at a distance of 42 cm for 5 minutes. After the exposure, infectivity of the virus was undetectable (Sachiko Matsuo, personal communication).

Immunization of mice

BALB / c mice were immunized by 4 intraperitoneal injections at 1 week interval (0.1 ml/mouse/dose), using either UV irradiated or unirradiated SE and/or RE fractions, which were mixed with an equal volume of Freund's complete adjuvant for the first injection or incomplete adjuvant for the subsequent injections. One week after the last injection, mice were individually bled and serum was separated for further test.

Plaque reduction neutralization test (PRNT or N)

This procedure was carried out as described by Hashimoto *et al.* (1971) with some modification. Serial two fold serum dilutions beginning from 1:10 were made using the diluent of 5% FCS in Eagle's medium and mixed with an equal volume of the seed virus diluted to yield 100-200 PFU/0.1 ml. For virus control the working dilution of the virus was mixed with an equal volume of the diluent. The mixtures were incubated for 90 min at 37°C in humidified atmosphere containing 5% CO_2. At the end of the incubation, the mixture was inoculated to the monolayer of BHK21 cells (0.1 ml/well) grown on 24-well polystyrene tissue culture plate. Adsorption was carried out at 37°C for 2 hr and the cells were covered with 1.25% methyl cellulose in the maintenance medium (cell growth medium from which serum concentration was reduced to 2%). Plates were incubated for 5 days in the same condition as above and cells were fixed with cold methanol, stained with 0.1% trypan blue in 0.9% NaCl at room temperature for 1 hr to reveal the plaques. The N was scored as positive when more than 50% plaque reduction was observed compared with the negative control of virus-diluent mixture. Reciprocal of the highest dilution of the test serum with positive plaque reduction was considered as N titer.

Sandwich ELISA

The modified procedure of Voller *et al.* (1976) was described in our previous communication (Shameem *et al.*, 1989).

Indirect micro ELISA

This was carried out according to Voller *et al.* (1976) using purified JE antigen (100 μg/m*l*). ELISA titer of test specimens was calculated by comparing the OD with those by serially diluted standard positive serum as described before (Igarashi *et al.*, 1981; Morita *et al.*, 1982). Standard anti-JE mouse serum was prepared by repeated intraperitoneal inoculation of purified JE virus (Srivastava *et al.*, 1987).

Hemagglutination-inhibition (HI) test

The procedure was carried out according to Clarke and Casals (1985) using goose red blood cells in virus adjusting diluent (VAD) at pH 6.6.

Statistical analysis

Student's t test was performed according to the standard procedure.

RESULTS

Mouse immunogenicity of SE and RE before UV-inactivation

In the initial experiment, mice were immunized with SE or RE fractions of both C6/36 and BHK21 cells without UV-inactivation. The antibody titer of individual serum from immunized mice was shown in Table 1. When antibody titers raised by SE and RE were compared, SE immunogen from C6/36 cells produced less N and HI titers than RE from the same cells, and these differences were statistically significant (Table 3). While, both SE and

Table 1 Immunogenicity of SE and RE before UV-irradiation, as shown by titers in log for N, ELISA and HI

Serum No.	Immunogen	N		ELISA		HI	
		Titer	Mean	Titer	Mean	Titer	Mean
1-1	C6/36 SE	1.30	1.68±0.29 (GMT 48)	3.85	4.28±0.40 (GMT 19,054)	1.90	2.05±0.31 (GMT 112)
1-2		1.60		4.72		1.90	
1-3		1.90		4.04		1.90	
1-4		1.90		4.49		2.51	
2-1	C6/36 RE	2.20	2.20±0.25 (GMT 158)	4.16	4.29±0.24 (GMT 19,498)	2.56	2.66±0.30 (GMT 457)
2-2		2.51		4.65		2.51	
2-3		1.90		4.22		2.51	
2-4		2.20		4.14		3.11	
3-1	BHK21 SE	1.90	2.05±0.21 (GMT 112)	4.49	4.49±0.01 (GMT 30,902)	2.20	2.36±0.22 (GMT 229)
3-2		2.20		4.48		2.51	
4-1	BHK21 RE	2.20	2.08±0.16 (GMT 120)	4.06	4.22±0.38 (GMT 16,595)	2.81	2.69±0.27 (GMT 490)
4-2		2.20		4.48		2.51	
4-3		2.20		4.72		2.51	
4-4		1.90		3.89		3.11	
4-5		2.20		3.96		2.51	

Note: GMT=Geometric mean titer

Table 2 Immunogenicity of SE and RE before UV-irradiation, as shown by titer ratio of ELISA/N and ELISA/HI

Serum No.	Immunogen	ELISA/N			ELISA/HI		
		Titer	Mean	GMT	Titer	Mean	GMT
1-1	C6/36 SE	2.55	2.60±0.40	398	1.95	2.22±0.41	166
1-2		3.12			2.82		
1-3		2.14			2.14		
1-4		2.59			1.98		
2-1	C6/36 RE	1.96	2.09±0.17	123	1.65	1.38±0.35	24
2-2		2.14			1.14		
2-3		2.32			1.71		
2-4		1.94			1.03		
3-1	BHK21 SE	2.59	2.44±0.22	272	2.29	2.13±0.23	134
3-2		2.28			1.97		
4-1	BHK21 RE	1.86	2.07±0.31	119	1.25	1.53±0.57	34
4-2		2.26			1.97		
4-3		2.52			2.21		
4-4		1.99			0.78		
4-5		1.76			1.45		

Table 3 Statistical analysis on N, ELISA and HI titer and titer ratio differences raised by SE and RE before UV-irradiation

Immunogens	log titer and titer ratio	P value
SE and RE of C6/36	N	$0.02 < P < 0.05$
	ELISA	>0.10
	HI	$0.02 < P < 0.05$
	ELISA/N	>0.10
	ELISA/HI	$0.02 < P < 0.05$
SE and RE of BHK21	N	>0.10
	ELISA	$0.02 < P < 0.05$
	HI	>0.10
	ELISA/N	>0.10
	ELISA/HI	>0.10

RE from C6/36 cells produced similar ELISA titers. In the case of immunogen from BHK21 cells, both SE and RE produced similar N and HI titers, while ELISA titer produced by RE was less than the titer by SE and the difference was statistically significant (Table 3). The antibody titers produced by immunogens from C6/36 and BHK21 cells were almost the same except that N titer by SE antigen from C6/36 cells was lower than the titer by other antigens (Table 1). Since immunogens of SE and RE from C6/36 cells and SE from BHK21 cells

possessed comparable ELISA titers as shown in Table 4, the results may indicate low immunogenicity of SE from C6/36 cells to induce N antibodies. Lower ELISA titer by RE from BHK21 cells may be explained by its slightly less ELISA titer (Table 4). The antibody titers were compared by their ratios (ELISA/N and ELISA/HI), and the results were shown in Table 2. The SE from C6/36 cells showed higher ELISA/HI ratio than RE from the same cells with statistically significant difference, while other ratios did not show significant difference between SE and RE. In this series of experiment a high mortality rate of mice around 40% was observed probably due to encephalitis caused by residual infective virus in the immunogens (data not shown). Therefore, it was conceivable that the antibody titers and their ratios in Tables 1 and 2 might reflect the effect of viral antigens produced in mice after virus replication, besides immunogenicity of inoculated specimens. Therefore, subsequent experiments were performed by immunogens which had been inactivated by UV-irradiation.

Mouse immunogenicity of SE and RE after UV-irradiation

Each immunogens of SE and RE from C6/36 and BHK21 cells were irradiated by UV as described in the Materials and Methods, and ELISA titers of these immunogens were shown in Table 4. The ELISA titer of SE from both C6/36 and BHK21 cells was reduced around 78%, while the titer reduction of RE was less (around 42-43%).

The antibody titers of individual mouse serum raised by UV-inactivated immunogens were shown in Table 5. Only the HI titer produced by RE from BHK21 cells was higher than the titer by SE from the same cell, and the difference was statistically significant (Table 7).

The titer ratio (ELISA/N and ELISA/HI) of the individual mouse serum was shown in Table 6. In this case, only the ELISA/HI produced by SE from C6/36 cells was higher than the titer by RE from the same cells, and the difference was statistically significant.

Comparison of the immunogenicity before and after UV-irradiation

The effect of UV-irradiation on the immunogen was evaluated by comparing the antibody titers (N, ELISA, HI) as well as their ratios (ELISA/N, ELISA/HI) raised by each immunogen before and after UV-irradiation. The statistically significant difference was observed only for the reduction in HI titer produced by RE from C6/36 cells and the increase in ELISA/N ratio produced by RE from BHK21 cells (Table 8). Other immunogenicities were apparently not affected by UV-irradiation.

Table 4 ELISA titer of immunogens before and after UV-irradiation

Immunogen		ELISA titer of immunogen		% Reduction
		before UV	after UV	in ELISA titer
C6/36	SE	232	50	78.45
C6/36	RE	206	116	43.69
BHK21	SE	229	50	78.15
BHK21	RE	186	107	42.48

Table 5 Immunogenicity of SE and RE after UV irradiation, as shown by titers in log for N, ELISA and HI

Serum No.	Immunogen	N		ELISA		HI	
		Titer	Mean	Titer	Mean	Titer	Mean
1-1	C6/36 SE	2.20	1.54±0.54 (GMT 35)	4.58	4.42±0.20 (GMT 26,302)	1.90	1.96±0.13 (GMT 91)
1-2		1.00		4.58		1.90	
1-3		1.00		4.11		1.90	
1-4		1.90		4.48		2.20	
1-5		1.60		4.35		1.90	
2-1	C6/36 RE	2.20	1.98±0.29 (GMT 95)	4.29	4.40±0.17 (GMT 25,118)	2.51	2.28±0.29 (GMT 191)
2-2		2.20		4.23		1.90	
2-3		1.90		4.51		2.20	
2-4		1.60		4.58		2.51	
3-1	BHK21 SE	2.20	1.84±0.39 (GMT 69)	4.64	4.43±0.23 (GMT 26,915)	2.51	2.20±0.22 (GMT 158)
3-2		1.60		4.51		1.90	
3-3		1.30		4.61		2.20	
3-4		2.20		4.35		2.20	
3-5		1.90		4.08		2.20	
4-1	BHK21 RE	1.90	1.90±0.24 (GMT 79)	4.35	4.50±0.12 (GMT 31,622)	2.51	2.66±0.39 (GMT 457)
4-2		1.90		4.58		3.11	
4-3		2.20		4.44		2.20	
4-4		1.60		4.61		2.81	

Discussion

From the results of our immunological studies it appears that the SE of JE virus is immunogenic in mice to induce N, ELISA and HI antibodies, and the immunogenicity of both antigens, SE and RE, were almost comparable. Although UV-irradiation totally inactivated infectivity and partially destroyed ELISA antigenicities in both SE and RE, they still retained their immunogenicities. The different UV-susceptibility of ELISA antigenicity between SE and RE from both C6/36 and BHK21 cells may be explained by different target size of the particle. The RE is the complete virion and possesses larger size and greater number of repetitive epitope units than SE, which is smaller size with less number of repetitive units. It is reasonable to imagine that RE with larger number of repetitive ELISA antigenic epitopes is more resistant to UV-irradiation than SE. Kimura-Kuroda and Yasui (1983) reported that at least five topographically distinct antigenic determinants including N, HI, and ELISA epitopes, were present on E protein and the epitopes of HI and N were separated from each other by monoclonal antibodies.

Recently, Mason *et al.* (1991) reported that the JE vaccinia virus recombinants possessing JE virus cDNA inserts produced particulate form of antigen containing M (membrane) and E proteins of JE virus. The antigen migrated in sucrose gradients slower than the complete virion, similar to the SE, and induce high level of anti-JE N antibodies and conferred protection against lethal JE virus infection.

Table 6 Immunogenicity of SE and RE after UV-irradiation, as shown by titer ratio of ELISA/N and ELISA/HI

Serum No.	Immunogen	ELISA/N			ELISA/HI		
		Titer	Mean	GMT	Titer	Mean	GMT
1-1	C6/36 SE	2.38	2.88±0.47	758	2.68	2.46±0.21	288
1-2		3.58			2.68		
1-3		3.11			2.21		
1-4		2.58			2.28		
1-5		2.75			2.45		
2-1	C6/36 RE	2.09	2.45±0.46	281	1.78	2.12±0.25	131
2-2		2.03			2.33		
2-3		2.61			2.31		
2-4		2.98			2.07		
3-1	BHK21 SE	2.44	2.60±0.50	398	2.13	2.23±0.28	170
3-2		2.91			2.61		
3-3		3.31			2.41		
3-4		2.15			2.15		
3-5		2.18			1.88		
4-1	BHK21 RE	2.45	2.59±0.32	389	1.84	1.84±0.31	69
4-2		2.68			1.47		
4-3		2.24			2.24		
4-4		3.01			1.80		

Table 7 Statistical analysis on N, ELISA and HI titer and titer ratio differences raised by SE and RE after UV-irradiation

Immunogens	log titer and titer ratio	P value
SE and RE of C6/36	N	>0.10
	ELISA	>0.10
	HI	>0.10
	ELISA/N	>0.10
	ELISA/HI	$0.02<P<0.05$
SE and RE of BHK21	N	>0.10
	ELISA	>0.10
	HI	$0.02<P<0.05$
	ELISA/N	>0.10
	ELISA/HI	>0.05

Table 8 Statistical analysis on the difference in antibody titers and titer ratios obtained by immunogens before and after UV-irradiation

Immunogens	P value				
	N	ELISA	HI	ELISA/N	ELISA/HI
SE of C6/36	>0.10	>0.10	>0.10	>0.10	>0.10
RE of C6/36	>0.10	>0.10	<0.001	>0.10	>0.10
SE of BHK21	>0.10	>0.10	>0.10	>0.10	>0.10
RE of BHK21	>0.10	>0.10	>0.10	0.02<P<0.05	>0.10

At present, inactivated JE vaccine has been manufactured from infected mouse brain homogenates, by purifying formaline-inactivated complete virion using primarily ultracentrifugation (Takaku *et al.*, 1968). Till now, little or no attention has been paid for the recovery of SE during JE vaccine preparation, and most of the SE would have been lost in the supernatant of ultracentrifugation. It would be worthwhile to consider the immunogenicity of SE in the crude vaccine preparation before ultracentrifugation in order to recover more immunogens.

Some flaviviruses have been shown to produce slowly sedimenting antigen along with rapidly sedimenting antigen or complete virion in the mouse brain homogenates or in tissue culture system (Igarashi *et al.*, 1963; Smith *et al.*, 1970; Shameem *et al.*, 1989). This information has permitted us to predict that some form of 'incomplete virion' is synthesized in the early period of replication of JE virus. In the case of dengue type 2 virus, another important member of flavivirus, Cardiff *et al.* (1971) argued that most of the incomplete virus or top component was associated with several unrelated phenomena, viz. defective interfering particles, adsorbing interfering particles, pleomorphic aggregates of coat structures, macromolecular capsid precursors and some naturally occurring particles which morphologically resemble infectious form except that they did not contain no core structure. Alternatively, SE may represent a protein synthesized during the course of viral replication which might be released into the medium, besides complete virion, sharing several biological properties possessed by envelope glycoprotein E.

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BASIC STUDIES ON THE MONGOLIAN GERBIL AS A SUSCEPTIBLE HOST TO FILARIAL INFECTION; COMPARATIVE STUDIES ON HEMATOLOGICAL FEATURES BETWEEN THE WILD-COLORED GERBIL AND THE COAT COLOR MUTANTS

MASUMI SHIMIZU[1], KAZUHIRO SHICHINOHE[1], SETSUKO TSUKIDATE[2] AND KOICHIRO FUJITA[2]

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Abstract: Hematological baseline parameters of the Mongolian gerbils were investigated to be compared between the wild-colored gerbil (agouti type) and the other coat color mutants such as white spotted-agouti, albino, black and white spotted-black type. Erythrocyte counts of the agouti type were higher than those of the coat color mutants. But, there was no significant difference. A frequent occurrence of polychromasia and basophilic stippling in circulating erythrocytes was known to be a particular feature of the Mongolian gerbil among laboratory animals. The polychromasia and the basophilic stippling were proved to be present in the coat color mutants in the same degree as the agouti type. As to sex dimorphism in erythrocytic values, it was only hemoglobin concentration that was observed significant sex-related difference in all coat color gerbils. In leukocytic values, quantitative sex-related difference was not seen in this experiment. A presence of basophils in peripheral blood was observed on all blood films of all coat color gerbils. These results confirmed that the coat color mutants of gerbils had the same unique hematological characteristics as an agouti type.

INTRODUCTION

The Mongolian gerbil (*Meriones unguiculatus*) has been recognized an useful rodent having considerable potential for laboratory studies, especially in the field of parasitology, as a susceptible host to filarial infection. The gerbil, ordinary used in laboratories has a wild coat color called an agouti type. We obtained some new coat color mutants from the gerbil and reported a comparative study on biological aspects among these mutants (Shimizu *et al.*, 1990). Some hematological data of agouti gerbils have been reported and it was ascertained that the blood of this species manifested some interesting characteristics not generally observed in other rodents (Mays, 1969; Dillon and Glomski, 1975a; Smith *et al.*, 1976; Termer and Glomski, 1978). But, according to the coat color mutant, only a limited data has been available that the white spotted-agouti type of mutants is with a slight anemia (Waring *et*

1 Department of Laboratory Animal Science, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113, Japan

2 Department of Medical Zoology, Faculty of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113, Japan

al., 1978). Hematological baseline parameters of the coat color mutants of gerbils were studied basically in order to analyze the susceptibility of gerbils to filarial infection. A comparative study on the blood picture among coat color mutants was carried out in the present work and showed some interesting hematological features of the coat color mutants of the gerbils as well as an agouti type.

Materials and Methods

The animals were bred under a conventional condition and housed 5 adults of the same sex in one cage. The room temperature and the humidity were maintained at 24±2°C and 60±5%, respectively. Ten of 3 to 5-month-old gerbils, such as agouti, white spotted-agouti, albino, black and white spotted-black, of both sexes were used in this experiment. Samples of blood were prepared from the retro-orbital venous plexus of the ether-anesthetized gerbils by means of hepalinized capillary pipettes. All samples were collected between 0900 and 1100 hours at 1-month intervals from the animals. Total erythrocyte and leukocyte counts were performed using hemocytometer under a light microscope. Hematocrit determinations were made by means of microcentrifugation and hemoglobin concentrations were determined spectrophotometrically by the cyanmethohemoglobin technique. Dry film blood smears were stained with May-Grunwald and Giemsa stain and differential counts were identified at least 100 leukocytes per an animal. Blood smears for reticulocyte counts were prepared by using Brecher's new methylene blue stain. One thousand of erythrocytes were examined and the number of cells containing a reticulum were recorded. The numbers of basophilic stippled erythrocytes, polychromatophilic cells and cells with Howell-Jolly bodies were also counted while examining 1,000 erythrocytes in the smears stained with May-Grunwald and Giemsa stain.

Table 1 Total erythrocyte counts and related values in each coat color mutant of Mongolian gerbils

Coat color	Sex	Erythrocytes ($\times 10^6/\mu l$)	Hemoglobin (g/dl)	Hematocrit (%)	Mean corpuscular volume (fl)	Mean corpuscular hemoglobin (pg)	Mean corpuscular hemoglobin concentration (%)
Agouti	Male	8.8±0.5	16.4±0.4†	48.6±1.0†	52.0±4.6	18.6±1.0	33.7±1.0
	Female	8.6±0.8	14.9±0.8	46.6±1.2	54.6±5.0	17.5±1.6	32.2±2.0
White spotted-agouti	Male	8.6±1.0*	15.7±0.6†	47.9±1.7†	56.3±5.7	18.5±1.8	32.9±0.7
	Female	7.7±0.6	14.9±0.6	45.7±0.8	59.5±3.9	19.5±1.5	32.7±1.2
Albino	Male	8.6±0.9	15.7±0.6*	47.9±2.0†	56.1±5.8	18.5±1.9	32.9±1.6
	Female	8.0±0.5	14.9±0.7	44.7±1.5	56.2±2.4	18.9±1.8	33.5±2.2
Black	Male	8.2±0.7	15.1±0.4*	45.2±1.1	55.7±5.3	18.6±1.6	33.8±1.6
	Female	8.0±0.9	14.6±0.5	45.8±2.3	57.9±9.0	18.4±2.4	32.0±1.6
White spotted-black	Male	8.2±0.7	15.6±1.1*	45.1±1.4	55.4±5.0	19.1±1.8	34.6±1.6
	Female	7.9±0.4	14.6±0.8	45.1±1.8	57.1±3.3	18.6±1.2	32.5±0.9

difference between male and female n =10, mean±standard deviation

* t test, $p<0.05$

† t test, $p<0.01$

Table 2 Incidence of erythrocytes with reticulum, basophilic stippling, polychromasia and Howell-Jolly body in each coat color mutant of Mongolian gerbils

Coat color	Sex	Reticulocytes (/1,000 RBC)	Basophilic stippled cells (/1,000 RBC)	Polychromatophilic cells (/1,000 RBC)	Cells with Howell-Jolly bodies (/1,000 RBC)
Agouti	Male	27.4±11.9	8.6±0.4	6.5±0.3	1.5±0.7
	Female	28.1± 6.3	12.8±2.9	6.5±2.2	1.0±1.0
White spotted-agouti	Male	37.9±10.2	6.1±1.8	3.6±0.6	0.6±0.5
	Female	31.2±13.0	9.0±3.7	5.9±2.0	0.6±0.5
Albino	Male	37.4± 7.8	14.0±4.6	6.8±1.8	1.1±1.0
	Female	34.6±13.1	11.0±3.1	5.3±2.7	1.5±1.2
Black	Male	38.0± 9.0	14.4±1.7	9.0±1.9	1.3±1.2
	Female	30.2± 5.4	12.5±4.0	6.7±2.6	0.7±0.7
White spotted-black	Male	34.5± 7.7	13.3±4.5	6.9±2.8	1.3±0.9
	Female	36.0± 8.3	14.3±4.2	6.5±2.6	1.1±0.7

n=10, mean±standard deviation

RESULT

The mean values and standard deviations for total erythrocyte counts as well as related data in the peripheral blood of each color mutant of Mongolian gerbils are presented in Table 1. The average erythrocyte counts for male gerbils were always higher than those for females in all color gerbils; the average counts for males were $(8.2\text{-}8.8)\times10^6/\mu l$ as compared to $(7.7\text{-}8.6)\times10^6/\mu l$ for females. However, the significant difference between sexes was proved only in the white-spotted agouti type of gerbil among all coat color gerbils. The agouti type of male was observed to have the highest value of total erythrocyte counts, but there is no significant difference among all coat color gerbils. No coat color-related significant difference in the hemoglobin content was observed, but sex-related difference was also evident in all coat color gerbils. The same tendency was seen in the hematocrit values of three coat color gerbils.

Table 2 shows the incidence of erythrocytes with cytologic features in each coat color mutant of gerbils. The average reticulocyte counts were (27.4-38.0)/1,000 erythrocytes (RBC) for males and (28.1-36.0)/1,000 RBC for females and sex-related difference was not seen in this case. That value of reticulocyte was lowest in the agouti type conversely to total erythrocyte counts. Polychromatophilic and stippled red blood cells were demonstrated frequently in all blood films in all coat color gerbils (Photos. 1, 2). The average counts of basophilic stippled cells were (6.1-14.4)/1,000 RBC for both males and females and those of polychromatophilic cells were (3.6-9.0)/1,000 RBC. It was observed in all blood films that one red blood cell had both polychromasia and stippling (Photo. 1). The erythrocytes with Howell-Jolly bodies were also observed (0.5-1.5)/1,000 RBC in all color gerbils (Photos. 2, 3).

Total numbers of circulating leukocytes and differentials in each coat color mutant of Mongolian gerbils are shown in Table 3. Total circulating leukocytes were $(12.4\text{-}15.6)\times10^3/\mu l$ for males and $(14.2\text{-}16.6)\times10^3/\mu l$ for females. In the circulating leukocyte counts, both sex-related and coat color-related differences were not seen in this study. The lymphocyte was observed most prevalent in all coat color gerbils of both sexes. Although the number of basophils and eosinophils accounted for only small propotion in the peripheral blood, it was

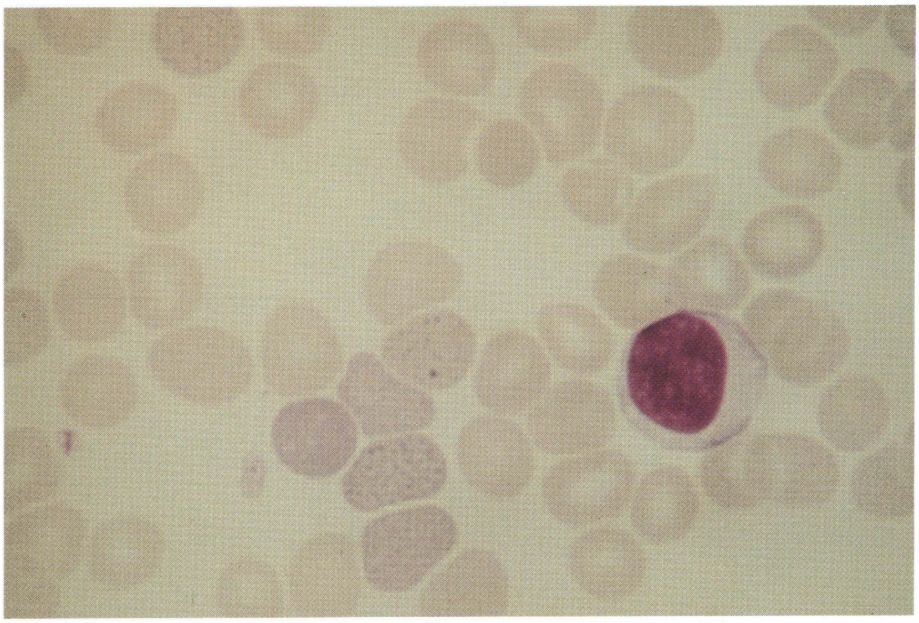

Photo. 1 Polychromatophilic and basophilic stippled erythrocytes near a lymphocyte on a blood film of a Mongolian gerbil. May-Grunwald and Giemsa stain.

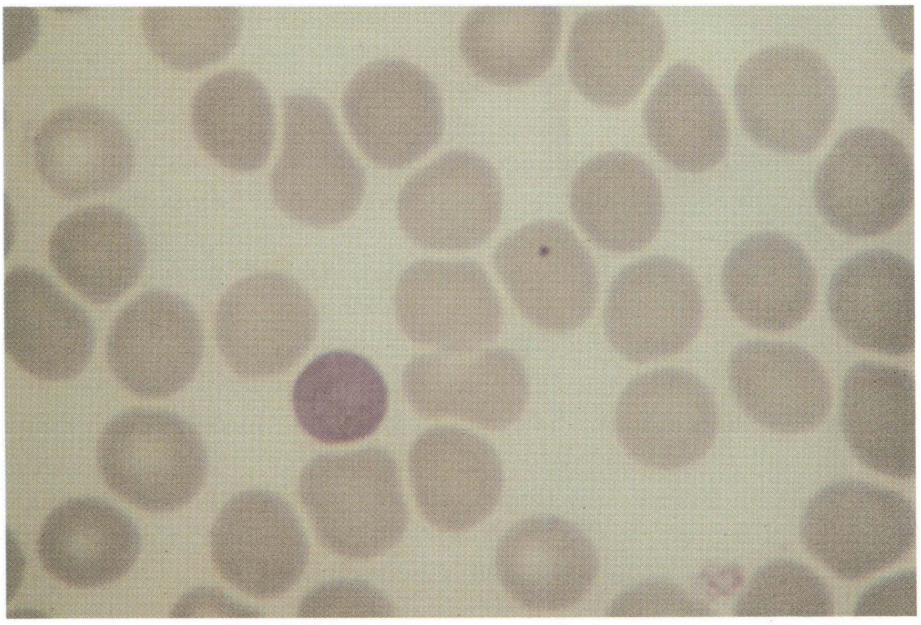

Photo. 2 An erythrocyte with polychromasia and with a Howell-Jolly body on a blood film of a Mongolian gerbil. May-Grunwald and Giemsa stain.

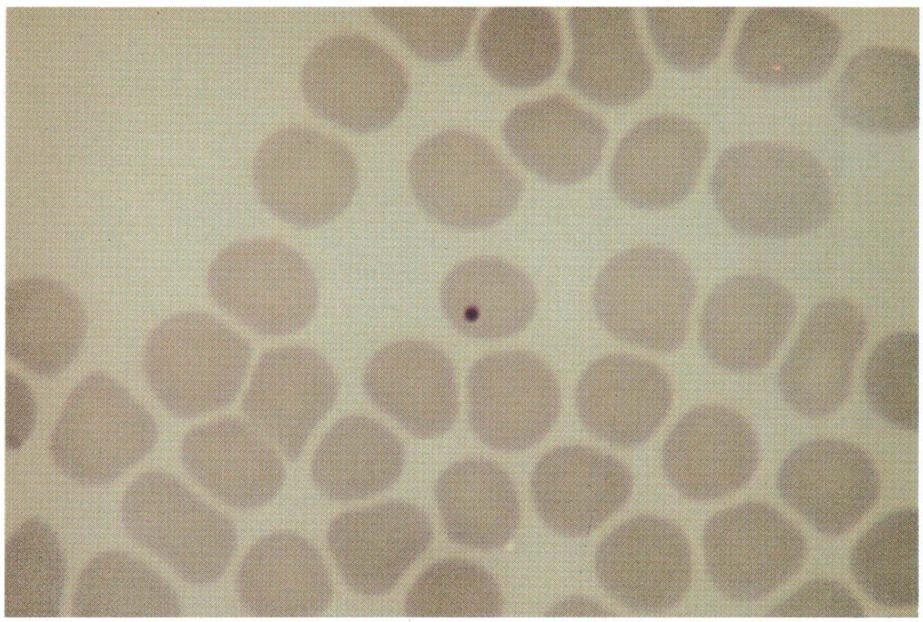

Photo. 3 A Howell-Jolly body in an erythrocyte on a blood film of a Mongolian gerbil. May-Grunwald and Giemsa stain.

Table 3 Total leukocyte counts and differentials in each coat color mutant of Mongolian gerbils

Coat color	Sex	Total leukocytes ($\times 10^3/\mu l$)	Basophils (%)	Eosinophils (%)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)
Agouti	Male	13.8±3.6	0.3±0.5	0.8±0.6	25.0±6.5	70.6±7.0	3.3±1.4
	Female	14.8±3.8	0.2±0.4	0.3±0.5	23.8±6.9	72.6±7.0	3.1±1.6
White spotted-agouti	Male	15.6±4.9	0.3±0.5	1.2±0.8	23.7±9.9	70.9±9.2	3.9±1.2
	Female	15.9±5.8	0.1±0.3	0.8±0.6	23.6±5.3	73.6±5.6	1.9±0.9
Albino	Male	15.5±3.6	0.4±0.5	1.3±0.7	20.3±3.6	75.5±3.8	2.5±1.6
	Female	14.8±4.8	0.2±0.6	1.5±1.5	21.8±4.0	74.5±4.2	2.0±1.4
Black	Male	12.4±2.6	0.2±0.4	0.4±0.5	30.0±9.3	67.9±8.8	1.5±0.7
	Female	16.6±5.6	0.1±0.3	0.4±0.7	26.0±8.6	71.1±8.7	2.4±1.3
White spotted-black	Male	15.6±2.9	0.1±0.3	0.1±0.3	30.7±6.9	66.8±6.7	2.3±0.8
	Female	14.2±4.4	0.1±0.3	0.2±0.4	32.5±9.6	64.8±9.2	2.4±1.4

n=10, mean±standard deviation

confirmed that these cells were present in the all blood films in all color mutants of gerbils.

Discussion

In this paper, we investigated hematological profiles in 4 kinds of the coat color mutants of gerbils and compared them to the agouti type. There was only one past report on a hamatological characteristics in a coat color mutant of a gerbil (Waring *et al.*, 1978). They noted that total erythrocyte counts of spotted gerbils were significantly lower than those of

an agouti type indicating the presence of a slight anemia. From their description about the location of white spots and about breeding data of the mutant, it seemed that their spotted gerbil was the same mutant as our white spotted-agouti type. But, their result of erythrocyte counts was different from ours. The total erythrocyte counts of agouti type were observed also higher than the other coat color gerbils in our experiment, however, there was no significant difference and an anemia of white spotted-agouti gerbils was not observed. The total erythrocyte counts of agouti type in our experiment were comparable to those of the other investigators (Ruhren, 1965; Mays, 1969; Dillon and Glomski, 1975a; Termer and Glomski, 1978), but, those of Waring *et al.* (1978) were about a half level of them.

As their own particular feature of the blood of the Mongolian gerbil, it was known the frequent occurrence of polychromasia and basophilic stippling in circulating erythrocytes throughout the life span of agouti gerbils (Ruhren, 1965; Dillon and Glomski, 1975a; Smith *et al.*, 1976; Termer and Glomski, 1978). In this study, it was proved that those cells were also present in the blood of the coat color mutants of gerbils as the agouti type. Those cytologic features were considered a property of immaturity of erythrocytes (Smith *et al.*, 1976). High incidence of those cells reflected that immature red cells emerged to the peripheral blood because of a brief life span of an erythrocyte (Dillon and Glomski, 1975b). Although gerbils have been known to have very wide sensitivity to various parasites compared with the other laboratory rodents, the cause of a high sensitivity was not investigated. Since a marked difference of the gerbil from the other rodents was the continuous presence of stippled and polychromatophilic cells in the adult gerbils as far as a hematological measurement, the brief span of blood cells may participate in a wide sensitivity of this animal.

Certain numbers of reticulocytes were also observed. Although there was no statistical difference, it was lowest in the agouti gerbil conversely to total erythrocyte counts.

As to sex dimorphism in total erythrocyte counts and related values, there have been some discussions. Male predominance of hemoglobin concentration, hematocrit, mean corpuscular volume or mean corpuscular hemoglobin was reported by some investigators (Mays, 1969; Dillon and Glomski, 1975a; Termer and Glomski, 1978). In this experiment, only hemoglobin concentration showed statistical sex-related difference in all coat color gerbils.

The possible existence of quantitative sex-related difference in the leukocytes has been a feature described for this rodent (Mays, 1969; Dillon and Glomski, 1975a). But, in this experiment, sex-related difference was not observed in total leukocyte counts in all coat color gerbils. This finding supported the result of the study by Termer and Glomski (1978). The predominant cells in the circulating leukocytes of the gerbils were lymphocytes, a characteristic common to other laboratory rodents (Wolford, 1986). On the other hand, a presence of basophils in peripheral blood was a specific characteristics of the gerbil and it was not observed in mice or rats. At the point of existence of both basophils in peripheral blood and mast cells in connective tissues or mucosal tunics of the gerbil (data not shown), this animal can be said more resemble to human being than mice or rats. The gerbil may be useful for analysis of the infection kinetics to various parasites, because the animal has both mast cells in connective tissues and a comparatively larger amount of basophils in peripheral blood.

Finally, this study confirmed that the coat color mutants had the same unique hematological characteristics as the wild-colored gerbil. Further detail hematological and other physiological experiment concerning this animal will help us to know why the gerbil is

very sensitive to various parasite infections.

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フィラリア感染好適宿主としてのMongolian gerbilの基礎的検討
—血液学的特性に関する被毛色間の比較—

清水　眞澄[1]・七戸　和博[1]・月舘　説子[2]・藤田紘一郎[2]

Mongolian gerbil（スナネズミ）の被毛色突然変異体（white spotted-agouti, albino, black, white spotted-black）の血液学的特性について，野生色（agouti）のものと比較検討した。赤血球数は統計学的な有意差はなかったが，agoutiが最も多かった。他の実験動物には見られないスナネズミ独特の性質として，多染性や好塩基斑点を持つ赤血球が多いことが知られているが，それらが被毛色突然変異体の末梢血中にも多数出現することが観察された。赤血球関連の検査値においてすべての被毛色で有意な性差が見られたのは，ヘモグロビン濃度のみであった。白血球数には性差が確認されなかった。白血球中で最も多くの割合を占めたのは，各被毛色ともリンパ球であった。すべての被毛色の各個体の末梢血中に好塩基球の存在が確認された。

以上のように被毛色突然変異体は，agoutiと同様にスナネズミ独自の血液学的性質を持っていることがわかった。

1　日本医科大学実験動物管理室（〒113 東京都文京区千駄木1－1－5）
2　東京医科歯科大学医学部医動物学教室（〒113 東京都文京区湯島1－5－45）

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CLINICAL STUDY OF MEBENDAZOLE THERAPY FOR STRONGYLOIDIASIS

KOUSHIN SHIKIYA[1], HIROYUKI UECHI[2], ATSUSHI SAITO[1] AND RYUJI ASATO[3]

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Abstract: A study was conducted of 225 patients in Okinawa Prefecture, Japan, to evaluate mebendazole (MBZ) therapy for *Strongyloides stercoralis*. Various schedules were used with the following results: 1) The eradication rates at 12 months after a single course of MBZ therapy (100 mg of a powder twice a day orally for 28 days) and a combination therapy (thiabendazole 500 mg powder form three times daily for 5 days followed by 100 mg of MBZ in powder form twice a day for 9 days, repeated once) were 85.0% (17 of 20 patients) and 100% (17 of 17 patients), respectively. 2) The eradication rates at 3-9 months after using MBZ alone in varying dosages were as follows: (a) 100 mg in powder form twice a day for 5 days, repeated at 1, 3 and 4 weeks following: 80.0% (24/30); (b) 100 mg in powder form twice daily for 5 days repeated at 1 and 3 weeks following: 83.3% (5/6); (c) 100 mg in powder form twice a day for 4 days repeated at 1, 3 and 4 weeks following: 96.0% (24/25); (d) a 100 mg tablet twice a day for 4 days repeated at 1, 3 and 4 weeks following: 90.6% (29/32). 3) The eradication rate at 24 days after a 2-course treatment (a 100 mg tablet twice daily for 4 days repeated 1 week later) was 93.8% (15/16). Although the 2-course treatment was effective, further study seems necessary as the follow-up time was too short and the number of patients was too few.

INTRODUCTION

The infection rate of *Strongyloides stercoralis* (*S. stercoralis*) in Okinawa Prefecture, Japan, was found to be less than 2% of the population when determined by the traditional methods of direct smear, filter paper culture and formalin-ether concentration. However, when stool samples were tested by a new technique, the agar plate method, the infection rate was found to be 8.1% (Arakaki *et al.*, 1988, 1990; Asato *et al.*, 1989). We attempted to evaluate the efficacy of treatment when using mebendazole (MBZ) alone and in combination with thiabendazole. However, liver disfunction developed when MBZ was used alone for a 28 day period and side effects also occurred in the combination therapy (Shikiya *et al.*, 1990a). We therefore reduced the period of administration of MBZ and repeated the dosage after intervals, but abnormal liver function was still noted (Shikiya *et al.*, 1990b, c). We finally reduced the dosage to a 4-day period that was repeated 1 week later. We are now

1 First Department of Internal Medicine, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-01, Japan
2 Okinawa Prefectural Nago Hospital, 1617-1 Nago, Nago, Okinawa 905, Japan
3 Okinawa Prefectural Institute of Public Health, 2085 Ozato, Ozato, Okinawa 901-12, Japan

reporting the results of the therapy with these various schedules.

Materials and Methods

Patients

A total of 225 Okinawan patients, 146 males and 79 females, with strongyloidiasis diagnosed by using the agar-plate culture method were included in this study.

Administration Schedules (Figure 1) (Table 1)

The patients were divided into 7 groups and different schedules of MBZ or a combination of MBZ and thiabendazole were administered as follows:

Group 1: 100 mg of MBZ in powder form was given twice a day orally for 28 days.

Group 2: thiabendazole powder (500 mg) was given three times daily for 5 days and for the following 9 days 100 mg of MBZ powder was given twice a day. This combination therapy was administered twice in succession.

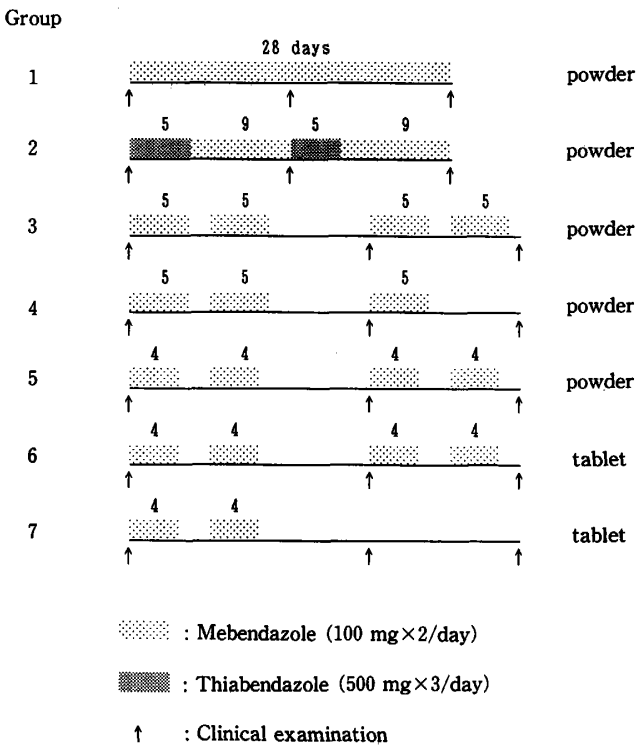

Figure 1 Administration schedules.

Group 3: MBZ powder was given twice a day for 5 days and was then repeated 1, 3 and 4 weeks later.

Group 4: MBZ powder was administered twice daily for 5 days and repeated 1 and 3 weeks later.

Group 5: MBZ powder was given twice a day for 4 days and repeated 1, 3 and 4 weeks later.

Group 6: MBZ in tablet form was given twice a day for 4 days and repeated 1, 3 and 4 weeks later.

Table 1 Sex and age distribution

Group		1	2	3	4	5	6	7	Total
Sex	Male	22 (66.7)	14 (53.8)	35 (74.5)	12 (92.3)	28 (62.2)	24 (53.3)	11 (68.8)	146 (64.9)
	Female	11 (33.3)	12 (46.2)	12 (25.5)	1 (7.7)	17 (37.8)	21 (46.7)	5 (31.2)	79 (35.1)
	Total	33(100.0)	26(100.0)	47(100.0)	13(100.0)	45(100.0)	45(100.0)	16(100.0)	225(100.0)
Age (yr)	40-49	6 (18.2)	3 (11.5)	5 (10.6)	0 (0.0)	3 (6.7)	2 (4.4)	1 (6.3)	20 (8.9)
	50-59	2 (6.1)	8 (30.8)	10 (21.3)	2 (15.4)	14 (31.1)	10 (22.2)	2 (18.5)	48 (21.3)
	60-69	13 (39.4)	11 (42.3)	11 (23.4)	7 (13.4)	17 (37.8)	12 (26.7)	7 (43.8)	78 (34.7)
	70-79	11 (33.3)	2 (7.7)	13 (27.7)	1 (7.7)	10 (22.2)	17 (32.8)	5 (31.3)	59 (12.9)
	80-89	1 (3.0)	2 (7.7)	8 (17.0)	3 (23.1)	1 (2.2)	4 (8.9)	1 (6.3)	20 (8.9)

No. of patients, (%)

Group 7: MBZ in tablet form was given twice a day for 4 days and repeated 1 week later.

Parasitological Examination

Fresh fecal samples were examined by using the agar-plate culture method. The first post-treatment fecal examination was done between 2 to 24 days after the last day of treatment in all groups. A second post-treatment fecal examination was done between 3 to 9 months after the last day of treatment in groups 1-6. In groups 1 and 2 a third post-treatment fecal examination was done 12 months after the last day of treatment.

Clinical Examination

Clinical examinations were done before, during and after treatment. The examinations included a clinical history, a complete blood count, serum-chemistry studies, serological studies and urinalysis. Serum-chemistry studies included liver function tests as follows: total bilirubin, gultamic oxaloacetic transaminase (GOT), gultamic pyruvic transaminase (GPT) and alkaline phosphatase (ALP).

RESULTS

Antiparasitic Effect (Table 2)

Patients who did not complete the course of treatment or who failed to submit post-treatment fecal samples were excluded.

The parasitological eradication rates at 12 months after treatment were 85.0% in group 1 and 100% in group 2. The parasitological eradication rates in groups 3 to 6 at 3-9 months after treatment were from 80.0 to 96.0%. The eradication rate in group 7 at 24 days after treatment was 93.8%.

Side Effects (Table 3)

Patients who did not complete the course of treatment were excluded.

Thirteen patients (54.2%) in the thiabendazole-MBZ group complained of side effects and therapy was discontinued for two patients because of severe nausea and dizziness which

Table 2 Eradication rates of *S. stercoralis* according to administration schedules

Group	during treatment	after treatment		
		2-24 days	3-9 months	12 months
1	90.3 (28/31)	81.8 (18/22)	77.3 (17/22)	85.0 (17/20)
2	96.0 (24/25)	100.0 (22/22)	100.0 (22/22)	100.0 (17/17)
3	89.1 (41/46)	100.0 (46/46)	80.0 (24/30)	—
4	84.6 (11/13)	92.3 (12/13)	83.3 (5/6)	
5	97.8 (44/45)	100.0 (45/45)	96.0 (24/25)	—
6	93.0 (40/43)	97.7 (42/43)	90.6 (29/32)	—
7	—	93.8 (15/16)	—	—

%, (No. of negative/No. of subjects)

Table 3 Incidence of side effects

Group (No. of subjects)	1 (27)	2 (24)	3 (47)	4 (13)	5 (45)	6 (45)	7 (16)
Heartburn	—	—	—	—	—	4.4 (2)	—
Nausea	—	20.8 (5)	—	—	—	2.2 (1)	—
Vomiting	—	4.2 (1)	—	—	—	—	—
Abdominal pain, Borborygmus	7.4 (2)	4.2 (1)	6.4 (3)	—	—	4.4 (2)	—
Diarrhea	3.7 (1)	—	10.6 (5)	—	2.2 (1)	4.4 (2)	6.3 (1)
Constipation	11.1 (3)	4.2 (1)	—	—	6.7 (3)	4.4 (2)	—
Anorexia	3.7 (1)	12.5 (3)	4.3 (2)	—	2.2 (1)	—	—
Headache	11.1 (3)	16.7 (4)	2.1 (1)	—	—	—	6.3 (1)
Dizziness or vertigo	—	8.3 (2)	2.1 (1)	—	6.7 (3)	4.4 (2)	—
Numbness of extremities	3.7 (1)	4.2 (1)	6.4 (3)	—	2.2 (1)	4.4 (2)	—
Blurring of vision	—	—	2.1 (1)	—	—	—	—
Fever	3.7 (1)	4.2 (1)	—	—	2.2 (1)	2.2 (1)	—
General fatigue	7.4 (2)	8.3 (2)	2.1 (1)	—	4.4 (2)	2.2 (1)	—
Itching	—	8.3 (2)	2.1 (1)	—	4.4 (2)	6.6 (3)	—
Exanthema	—	12.5 (3)	—	—	4.4 (2)	4.4 (2)	—
Arthralgia, Lumbago	3.7 (1)	8.3 (2)	8.5 (4)	—	4.4 (2)	—	—
Urinary frequency	7.4 (2)	—	2.1 (1)	—	4.4 (2)	—	—
Others	7.4 (2)	8.3 (2)	—	—	—	—	—
Total	40.7(11)	54.2(13)	34.0(16)	0.0 (0)	31.1(14)	31.1(14)	12.5 (2)

%, (No. of patients)

Table 4 Incidence of liver disfunction

Group (No. of subjects)	1 (28)	2 (22)	3 (47)	4 (13)	5 (45)	6 (45)	7 (16)
GOT ↑	—	—	4.3 (2)	—	—	4.4 (2)	6.3 (1)
GPT ↑	7.1 (2)	9.1 (2)	—	7.7 (1)	—	6.7 (3)	6.3 (1)
ALP ↑	—	—	2.1 (1)	—	—	—	—
T.bil ↑	10.7 (3)	4.5 (1)	2.1 (1)	—	—	2.2 (1)	—
GOT, GPT ↑	28.6 (8)	27.3 (6)	29.8(14)	23.1 (3)	48.9(22)	51.1(23)	12.5 (2)
GOT, T.bil ↑	—	—	—	—	2.2 (1)	—	—
GOT, GPT, ALP ↑	—	—	6.4 (3)	—	2.2 (1)	2.2 (1)	—
GOT, GPT, T.bil ↑	17.9 (5)	4.5 (1)	—	—	2.2 (1)	2.2 (1)	—
GOT, GPT, T.bil, ALP ↑	7.1 (2)	4.5 (1)	6.4 (3)	—	2.2 (1)	—	—
Total	71.4(20)	50.0(11)	51.1(24)	30.8 (4)	57.8(26)	68.9(31)	25.0 (4)

%, (No. of patients)

developed 2 weeks after treatment was begun.

Side effects were observed in 40.7% of group 1, 34.0% of group 3 and 0% of group 4. Incidence of side effects was not significantly different between group 5 (powder) and group 6 (tablet). Side effects were observed in 12.5% of group 7. No special treatment was given

for the side effects.

Liver Disfunction

Patients who did not complete the course of treatment were excluded.

Table 4 shows the incidence of abnormal liver function (elevation of serum total bilirubin and/or GOT and/or GPT and/or ALP) which occurred during treatment and from 2 to 24 days after treatment.

Liver disfunction was observed in 71.4% of group 1 and in 50% of group 2 but only one patient had malaise and jaundice.

In group 1, one patient had a total bilirubin of 6.9 mg/d*l* (normal: 0.3-0.8 mg/d*l*), a GOT of 898 IU/L (normal: 10-32 IU/L) and a GPT of 1,215 IU/L (normal: 7-26 IU/L) at 1 week after the last dosage; her only symptom was itching. Of the 20 patients in this group who experienced liver disfunction, 13 patients received a lymphocyte stimulation test and were negative for MBZ. None of the patients with liver disfunction had allergic reactions such as skin rash, fever and eosinophilia.

In group 2, one patient had a GOT of 77 IU/L and a GPT of 174 IU/L at 1 week after the last treatment but had no symptoms.

The abnormal liver function of all patients in both groups 1 and 2 showed a return to normal in subsequent tests.

Since these liver disfunctions seemed to be dose-dependent, we reduced the length of periods of administration of MBZ and repeated it after intervals. But because 51.1% of the patients in group 3 displayed a liver disfunction, therapy for the 13 patients in group 4 was interrupted before the 4th course.

Incidence of liver disfunction was not significantly different between group 5 (powder) and group 6 (tablet). Although the GOT after the second course in group 5 (30.3±32.2 IU/L) was significantly higher than that in group 6 (22.9±12.7 IU/L), ($p<0.05$) according to the Wilcoxan 2-sample test, there were no significant differences in the GOT of the two groups after the fourth course (118.0±146.2 vs. 84.1±94.4) and in the GPT after either the second (28.0±63.8 vs. 19.1±16.6) or fourth course (137.0±162.2 vs. 119.0±161.1). The GOT and GPT after the second course (GOT: 30.3±32.2, 22.9±12.7; GPT: 28.0±63.8, 19.1±16.6) were significantly lower than after the fourth course (GOT: 118.0±146.2, 84.1±94.4; GPT: 137.0±162.2, 119.0±161.1) in both groups ($p<0.05$). The incidence of liver disfunction in group 7 was lower than in the other groups.

Discussion

We decided to use MBZ for a 28 day period for strongyloidiasis because MBZ is effective against the parasite (Mraval *et al.*, 1983; Wilson *et al.*, 1983), because the drug's side effects are mild and of low frequency (Bekhti *et al.*, 1977; Keystone *et al.*, 1979; Wilson *et al.*, 1978), and because *S. stercoralis* has a 17 to 27-days larval development period in the intestinal tissue (Grove, 1989; Mraval *et al.*, 1983). However, a high incidence of liver disfunction had developed in our previous study and because the liver disfunction seemed to be dose-dependent (Shikiya *et al.*, 1990a), we reduced the administration period of MBZ and repeated it after intervals. Although the incidence of liver disfunction tended to decrease, liver disfunction was still observed in groups 3 and 4 (Shikiya *et al.*, 1990b). Since *S. stercoralis* is located

mainly in the small intestine, we used powdered MBZ in order to more effectively reach the parasites. Although we considered that a powder would be easily absorbed and therefore liver disfunction would occur in high incidence, the incidence of liver disfunction and other side effects, as well as the eradication rate of the parasite were similar for both the powder and tablet forms of the drug. Since a significant difference was observed in liver disfunction when examined between the second course and the fourth course in groups 5 and 6 (Shikiya *et al.*, 1990c), we finally reduced the period of administration to 4 days (a 100 mg tablet twice a day) and repeated it only once a week later.

As high doses of MBZ produces liver disfunction (Berhti *et al.*, 1987; Davis *et al.*, 1986), many investigators have tried low-dose MBZ treatment. Abadi (1985) reported that a single 500 mg dose of MBZ was not effective for a strongyloidiasis patient. Goldsmid (1974) using 100 mg of MBZ twice a day for 3 days, reported a cure rate at 7-10 days after treatment of 88% (7 of 8 patients). Musgrave *et al.*, (1979) tried 100 mg of MBZ twice a day for 4 days and described a cure rate at 10-20 days after therapy of 67% (14 of 21 patients).

Although our study showed that one 100 mg MBZ tablet twice a day for 4 days with a second course of treatment one week later resulted in a 93.8% eradication rate (15 of 16 patients) and in a 25% incidence of liver disfunction (4 of 16 patients), further study seems to be necessary as, in most studies to date, the follow-up time has been too short and the number of patients too few.

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Mebendazoleによる糞線虫症治療に関する臨床的研究

志喜屋孝伸[1]・上地　博之[2]・斎藤　　厚[1]・安里　龍二[3]

Mebendazole (MBZ)を用いて225例の糞線虫症患者を治療し，下記の結果を得た。なお，MBZ (100 mg) は1回1錠を1日2回経口投与し，[　] 内は肝機能障害出現頻度を示す。

1．MBZ（粉末）28日間投与の治療1年後の駆虫率：85.0%（20例中17例），[71.4%]

2．Thiabendazole（粉末）を1回1錠，1日3回の5日間投与後，MBZ（粉末）を9日間投与し，それを2コース繰り返す方法の治療1年後の駆虫率：100%（17/17），[50%]

3．下記の投与法による3-9カ月後の駆虫率は以下のとおりであった。

1）MBZ（粉末）5日間の4コース投与：80.0%（24/30），[51.5%]

2）MBZ（粉末）5日間の3コース投与：83.3%（5/6），[30.8%]

3）MBZ（粉末）4日間の4コース投与：96.0%（24/25），[57.8%]

4）MBZ（錠剤）4日間の4コース投与：90.6%（29/32），[68.9%]

4．MBZ（錠剤）4日間の2コース投与24日後の駆虫率：93.8%（15/16），[25%]

以上のように，MBZ 4日間の2コース投与では肝障害の出現頻度が低くなり，駆虫率は高いが症例数が少なく，観察期間が短いのでさらに検討が必要であると思われる。

1　琉球大学医学部第一内科（〒903-01 沖縄県中頭郡西原町上原207）

2　沖縄県立名護病院（〒905 名護市名護1617-1）

3　沖縄県公害衛生研究所（〒901-12 沖縄県島尻郡大里村大里高嶺原2085）

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MANAGEMENT OF *PNEUMOCYSTIS CARINII* PNEUMONIA IN PATIENTS WITH CONVENTIONALLY CAUSED IMMUNE SUPPRESSION

TATSUYA KATSUMATA, SHIGERU KOHNO, HIRONOBU KOGA,
YUKO YOSHITOMI, HARUKO MATSUDA, KOTARO MITSUTAKE,
YASUHITO HIGASHIYAMA, YOSHITSUGU MIYAZAKI AND KOHEI HARA

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Abstract: Ten non-AIDS patients with *Pneumocystis carinii* pneumonia were studied. While the 2 patients with adult T cell leukemia had longer prodromes, the other 8 patients had acute onset. At presentation a chest radiograph revealed an abnormal bilateral diffuse shadow in all cases. In 8 patients, diagnostic material was obtained by transbronchial lung biopsy and/or bronchoalveolar lavage, and in 2 patients at postmortem. At the time of diagnosis the serum lactate dehydrogenase value was much higher than prior to the acute illness, and the $AaDO_2$ gradient was highly increased: These appear to be useful as markers for an initial diagnosis. Other opportunistic organisms were isolated in 5 patients. The concomitant use of pentamidine and cotrimoxazole was relatively well tolerated, but with a high incidence of treatment failure. Corticosteroids appeared to be effective as an adjunctive therapy.

INTRODUCTION

Pneumocystis carinii pneumonia (PCP) is the most common opportunistic infection which occurs and is a significant cause of death among patients with acquired immunodeficiency syndrome (AIDS). In Japan, the prevalence of AIDS is still low, and PCP is an opportunistic infection seen most frequently in association with certain malignancies or immunosuppressive therapy (Macfarlane and Finch, 1985; Engelberg *et al.*, 1984).

Diagnosis of PCP usually requires invasive methods, such as fiber optic bronchoscopy, although various noninvasive tests, such as chest radiographs and gallium scans, have been used to diagnose this infection (Oka *et al.*, 1985; Cordonnier *et al.*, 1984). Recent studies have shown that the serum LDH value and $P(A\text{-}a)O_2$ gradient might be useful as markers for diagnosis and prognosis of PCP in cases with AIDS (Zaman and White, 1988; Garay and Greene, 1989).

Several studies have demonstrated that pentamidine has a greater incidence of significant adverse reactions, and is no more effective in the treatment of PCP, than cotrimoxazole.

Second Department of Internal Medicine, Nagasaki University School of Medicine
Correspondence to: Tatsuya Katumata MD, PhD, Second Department of Internal Medicine, Nagasaki University School of Medicine, 7-1 Sakamoto-machi, Nagasaki 852, Japan

Table 1 Details of patients with pneumocystis pneumonia

Patient	Sex	Age (years)	Underlying disease	Duration of immunosuppressive therapy	Clinical prodrome and duration	
1	M	63	malignant lymphoma	3 months	cough, fever	3 days
2	M	78	malignant lymphoma	2.5 months	cough,	3 days
3	M	69	adult T cell leukemia	1 week	cough, fever, dyspnea	2 weeks
4	M	47	adult T cell leukemia	2 weeks	cough, fever, dyspnea	3 weeks
5	F	73	lung cancer	4 months	dyspnea	2 days
6	M	32	renal transplantation	10 months	cough, dyspnea	3 days
7	F	53	nephrotic syndrome	2 months	asymptomatic	—
8	M	75	sarcoidosis	2 months	asymptomatic	—
9	M	51	dermatomyositis	7 months	dyspnea	2 days
10	F	44	systemic lupus erythematodes	18 months	asymptomatic	—
Average		58.5 ±14.6		4.9 ±5.2 months		6.9 ±7.0 days

Pentamidine has therefore been used in patients with PCP who have failed to respond to cotrimoxazole or who have sustained adverse reactions to this drug combination. PCP cases from conventionally caused immune suppression are reported to be more fulminant, less likely to relapse, with fewer adverse effects and a greater incidence of treatment failure than those from AIDS (Drake *et al.*, 1985; Salamone and Cunha, 1988; Pearson and Hewlett, 1985; Kluge *et al.*, 1978; Kovacs *et al.*, 1984; Sattler *et al.*, 1988). Therefore in the non-AIDS population, the concomitant use of pentamidine and cotrimoxazole has yet to be examined.

We present our experience in fulminant cases of PCP from conventionally caused immunosuppression admitted to Nagasaki University Hospital during the period 1976-1989.

Patients and Methods

The hospital charts of patients with confirmed PCP between 1976 to 1989 were reviewed. Only those patients with microbiologically documented disease were included. The following data was recorded in all cases: age, sex, underlying disease, use of immunosuppressive drugs, nature and duration of the problem leading to diagnosis, arterial blood gas with calculation of the $P(A\text{-}a)O_2$ gradient, lactate dehydrogenase (LDH) value, chest radiograph findings, treatment, and outcome. Diagnostic procedures included fiber optic bronchoscopy with bronchoalveolar lavage (BAL), transbronchial lung biopsy (TBLB), and postmortem examination. Fiber optic bronchoscopy was performed under local anaesthesia with parenteral sedation. The bronchoscope was wedged into the subsegmental bronchus in the main region of radiographic abnormality. Bronchoalveolar lavage was performed using at least two

Table 2 Diagnostic information

Patient	LDH(IU/L)			PaO_2 (torr) at diagnosis	$P(A-a)O_2$ (torr) at diagnosis	Initial chest radiograph	Definitive diagnosis by
	1-2 months prior to diagnosis	At diagnosis	Increase				
1	478	896	418	38.5(RA)	72.0	bilateral reticular	TBLB
2	587	1,132	545	44.5(RA)	63.5	bilateral reticular and infiltrates	BAL
3	472	663	191	58.2 ($O_2$3l/min)	122	bilateral reticular and infiltrates	BAL
4	—	1,170	—	54.4(RA)	57.0	bilateral reticular and infiltrates	BAL TBLB
5	400	734	334	32.6(RA)	75.6	bilateral reticular	BAL TBLB
6	322	790	468	59.7(RA)	49.0	bilateral reticular	BAL
7	346	1,455	1,109	31.9(RA)	70.1	diffuse ground glass	BAL
8	309	1,723	1,414	32.6(RA)	82.5	bilateral reticular	BAL
9	400	1,173	773	40.0(RA)	74.6	bilateral reticular and infiltrates	post mortem
10	353	589	236	115 ($O_2$11/min)	26.3	bilateral reticular	post mortem
Average	407 ±85.1	1,030 ±347	610 ±391		69.3 ±23.4		

*RA: room air

20 m*l* aliquots of sterile normal saline. Transbronchial lung biopsy was performed under the guidance of fluoroscopy. The fluid obtained by lavage was centrifuged at 1,500 rpm for 5 min. The deposit was spread onto several glass microscope slides and stained to reveal *Pneumocystis carinii* cysts using Gomori's methenamine silver stain. All specimens were also stained by Gram's method. Routine aerobic and anaerobic bacterial cultures were performed on all samples, as were cultures for mycobacteria and fungi. All patients, except for one patient diagnosed as PCP at postmortem examination, were treated with a high dosage (8-16 g/day) of cotrimoxazole given orally together with 4 mg/kg/day of pentamidine isethionate given either intravenously or intramuscularly. Two of the patients were treated with 600 mg/day of pentamidine isethionate by inhalation and intravenous corticosteroid adjunctively.

Results

Ten patients met the criteria for inclusion in this study. There were 7 males and 3 females, with ages ranging from 32 to 78. Four patients had an underlying hematological disorder, 2 had renal disease, 2 had collagen disease, 1 had lung cancer, and 1 had sarcoidosis.

Table 3 Therapy and outcome

Patient	Other opportunistic organism	Therapy	Adverse reaction	Intravenous Corticosteroid therapy	Mechanical ventilation	Outcome of PCP
1	(−)	Cotrimoxazole (po) Pentamidine (im)	(−)	(−)	(−)	poor
2	(−)	Cotrimoxazole (po) Pentamidine (im)	(−)	(−)	(−)	poor
3	(−)	Cotrimoxazole (po) Pentamidine (im)	hypoglycemia hypotension	(−)	(−)	poor
4	(−)	Cotrimoxazole (po) Pentamidine (im)	(−)	(−)	(−)	well
5	*Haemophilus influenzae*	Cotrimoxazole (po) Pentamidine (im)	(−)	(−)	(−)	poor
6	*Candida albicans*	Cotrimoxazole (po) Pentamidine (iv, aer)	thrombopenia liver dysfunction	(+)	(+)	well
7	*Candida albicans*	Cotrimoxazole (po) Pentamidine (iv, aer)	(−)	(+)	(+)	well
8	(−)	Cotrimoxazole (po) Pentamidine (iv)	(−)	(−)	(+)	poor
9	Aspergillus spp. Cytomegalovirus	Cotrimoxazole (po)	(−)	(−)	(−)	poor
10	*Candida albicans* Cytomegalovirus	(−)	(−)	(−)	(−)	poor

*aer: aerolized, im: intramusculaly, iv: intravenously, po: per oral

All patients had received immunosuppressive therapy, such as corticosteroids. Eight patients, all but the two with adult T cell leukemia, had received the therapy for more than 2 months (Table 1). Three cases were asymptomatic when chest radiography revealed an abnormal shadow, and 5 patients had a rapid progression of cough, dyspnea, and fever, over 2-3 days. The patients with adult T cell leukemia had longer prodromes (Table 1).

The mean $P(A\text{-}a)O_2$ gradient at presentation was 69.3±23.4 torr, and the mean LDH value at presentation was 1,030±347 IU/L. To assess whether the elevated LDH values were a result of the infection or reflected other aspects of the status of the patients, we reviewed measurements prior to the acute illness. Previous values, determined 1-2 months before the diagnosis of PCP, were available in 9 patients, Comparison of these values with those at the time of diagnosis of PCP showed that all 9 patients had an increased in LDH values of more than 190 IU/L, with a median increase of 610±391 IU/L (Table 2). At the time of presentation, chest radiography revealed a bilateral reticular shadow in 5 patients, a bilateral reticular shadow and infiltrates in 4, and an AIDS-like diffuse ground glass shadow in 1 (Table 2). Two patients had *Pneumocystis carinii* cysts demonstrated at postmortem examination, but in all other patients diagnostic material was obtained while alive by TBLB and/or BAL (Table 2).

Coexisting pulmonary infections were diagnosed in 5 patients. *Candida albicans* was recognized in 3 patients, Cytomegalovirus in 2, *Haemophilus influenzae* in 1, and *Aspergillus* spp. in 1 (Table 3). Nine patients received treatment with a high dosage of cotrimoxazole and pentamidine isethionate. In 3 patients, mechanical ventilation was also used, and in 2 of these, intravenous corticosteroid and aerozolized pentamidine were prescribed adjunctively. Six patients died of progressive pneumonia. Although the other 3 patients recovered from

PCP, they died subsequently due to underlying hematological malignancy, heart failure, and multi organ failure (Table 3).

Discussion

PCP associated with AIDS often presents in an indolent fashion, with symptoms manifest for several weeks prior to presentation. Non-AIDS PCP has a more acute onset, with pulmonary symptoms progressing rapidly to respiratory failure within 1 week (Macfarlane and Finch, 1985; Engelberg *et al.*, 1984). In the present study, all patients were PCP cases from conventionally caused immunosuppression, and 8 patients had a rapid progression of symptoms for less than 2-3 days, with the patients with adult T cell leukemia having longer prodromes. In one study, patients with hematological disorders had variable prodromes ranging from 2 days to 4 weeks, which is in accordance with our results (Carter *et al.*, 1988).

Recent studies have suggested that the serum LDH value and $P(A-a)O_2$ gradient have diagnostic and prognostic implications in patients with PCP associated with AIDS (Garay and Greene, 1989). The serum LDH value is elevated in most AIDS patients when PCP is present, and usually increases with the development of the infection and decreases again with recovery, although the mechanism for this change is unknown. An association between the LDH value and survial from PCP was also noted, with survivors having significantly lower mean serum levels than those who died (El-sadr and Simberkoff, 1988). Higher values of LDH appeared to reflect more extensive interstitial inflamation. A reduction in the diffusing capacity for carbon monoxide is one of the characteristic abnormalities of pulmonary function in patients with PCP, which causes alveolar-capillary block. This abnormality is attributable to thickening of the alveolar-capillary membrane by attachment of this parasite to the epithelial surface (Sankary *et al.*, 1988). The mechanism of the increased $P(A-a)O_2$ gradient has not been fully mentioned. In our study, the LDH value and $P(A-a)O_2$ gradient at presentation were significantly elevated, and there was an increase in the LDH value at presentation over the previous values. Therefore the elevated LDH values appeared to be a result of PCP. When the patients with good outcome of PCP were compared with those with failure, the mean LDH value was 969 ± 287 vs. $1,000\pm392$ IU/L, and the mean $P(A-a)O_2$ gradient was 62.0 ± 9.48 vs. 74.1 ± 28.2 Torr. However there was no significant difference between the two groups. The extremly high degree of the elevation in the LDH value and the increase in $P(A-a)O_2$ gradient appeared to reflect the severity of the disease, which might account for the poor outcome in our study group. In the present study, we conclude that these values might be useful as markers for diagnosis of PCP in non-AIDS patients also.

PCP usually presents on chest radiograph as a diffuse bilateral, progressively coalescing pneumonia that in its earliest stages often spares the peripheral lung fields. PCP presenting as a pulmonary nodule or a cavity is rare, and mediastinal lymphadenopathy and pleural effusion are not believed to occur with this infection (Barrio *et al.*, 1986). In our study, chest radiography revealed a typical diffuse bilateral, progressively coalescing pneumonia, and in 5 patients bilateral diffuse infiltrates were already visible at presentation. This might suggest an acute onset of the disease in our population and the difficulty in making an early diagnosis.

It is necessary for the adequate management of PCP to obtain a definitive diagnosis at an early stage. Non-invasive methods, such as sputum induction with the use of nebulised hypertonic saline, are safe, but the sensitivity is relatively low (Yoshida *et al.*, 1978; Pitchenik

et al., 1986). In most studies, transthoracic needle biopsy has not been recommended because of its high incidence of complications. Open lung biopsy has been shown to be more reliable, but is highly invasive, and this method is currently of limited value and has not been used widely for the diagnosis of PCP in Japan (Pass *et al.*, 1986; Shorter *et al.*, 1988). Several studies have demonstrated that fiber optic bronchoscopy with BAL and TBLB is a safe and sensitive method for the initial diagnosis of PCP as well as other opportunistic infections (Oka *et al.*, 1985; Cordonnier *et al.*, 1985). In the present study, 7 BALs and 3 TBLBs were performed in 8 patients who were diagnosed as having PCP, with no complication. We suggest that fiber optic bronchoscopy, especially with BAL, is the most safe, sensitive, and rapid method for the initial diagnosis of PCP.

In half of the patients, coexisting pulmonary infections were recognized. In patients 6 and 7, *Candida albicans* was recognized for the first time in BAL, which was performed after recovering from PCP. In these cases, fluconazole was given for the treatment of the fungi, but it was supposed that prophylactic therapy might have prevented this infection. In PCP cases in the immunocompromised host, great care must also be given to coexisting infections.

Pentamidine isethionate was the first drug demonstrated to have efficacy in the treatment of PCP. Experience in immunocompromised patients demonstrated about 70% efficacy, but with substantial toxicity. Adverse reactions include renal failure, liver dysfunction, hypoglycemia, hyperglycemia, pain and swelling at the injection site, hematologic disturbances, hypotension, and pancreatitis (Salamone and Cunha, 1988; Pearson and Hewlett, 1985; Kluge *et al.*, 1978; Zuger *et al.*, 1986; Helmick and Green, 1985; Belehu and Naafs, 1982; Stahl-Bayliss *et al.*, 1986; Stoner, 1988; Montgomery *et al.*, 1989). Cotrimoxazole became the preferred therapy, with pentamidine an alternative, in cases of failure and for those with severe adverse reactions (Kovacs and Masur, 1988; Furio *et al.*, 1988). It has been suggested that a combination of cotrimoxazole and pentamidine is no more effective and may be harmful in the treatment of PCP. PCP in the non-AIDS population, however, is reported to be more fulminant, with greater incidence of treatment failure and fewer adverse reactions than those from AIDS (Drake *et al.*, 1985; Salamone and Cunha, 1988; Pearson and Hewlett, 1985; Kluge *et al.*, 1978; Kovacs *et al.*, 1984; Sattler *et al.*, 1988). It therefore seems to be useful to examine the efficacy and the incidence of adverse reactions in the concomitant use of pentamidine and cotrimoxazole in the non-AIDS population. PCP cases with acute onset tend to progress to hypoxemic respiratory failure requiring mechanical ventilation. The mortality rate for these patients is significantly high, despite optimal medical management. Several studies have demonstrated a possible role of intravenous corticosteroids in adjunctive therapy in cases with acute life-threatening respiratory failure secondary to PCP. It was also speculated in some studies that adjunctive corticosteroid therapy might obviate the use of mechanical ventilation (Gallacher *et al.*, 1989). The outcome of patients with corticosteroid therapy and the role of mechanical ventilation have yet to be examined. In the present study, pentamidine together with cotrimoxazole was prescribed for 9 patients. Although one patient showed thrombocytopenia and liver dysfunction and one other showed hypotension and hypoglycemia, seven patients sustained mild adverse reactions from the therapy and tolerated it well. Three of the 9 patients given this treatment recovered from PCP, which is a relatively poor outcome. This might be due to the severity of the underlying diseases and failure in making an ealier diagnosis. The concomitant use of pentamidine and cotrimoxazole in our study seemed no more harmful, but no more effective, than therapy with cotrimoxazole

followed by pentamidine. Two patients treated with intravenous corticosteroids and mechanical ventilation have recovered from PCP, and we suggest the possible efficacy of this regime. However, we acknowledge the low reliability of this conclusion due to the small population size and the need for evaluation in a larger patient population.

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AIDS 以外の免疫抑制患者におけるニューモシスチスカリニ肺炎の管理

勝又　達哉・河野　　茂・古賀　宏延・吉富　祐子・
松田　治子・光武耕太郎・東山　康仁・
宮崎　義継・原　　耕平

AIDS 以外の免疫抑制状態に，ニューモシスチスカリニ肺炎を発症した，10症例に臨床的検討を加えた。発症様式では，成人T細胞白血病の２例は前駆症状が長かったが，他の症例では急性の発症を示した。発症時の胸部レントゲンでは，全例において両側びまん性の陰影を認めた。確定診断は，８例において経気管支的肺生検，または気管支肺胞洗浄によって，２例において剖検によってなされた。診断時には，LDH の値は発症前の値に比べて高度に上昇しており，肺胞気動脈血酸素分圧較差も高度に開大しており，これらの値が早期診断に有用であることが示唆された。他の日和見感染の合併は，５例に認められた。治療においては，ペンタミジンと ST 合剤の併用は，副作用は少なかったものの，有効率も低かった。コルチコステロイドは，補助療法として有用と考えられた。

長崎大学医学部第二内科学教室（〒852 長崎市坂本町７－１）

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TWO NEW BLACKFLY SPECIES OF *SIMULIUM* (*SIMULIUM*) FROM JAVA, INDONESIA (DIPTERA: SIMULIIDAE)

HIROYUKI TAKAOKA[1] AND UPIK KESUMAWATI HADI[2]

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Abstract: Two new blackfly species, *Simulium* (*Simulium*) *sigiti* sp. nov. and *S.* (*S.*) *javaense* sp. nov. are described based on the female, male, pupal and larval specimens collected from Java. *Simulium* (*S.*) *sigiti* is characterized by the presence of a pit-like cuticular organ at the base of pupal gill, as well as shortened pupal filaments. This is the first species of the *tuberosum* group from Java. The variety of *S.* (*S.*) *iridescens* De Meijere, 1913 is here elevated to the species status and is given a new specific name *S.* (*S.*) *javaense* sp. nov. This new species is easily distinguished from *S.* (*S.*) *iridescens* by the inflated gill filaments, the absence of tubercles on the cephalic and thoracic integuments of the pupa, and the russet body coloration of the larva.

INTRODUCTION

The simuliid fauna of the Sunda Islands has not been studied since Edwards (1934) described 11 new species from Sumatra, Java and Bali, making a total of 19 taxa (including two subspecies) for this archipelago.

Recently, we made a preliminary survey on the blackflies in East and West Java and collected a total of 16 species of *Simulium* Latreille s. l. including several new species. This paper treats two new species belonging to the subgenus *Simulium* Latreille s. str.

The classification follows that of Crosskey (1969). Collecting and rearing methods, as well as dissection of anatomical parts for description, were mentioned in Takaoka (1983).

The holotype, allotype and some paratype specimens will be deposited at the Department of Parasitology and Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University, and some other paratypes at the Department of Entomology, Bogor Museum of Zoology, Bogor, Indonesia.

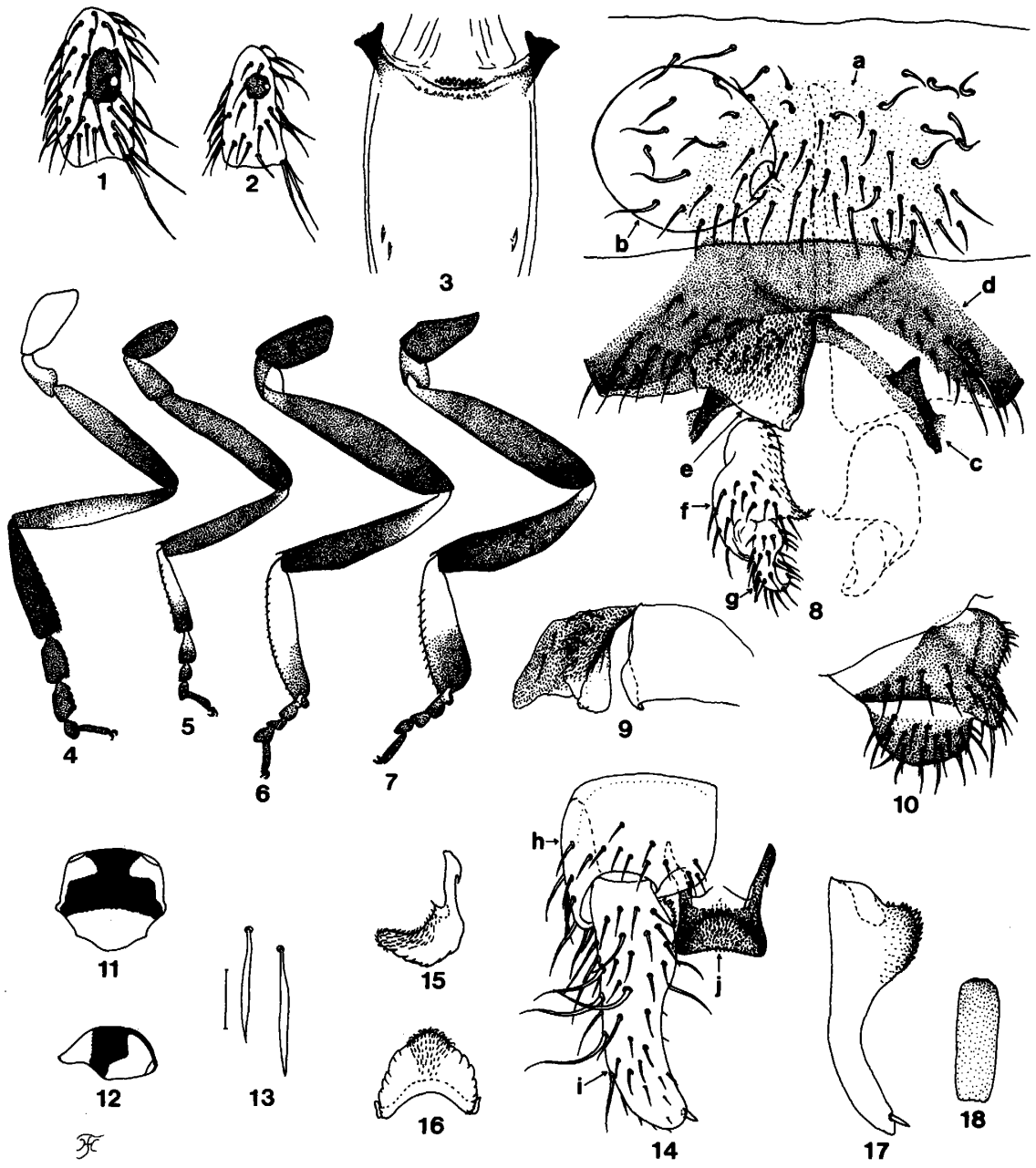
DESCRIPTION

Simulium (*Simulium*) *sigiti* sp. nov.

Female. Body length 2.1 mm. *Head.* Narrower than width of thorax. Frons black, shiny,

1 Division of Medical Zoology, Medical College of Oita, Hazama, Oita 879-55, Japan
2 Entomology Laboratory, Department of Parasitology and Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University, Taman Kencana 3, Bogor 16151, Indonesia

and with several dark stout hairs along lateral margins; frontal ratio (width at top of eyes and that just above antenna to length) 1.3:1:1.3; frons-head ratio (width of frons at top of eyes to maximal width of head) 1:4.1. Clypeus black, shiny, probably greyish pruinose and with scattered dark stout hairs. Antenna composed of 2+9 segments, dark brown except scape, pedicel and base of 1st flagellar segment pale. Maxillary palp brownish black, composed of 5 segments with proportional length of 3rd, 4th and 5th segments 1:1:1.7; 3rd segment (Fig. 1) of moderate size; sensory vesicle medium in size, elliptical, with rugged surface, 0.34× length of 3rd segment, and with small round opening a little distad to the center. Maxillary lacinia with 17 inner and 17 outer teeth. Mandible with ca. 30 inner and 12 outer teeth. Cibarium (Fig. 3) with numerous minute tubercles. *Thorax.* Scutum black, shiny, not patterned and covered moderately with recumbent, dark brown pubescence, interspersed with long, upstanding dark hairs on prescutellar area. Scutellum black, with long dark hairs. Postscutellum black, shiny, silvery iridescent when viewed in certain angle of lights, and without hairs. Pleural membrane bare. Katepisternum longer than depth, and bare. *Legs.* Foreleg (Fig. 4): coxa pale yellow; trochanter dark yellow; femur dark yellow basally, gradually becoming dark medially and black on distal 1/2 or 1/3; tibia black with outer median portion largely pale white and in lights sheeny; basitarsus entirely black, dilated (W:L=1:4.8), and with short dorsal hair crest; rest tarsal segments black. Midleg (Fig. 5): blackish except basal 1/2 of trochanter, base of tibia, basal 2/3 or more of basitarsus and basal 1/2 of 2nd tarsomere whitish yellow; tibia largely sheeny on posterior surface in lights. Hind leg (Fig. 6): blackish with trochanter and base of femur dark yellow, and basal 1/3 of posterior surface of tibia, basal 3/5 of basitarsus and basal 1/2 of 2nd tarsomere whitish yellow; tibia largely sheeny on posterior surface in lights, and slightly narrower than femur; basitarsus W:L=1:5, calcipala short, width to that of basitarsal tip is 1:1.9, W:L ratio 1:1; pedisulcus distinct. All tarsal claws simple, without subbasal or basal tooth. Femora and tibiae of all legs covered with simple hairs only. *Wing.* Length 2.0 mm; costa with spinules and hairs; subcosta haired; basal section of vein R bare; hairs at base of stem vein dark brown; basal cell absent. *Abdomen.* Basal scale black with fringe of dark hairs; 2nd segment dark brown with large, dorsolateral whitish iridescent spots broadly connected to each other in the middle; tergites 3, 4 and 5 small, dark brown and shining, tergites 6-9 large, dark brown, shining and with dark hairs. *Genitalia* (Figs. 8-10). Ventral surface of abdominal segment 7 with large, weakly developed sternite medially (Fig. 8a) and moderately haired. Sternite 8 (Fig. 8d) well sclerotized, bare medially but with ca. 13 short and long stout hairs laterally on each side; anterior gonapophyses (Fig. 8e) triangular in shape, membraneous, covered with ca. 12 minute setae as well as numerous microsetae except narrow portion along posterior margin bare; inner borders slightly curved, narrowly sclerotized, and folded dorsally as shown in Fig. 9. Genital fork (Fig. 8c) of inverted-Y form, with well sclerotized stem; arms slender, each with strongly sclerotized distal ridge and distinct projection directed anterodorsally. Paraproct (Fig. 10) slightly shorter than wide, with ventral margin concave medially, and covered with ca. 15 short stout hairs in lateral view; paraproct in inside view somewhat broadly sclerotized along ventral margin and covered sparsely with microsetae. Cercus (Fig. 10) rounded posteriorly, ca. 0.5× as long as wide, and covered with numerous short hairs. Spermatheca (Fig. 8b) nearly globular in shape, well sclerotized with weakly defined reticulate pattern, and with minute internal setae; tube and small adjacent area of spermatheca unsclerotized.



Figures 1-18 Adults of *Simulium* (*Simulium*) *sigiti* sp. nov. 1, third segment of female maxillary palp; 2, third segment of male maxillary palp; 3, cibarium; 4, foreleg of female; 5, midleg of female; 6, hind leg of female; 7, hind leg of male; 8, ventral view of female genitalia——a, 7th sternite; b, spermatheca; c, genital fork; d, 8th sternite, e, anterior gonapophysis; f, paraproct; g, cercus; 9, anterior gonapophyses showing dorsally folded inner margin; 10, paraproct and cercus in side view; 11, male scutum in dorsal view; 12, male scutum in side view; 13, scale-like hairs on outer surface of male hind femur (scale, 0.02 mm); 14, male genitalia in ventral view (coxite and style on right side, median sclerite and parameres omitted)——h, coxite; i, style; j, ventral plate; 15, ventral plate in lateral view; 16, ventral plate in end view; 17, style viewed ventromedially showing basal protuberance with numerous spines; 18, median sclerite.

Male. Body length 2.4 mm. *Head.* Width slightly wider than thorax. Upper eye consisting of large facets in 20 horizontal and vertical rows. Clypeus black, whitish grey pruinose, strongly silvery iridescent when illuminated, and covered sparsely with dark brown hairs. Antenna composed of 2+9 segments, dark brown except scape and pedicel dark yellow and base of 1st flagellar segment pale yellow; 1st flagellomere somewhat elongated (W:L=1:2.1). Maxillary palp composed of 5 segments with proportional length of 3rd, 4th and 5th segments 1:1.1:2.3; 3rd segment (Fig. 2) of normal size with small, globose sensory vesicle which is 0.2× length of 3rd segment; small rounded opening situated medially. *Thorax.* Scutum black, with white pruinose pattern composed of an anterior pair of triangular spots with rounded apex on shoulders extending posteriorly along lateral margins up to base of wing and a large transverse spot entirely covering prescutellar area which is not contiguous to anterior spots (Figs. 11 and 12), —these pruinose areas with silvery iridescence when illuminated: scutum uniformly covered with dark brown recumbent pubescences (in lights these pubescences appear bright coppery), interspersed with long upright hairs on prescutellar area. Scutellum black, shiny, white pruinose and with several upright dark hairs. Postscutellum black, shiny, white pruinose and without hairs. Pleural membrane and katepisternum as in female. *Legs.* Coloration as in female except hind tibia black with base pale whitish yellow, and hind basitarsus with less pale portion (basal 1/2 or a little more); in addition to simple hairs, scale-like hairs (Fig. 13) are seen densely on outer surface of femora and on both sides of tibiae of hind leg; fore basitarsus somewhat dilated (W:L ratio 1:4.4); hind basitarsus (Fig. 7) much enlarged (W:L=1:4.5), widening toward basal 1/2, then nearly parallel sided, and its greatest width equal to those of hind femur and tibia. Calcipala small (W:L ratio=1:1) and ca. 0.22× as wide as basitarsal width. Pedisulcus well marked on 2nd tarsomere. *Wing.* Length 1.9 mm; other features as in female except subcosta bare. *Abdomen.* Basal scale blackish with long dark hairs. Terga black, with dark hairs; segments 2, 6 and 7 each with a pair of silvery iridescent areas dorsolaterally, those on segment 2 connected broadly to each other in the middle. *Genitalia* (Figs. 14-18). Coxite in ventral view (Fig. 14h) as long as wide and ca. 0.6× length of style; style (Fig. 14i) length ca. 3× greatest width at basal 1/4, spatulate, curved inwards, slightly tapering apically and with a subterminal spine; style (Fig. 17) with basal protuberance produced dorsomedially, bearing numerous spines on its surface. Ventral plate (Fig. 14j) with base nearly quadrate in shape, having ventrally produced hairy process with weakly toothed posterolateral margins on proximal 2/3; posterior margin when viewed ventrally slightly concave; basal arms slightly diverging from each other. Median sclerite (Fig. 18) well sclerotized at base, plate-like and nearly parallel sided. Parameres each with numerous small hooks.

Pupa. Body length (excluding gill filaments) 2.5 mm. *Head.* Integument dark golden yellow, sparsely covered with large, disc-like tubercles of various sizes (Fig. 20), larger ones appearing to have more distinct depression in the center than smaller ones; most tubercles fringed with several microtubercles; head with 1 facial and 2 frontal pairs of slender, simple trichomes. *Thorax* (Fig. 21). Anterior 1/2 of thoracic integument dark golden yellow, sparsely covered with large disc-like tubercles (Fig. 21b) similar to those on the head, though the larger groups of tubercles much smaller, and central depression of tubercles less distinct, while posterior 1/2 of thorax pale greyish brown and covered moderately with small, conical processes (Fig. 21c); thorax with 2 dorsal and 2 lateral pairs of simple trichomes on anterior

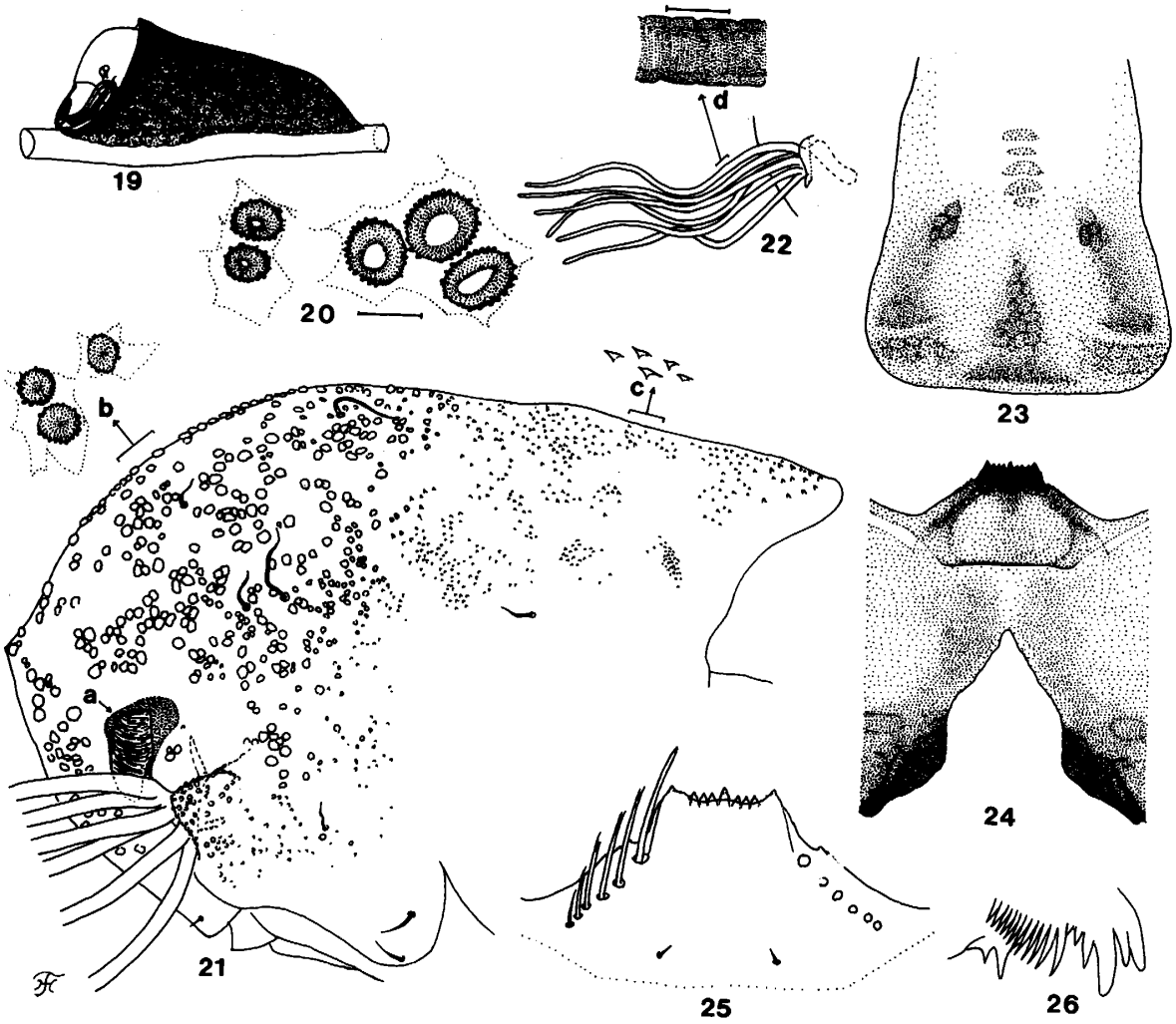
1/2 as well as 1 lateral pair of trichomes on posterior 1/2 (Fig. 21); at base of gill, there is a large, pit-like cuticular organ (Fig. 21a). Gill (Fig. 22) with 6 very short, slender and dark brown filaments in pairs, almost sessile, and slightly tapering apically; all filaments subequal to each other in length (ca. 0.8-1.0 mm) and thickness, and generally extending close together forwards, downwards, and then inwards just in front of head (in some specimens 1 or 2 filaments, specially of upper and lower pairs, curved upwards or downwards and directed backwards); cuticle of filaments with well-defined annular furrows, and covered uniformly with minute tubercles (Fig. 22d). *Abdomen.* Tergum 2 on each side with 1 long and 5 short simple setae, 4 of which are stout and spinous. Terga 3 and 4 each with 4 hooked spines along posterior margin on each side. Terga 6, 7, 8 and 9 each with a cross row of spine-combs on each side, though spine-combs on segment 6 weakly developed (in some pupae, tergum 6 without spine-combs). Tergum 9 lacking terminal hooks. Sternum 4 with a bifid hook and a minute simple seta close together submedially on each side. Sternum 5 with a pair of bifid hooks submedially on each side. Sterna 6 and 7 each with a pair of bifid inner and simple outer hooks widely spaced on each side. Grapnel-like hooklets absent. *Cocoon* (Fig. 19). Simple, slipper-shaped, thickly woven, with a thick anterior rim; there is a connecting band across the front floor but not forming a definite front wall.

Mature larva. Body length 5.0-5.2 mm. Body color dark brown (though annular interspaces of abdominal segments 1-5 narrowly discolored in the alcoholic specimens). Cephalic apotome (Fig. 23) pale widely on anterior 1/2, and light brown on posterior 1/2, with darkened areas medially, laterally and posteriorly; head spots more or less positive. Antenna composed of 3 segments and apical sensillum, longer than stem of cephalic fan; length ratio of segments (from base to tip) 1.5:1.6:1. Cephalic fan with ca. 42 main rays. Mandible (Fig. 26) with a large and a medium mandibular serration but without supernumerary serration. Hypostomal teeth 9 in number, small with median and corner teeth longer than others; 6 hypostomal bristles lying subparallel to lateral border on each side (Fig. 25). Postgenal cleft (Fig. 24) nearly parallel-sided near base, then converging apically, and ca. 2× length of postgenal bridge. Thoracic cuticle bare. Abdominal cuticle bare except dorsolateral areas on last segment moderately covered with short, discolored setae. Rectal gill of 3 lobes each with 2-3 small secondary lobules ventrally. Anal sclerite X-shaped, with broadened anterior arms which are 0.54× length of posterior ones. Posterior circlet with ca. 78 rows of hooklets with up to 14 hooklets per row. Ventral papillae absent.

Type specimens. Holotype ♀, slide mounted, reared from pupa collected from Puncak, West Java, 10.I.1991, H. Takaoka and U.K. Hadi. Allotype ♂, slide mounted, reared from pupa, same data as holotype. Paratypes, 1 ♀, 1 ♂ reared from pupae, 4 pupal skins and associated cocoons, and 2 mature larvae, all slide mounted and same data as holotype.

Distribution. West Java.

Ecological notes. The pupae and larvae were found on slender roots of trees trailing in the water of a small shaded stream (less than 0.5 m wide; 1,250 m in altitude) running down through the sloped natural forest near the upper border of open field cultivated for tea plantation. This species was collected together with *S.* (*S.*) *iridescens* and two *Gomphostilbia*



Figures 19-26 Pupa and larva of *Simulium* (*Simulium*) *sigiti* sp. nov. 19, pupa and cocoon attached to the slender plant root; 20, enlargement of disc-like tubercles on pupal head (scale, 0.02 mm); 21. lateral view of pupal thorax——a, pit-like organ; b, enlargement of disc-like tubercles on anterior half (same scale as in Fig. 20); c, enlargement of conical processes on posterior half (same scale as in Fig. 20); 22, pupal gill filaments——d, enlargement of filament showing annular furrows (scale, 0.02 mm); 23, larval cephalic apotome; 24, ventral view of head capsule showing postgenal cleft; 25, larval hypostomium; 26, apical tip of larval mandible.

species.

Remarks. This specific name was given in honor of Dr. Singgih H. Sigit, Professor and Dean, Faculty of Veterinary Medicine, Bogor Agricultural University for his contributions to medical entomology.

This new species can be assigned to the *tuberosum* group of *Simulium* (*Simulium*) defined by Rubtsov (1959-1964) by having the spinous basal protuberance of the style, quadrate ventral plate with ventral projection and toothed posterolateral borders, pruinose

scutal pattern in the male, simple claws and unpatterned scutum in the female, and pupal gill with six filaments.

Although the female, male and larva of this species are very similar to those of most other known species of the *tuberosum* group, the pupa of *S.* (*S.*) *sigiti* sp. nov. is very distinct in possessing a pit-like organ at the base of gill (Fig. 21a), as well as the short, slender gill filaments which are subequal to each other in length and thickness. The similar pit-like cuticular structure near the base of pupal gill has been reported in the *clathrinum* group of *Simulium* (*Morops*) from Australasian region (Smart and Clifford, 1965; Crosskey, 1967). However, none of the other known species of the *tuberosum* group has such an interesting cuticular structure except *S.* (*S.*) *nigrifacies* Datta, 1974 described from the male and pupal specimens collected from Darjeeling, West Bengal, India, which seems to have the similar pit-like organ at the base of pupal gill judging from the illustration made by Datta (1974). The pupal gill filaments of the latter species are also shortened (1.2 mm long) like this species but the relative thickness of filaments are different, decreasing from above downwards with the outer filament of upper pair being the thickest of all, as in most other species of this group. In addition, the pupa of *S.* (*S*). *nigrifacies* has spine-combs on the abdominal segments 8 and 9 only.

Simulium (*S.*) *puliense* Takaoka, 1979 from Taiwan seems to be close to this new species by having the somewhat shortened pupal gill filaments (ca. 1.5 mm long) as well as the similar arrangement of spine-combs on the pupal abdomen. However, unlike this new species, pupal filaments of the Taiwanese species are different in relative thickness, i.e., outer filament of upper pair is a little thicker than other five filaments which are subequal in thickness to each other.

The pupa of *S.* (*S.*) *aeneifacies* which was described from Sabah (Edwards, 1933) and later assigned to the same species group by Takaoka (1983) is yet unknown. The comparison of female genitalia (Takaoka, 1983) shows that there are differences in the number of hairs on the eighth sternite and anterior gonapophyses, and in the shape of paraproct.

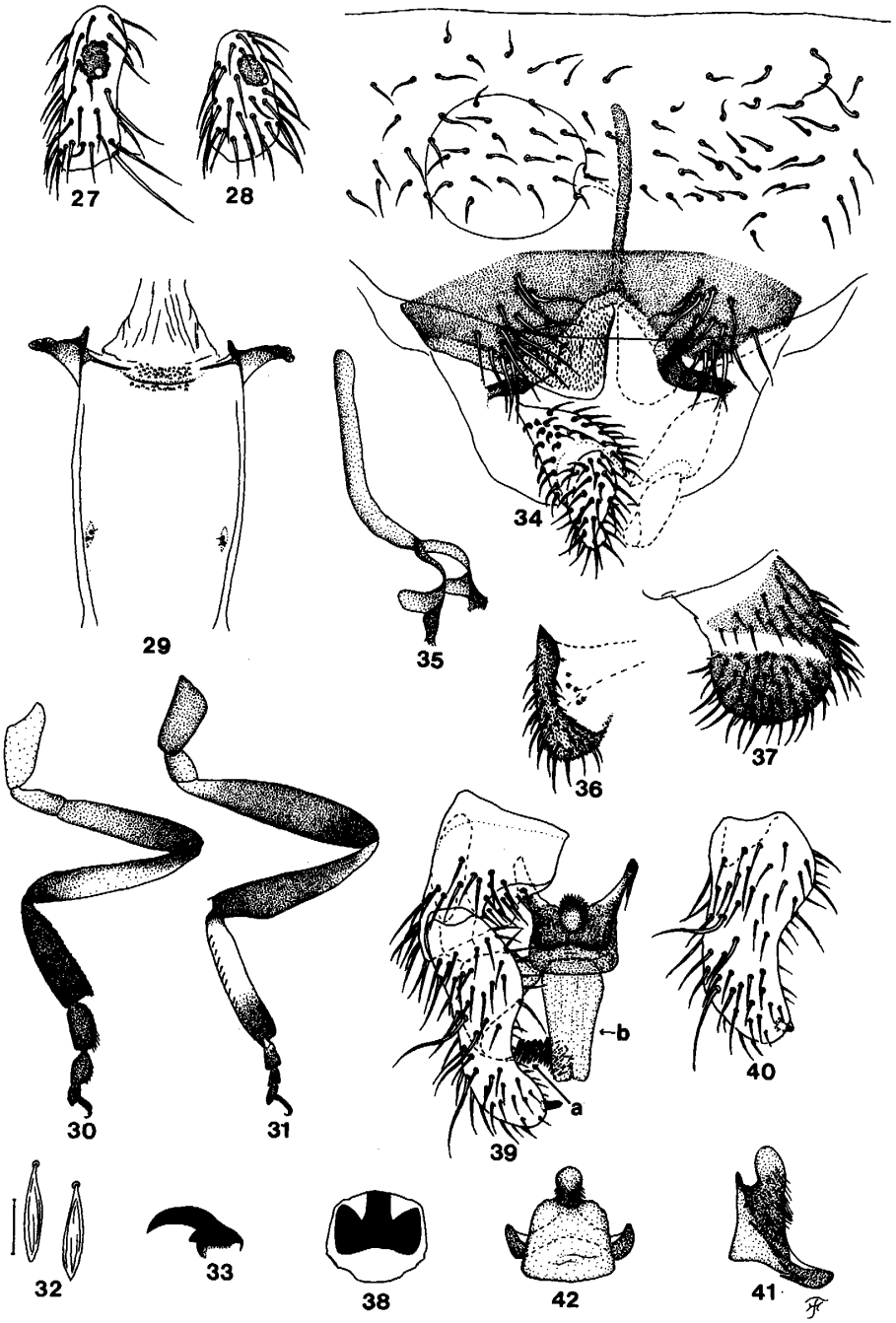
Simulium (*Simulium*) *javaense* sp. nov.

Simulium iridescens De Meijere 1913 var. ?: Edwards, 1934, Arch. Hydrobiol., Suppl., 13, 114 (male, pupa and larva).

Female. Body length 2.4 mm. *Head.* Narrower than width of thorax. Frons black, shiny, thinly greyish pruinose and with several dark stout hairs along lateral margins; frontal ratio 1.2:1:1.1; frons-head ratio 1:3.8. Clypeus black, shiny, greyish pruinose and with scattered dark stout hairs; silvery iridescent when illuminated. Antenna composed of 2+9 segments, dark brown except scape, pedicel and base of 1st flagellar segment pale. Maxillary palp brownish black, composed of 5 segments with proportional length of 3rd, 4th and 5th segments 1:1.1:1.8; 3rd segment (Fig. 27) not enlarged; sensory vesicle small, elliptical, with rugged surface, 0.22× length of 3rd segment, and with small round opening. Maxillary lacinia with 14 inner and 14 outer teeth. Mandible with ca. 26 inner and 13 outer teeth. Cibarium (Fig. 29) with numerous minute tubercle. *Thorax.* Scutum black, shiny, thinly grey pruinose, not patterned and covered moderately with recumbent, dark brown pubescence, interspersed with long, upstanding dark hairs on prescutellar area. Scutellum black, with

long dark hairs. Postscutellum black, shiny, silvery iridescent when viewed in lights, and without hairs. Pleural membrane bare. Katepisternum longer than depth, and bare. *Legs.* Foreleg (Fig. 30): coxa pale yellow; trochanter dark yellow with distal 1/3 brown; femur brown basally, and much darkened distally; tibia black with outer median portion largely pale white and in lights sheeny; basitarsus entirely black, dilated (W:L=1:3.7), and with thick dorsal hair crest; rest tarsal segments black. Midleg: blackish except basal 4/5 or more of basitarsus and basal 1/2 of 2nd tarsomere whitish yellow; tibia largely sheeny on posterior surface in lights. Hind leg (Fig. 31): blackish with trochanter and base of femur dark yellow, and base of tibia, basal 2/3 of basitarsus and basal 1/2 of 2nd tarsomere whitish yellow; tibia largely sheeny on posterior surface in lights, and slightly narrower than femur; basitarsus W:L=1:5.2, calcipala short, width to that of basitarsal tip is 1:2, W:L ratio 1:0.7; pedisulcus distinct. All tarsal claws each with small, subbasal tooth (Fig. 33). Femora and tibiae of all legs covered densely or moderately with scale-like hairs (Fig. 32) as well as simple hairs on outer surface. *Wing.* Length 2.0 mm; costa with spinules and hairs; subcosta bare; basal section of vein R bare; hairs at base of stem vein dark brown; basal cell absent. *Abdomen.* Basal scale black with fringe of dark hairs; 2nd segment dark brown with large, dorsolateral whitish iridescent spots broadly connected to each other in the middle; tergites 3 and 4 small, dark brown and shining, tergites 5-9 large, dark brown, shining and with dark hairs. *Genitalia* (Figs. 34-37). Ventral surface of abdominal segment 7 widely expanded posteriorly, covering sternite 8 almost entirely, and moderately haired on anterior 1/2 but bare on posterior 1/2; plate-like sternite undeveloped. Sternite 8 well sclerotized, bare medially and laterally but with 14 or 15 long stout hairs submedially on each side; anterior gonapophyses triangular in shape, rounded on posterointernal tip, membraneous, covered with a few minute setae as well as numerous microsetae except narrow portion along posterior margin bare; inner borders nearly straight and narrowly sclerotized. Genital fork of inverted-Y form, with well sclerotized stem; arms slender, each with strongly sclerotized distal ridge and distinct projection directed anterodorsally (well discernible when viewd laterally—Fig. 35). Paraproct narrowed ventroposteriorly in lateral view (Fig. 37), covered uniformly with numerous short hairs on outer and ventral surfaces and also narrowly on inner surface (Fig. 36); when viewed from inside, paraproct (Fig. 36) widely transparent with several minute setae medially and not forming wide, darkly sclerotized plate, although narrowly sclerotized along ventral margin near base. Cercus (Fig. 37) rounded posteriorly, ca. 0.5× as long as wide, and covered with numerous short hairs. Spermatheca nearly globular in shape, well sclerotized with weakly defined reticulate pattern, and with minute internal setae; tube and small adjacent area of spermatheca unsclerotized.

Male. Body length 2.5 mm. *Head.* Width as wide as thorax. Upper eye consisting of large facets in ca. 18 horizontal and vertical rows. Clypeus black, whitish grey pruinose, strongly iridescent when illuminated, and covered sparsely with dark brown hairs. Antenna composed of 2+9 segments, entirely dark brown with base of 1st flagellar segment pale; 1st flagellomere somewhat elongated (W:L=1:1.7). Maxillary palp composed of 5 segments with proportional length of 3rd, 4th and 5th segments 1:1.1:2.1; 3rd segment (Fig. 28) of normal size with small, elliptical sensory vesicle which is 0.3× length of 3rd segment. *Thorax.* Scutum black, with white pruinose pattern composed of an anterior pair of triangular spots, a large transverse spot on prescutellar area which is contiguous to anterior spots by a narrow band along

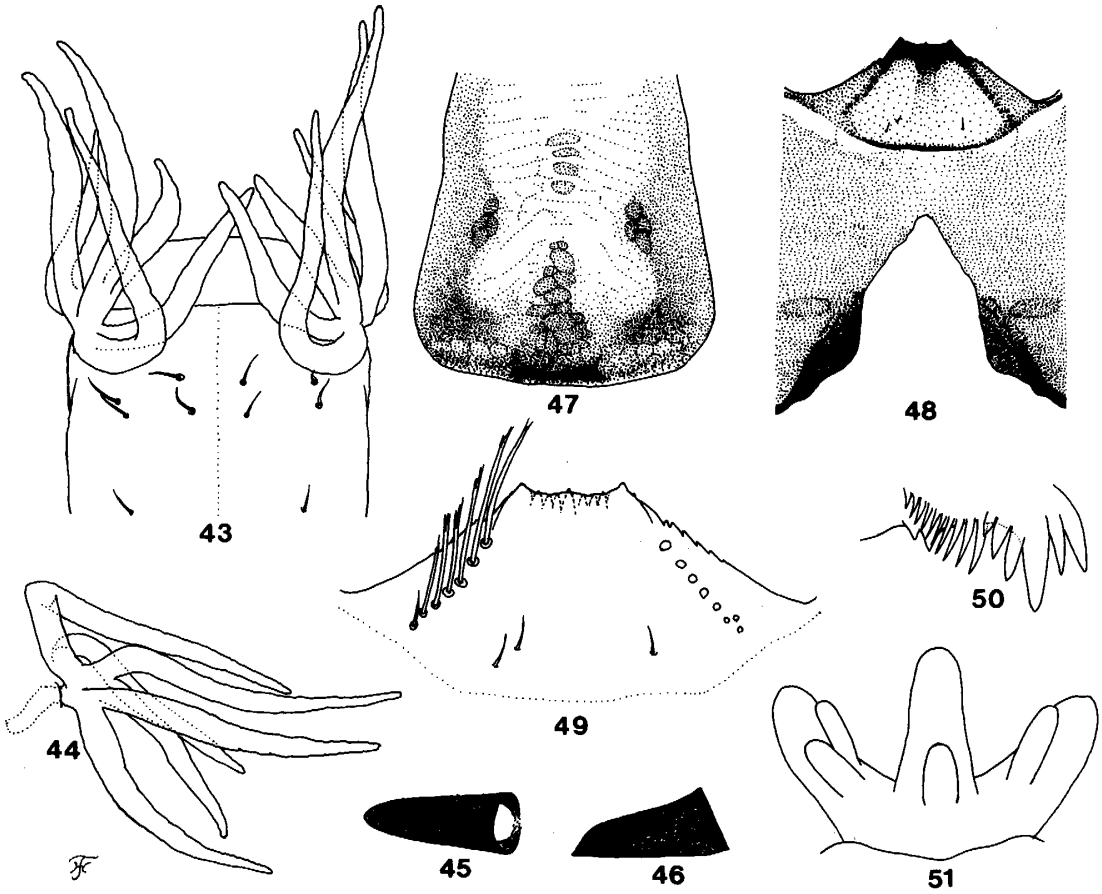


Figures 27-42 Adults of *Simulium* (*Simulium*) *javaense* sp. nov. 27, third segment of female maxillary palp; 28, third segment of male maxillary palp; 29, cibarium; 30, female foreleg; 31, female hind leg; 32, scale-like hairs on outer surface of hind femur (scale, 0.02 mm); 33, tarsal claw of female hind leg; 34, ventral view of female genitalia; 35, genital fork in lateral view; 36, paraproct in inside view; 37, paraproct and cercus in outside view; 38, male scutum in dorsal view; 39, ventral view of male genitalia *in situ* (coxite, style and paramere on right side omitted)——a, paramere; b, median sclerite; 40, style viewed ventrolaterally; 41, ventral plate in lateral view; 42, ventral plate in end view.

lateral margins (Fig. 38)—these pruinose areas with silvery iridescence when illuminated; scutum uniformly covered with dark brown recumbent pubescences (in lights these pubescences appear bright coppery), interspersed with long upright hairs on prescutellar area. Scutellum, postscutellum, pleural membrane and katepisternum as in female. *Legs.* Only following observations were possible due to incomplete condition of legs in dissected specimens: coloration probably as in female; scale-like hairs on outer surface of femora and tibiae also as in female; fore basitarsus somewhat dilated (W:L ratio 1:4.5); hind basitarsus enlarged, probably as wide as or wider than hind tibia, with distinct calcipala; pedisulcus well marked on 2nd tarsomere. *Wing.* Length not measured; other features as in female. *Abdomen.* Basal scale blackish with long dark hairs. Terga black, with dark hairs; segments 2, 4, 5, 6, 7 each with a pair of silvery iridescent areas dorsolaterally, those on segment 2 connected broadly to each other in the middle. *Genitalia* (Figs. 39-42). Coxite in ventral view 1.5× as long as wide and ca. 0.77× length of style; style (Fig. 40) length 2.2× greatest width at basal 1/4, spatulate, curved inwards, tapering apically and with a subterminal spine. Ventral plate: in ventral view nearly quadrate in shape with short basal arms diverging from each other; in lateral view sharply increasing its thickness from anterior to posterior margins thus appearing triangular and with thumb-like projection directed ventrally in the center which is setose on anterior surface but bare on its apex (Fig. 41); in end view trapezoid, slightly narrowed apically, with apical margin concave and irregularly dentate medially and almost smooth and bare (a few scattered minute setae in one specimen) on posterior surface (Fig. 42). Median sclerite (Fig. 39b) of shoehorn shape, thin, and slightly narrowed apically. Parameres (Fig. 39a) each with numerous small hooks.

Pupa. Body length (excluding gill filaments) 2.2-2.5 mm. *Head* and *thorax.* Integument dark yellow, almost bare except posterior 1/2 of thoracic integument covered moderately with small tubercles; head with 1 facial and 2 frontal pairs of simple trichomes, and thorax (Fig. 43) with 5 pairs of simple trichomes dorsally, all subequal in length. Gill (Figs. 43 and 44) with 6 greyish, short, inflated filaments in pairs, shortly stalked, and tapering apically; all filaments subequal to each other in length and thickness; 1 of 2 filaments of upper and middle pairs directed inwardly and downwardly just in front of head, and 1 of upper paired-filaments directed upwardly and abruptly bent forwardly; cuticle of filaments becoming somewhat rugged towards apex and with rough annulations but not forming well-defined reticulate patterns, and covered uniformly with minute tubercles. *Abdomen.* Tergum 2 on each side with 1 long and 5 short simple setae, 3 of the latter stouter than other 2 setae. Terga 3 and 4 each with 4 hooked spines along posterior margin on each side. Terga 7 and 8 each with a cross row of 3-5 spine-combs on each side. Tergum 9 with comb-like groups of minute spines but devoid of spine-combs like those on segments 7 and 8, and also lacking terminal hooks. Sternum 5 with a pair of bifid hooks close together submedially on each side. Sterna 6 and 7 each with a pair of bifid or simple hooks widely spaced on each side. Grapnel-like hooklets absent. *Cocoon* (Figs. 45 and 46). Simple, slipper-shaped, thickly woven, with a thick anterior rim; there is a connecting band across the front but not forming a definite front wall.

Mature larva. Body length 4.5-5.2 mm. Body color greyish with broad russet band dorsally on each abdominal segment, those bands often discolored medially to varying extents.



Figures 43-51 Pupa and larva of *Simulium* (*Simulium*) *javaense* sp. nov. 43, dorsal view of head and anterior half of thorax of the pupa showing inflated gill filaments and thoracic trichomes; 44, pupal gill filaments in lateral view; 45, cocoon in dorsal view; 46, cocoon in lateral view; 47, larval cephalic apotome; 48, ventral view of larval head-capsule showing postgenal cleft; 49, larval hypostomium; 50, apical tip of larval mandible; 51, ventral view of larval rectal gill showing small finger-like secondary lobules.

Cephalic apotome(Fig. 47) pale yellowish on anterior 1/2, also pale or light brown on posterior 1/2, and much darkened on peripheral areas except near anterior border; head spots more or less positive except submedial cross spots near posterior border turned to negative. Antenna composed of 3 segments and apical sensillum, longer than stem of cephalic fan; length ratio of segments (from base to tip) 1.9:2.1:1. Cephalic fan with ca. 40 main rays. Mandible (Fig. 50) with a large and a medium mandibular serration but without supernumerary serration. Hypostomal teeth 9 in number, small with median and corner teeth longer than others; 7 or 8 hypostomal bristles diverging posteriorly from lateral border on each side (Fig. 49). Postgenal cleft (Fig. 48) deltoid in shape, widening posteriorly, ca. 2.5× length of postgenal bridge. Thoracic cuticle bare. Abdominal cuticle bare except dorsolateral areas on last segment moderately covered with short, discolored setae. Rectal gill (Fig. 51) of 3 lobes each with 1-3 small secondary lobules ventrally. Anal sclerite X-shaped, with broadened anterior arms which are 0.52× length of posterior ones. Posterior circlet with ca.

90 rows of hooklets with up to 16 hooklets per row. Ventral papillae absent.

Type specimens. Holotype ♀, slide mounted, reared from pupa collected near Pujon, East Java, 19. XII. 1990, H. Takaoka. Allotype ♂, slide mounted, dissected from pupa, same data as holotype. Paratypes, 2 ♀♀ reared from pupa, 1 ♂ dissected from pupa, 3 pupal skins and associated cocoons, and 5 mature larvae; all slide mounted except 1 ♀ and 3 mature larvae in alcohol, same data as holotype.

Ecological notes. The pupae and larvae were found attached to slender plant roots and sticks in a small fast-flowing stream (width ca. 0.3 m) covered densely with shrubs on the hilly slope just above the mountainous road climbing from Kediri to Batu. The stream crosses this road before joining the main river which runs parallel to the road. This species was collected together with *S.* (*S.*) *iridescens* and *S.* (*Gomphostilbia*) spp.

Distribution. East Java.

Remarks. Edwards (1934) reported this species as a variety of *Simulium* (*Simulium*) *iridescens* de Meijere, 1913 based on the male, pupal and larval specimens taken from East Java. The male of this species is very similar to that of *S.* (*S.*) *iridescens* in many features including the genitalia, as pointed out by Edwards (1934). There seems to be a slight difference in the size of the triangular pruinose scutal spots on the shoulders which are in *S.* (*S.*) *iridescens* much larger, leaving smaller median and submedian areas black. In the female, it is also very difficult to separate this from the latter, although the eighth sternite of *S.* (*S.*) *iridescens* seems to bear a little fewer stout hairs.

On the other hand, this new species is readily distinguished from *S.* (*S.*) *iridescens* by the inflated gills on the pupal stage. Further, there is a difference in the cephalic integument which is smooth in this species but is covered moderately with small tubercles in the latter species. The larva of this species is also very similar to that of *S.* (*S.*) *iridescens* but differs from the latter by the presence of dorsal russet bands on abdomen.

The male of *S.* (*S.*) *javaense* shows similarities to *S.* (*S.*) *nebulicola* Edwards, 1934 described from a male taken at Tosari, East Java, but differs from the latter in the shape of the genitalia, as well as coloration of legs. The pupa of the latter species recently collected by us has the six slender gill filaments similar to that of *S.* (*S.*) *iridescens*.

This new species appears to be closely related to the species of the *melanopus* group, reported from the Philippines, North Borneo and North Sulawesi (Takaoka, 1983; Takaoka and Roberts, 1988) in having the similar male scutal pattern, the unpatterned female scutum, the female claws with a subbasal tooth, six-filamented pupal gills and the larval postgenal cleft deep but not reaching the posterior border of hypostomium. However, the shape of the ventral plate easily separates the male of this species from those of the *melanopus* group, and the female lacks a ventrointernal sclerotized plate on the paraproct which is found in all the member species of this group. The slipper-shaped cocoon also separates this species from the *melanopus* group, the cocoons of which are all shoe-shaped.

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We thank Dr. Singgih H. Sigit, Professor of Department of Parasitology and Pathology and Dean of Faculty of Veterinary Medicine, Bogor Agricultural University for his generosities and kindness in providing laboratory space and facilities and arranging collecting trips during this investigation. Our appreciation also goes to all the staffs of Entomology Laboratory, Department of Parasitology and Pathology, Bogor Agricultural University for their help in various ways. The senior author would like to thank Dr. D.M. Davies, Professor Emeritus, McMaster University, Ontario, and Dr. H. Takahashi, Tokyo for their encouragement and suggestion.

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ジャワ島産アシマダラブユ亜属の2新種について

高岡　宏行[1]・Upik Kesumawati Hadi[2]

1990年12月から1991年1月にかけて，ジャワ島において吸血性昆虫ブユの採集調査を行い，得られた標本を検討した結果，数種の新種が含まれていることが分かった。本論文では，ブユ属アシマダラブユ亜属に属する2新種の記載を行った。1種は，*tuberosum* グループに属し，蛹の呼吸管基部近くの外皮に小孔を有し，呼吸管糸が6本とも極めて短いなど，他の近似種に見られない特徴を有する。本グループに属するブユ種は，これまでにインドネシアからは報告がなく，本種が初めての記録である。他の1種は，これまで *Simulium iridescens* の変種とされていたブユであるが，蛹の呼吸管糸が袋状に大きくなっているなど，幾つかの形態的な違いがあることから，*S. iridescens* から独立させ，新種名を与えて記載を行った。

1　大分医科大学医動物学教室（〒879-56 大分県大分郡挾間町医大ケ丘1-1）
2　インドネシア国ボゴール農科大学獣医学部寄生虫・病理学部門昆虫学研究室

皮膚リーシュマニア症の治療
—Promastigote型原虫に対するアンチモン剤とMetronidazoleの薬効の比較—

矢後　文子

平成3年8月7日受付／平成3年10月1日受理

緒　言

リーシュマニア症の第一選択剤は，重金属であるアンチモン剤とされている（熱帯病治療薬の開発研究班，1990; Paul *et al.*, 1984; WHO, 1990）。かつて重金属は，医薬品として頻用されていたが，安全で有効な薬品が，開発されるようになったので，治療薬としての価値は激減した。しかし，現在でも使用されている，数少ない重金属にアンチモン剤がある。同剤は，化学的にも生物学的にもヒ素剤に類似し，重篤な副作用を伴う物質（グットマン，ギルマン，1975）なので，皮膚リーシュマニア症患者への投薬を，躊躇する医師も多い。内蔵リーシュマニア症と異なり，生命を脅かさない皮膚リーシュマニア症の治療に，時にはショック症状などの副作用を伴う治療薬は適当ではなく，より安全で日本でも入手し易い薬剤が望まれている。この度，サウジアラビアで皮膚リーシュマニア症に罹患し，5価のアンチモン剤を，10日間にわたり合計5,700 mg筋注後，約3カ月経過しても，皮疹から原虫が検出された患者を治療する機会を得た。本例以外にも，アンチモン剤の皮膚リーシュマニア症に対する有効性に，疑問が持たれる症例（Convit and Kerdel-Vegas, 1965; Rahim and Tatar, 1966; 土田ら，1989）がある。そこで，現在日本で使用されている代表的な抗原虫薬であるmetronidazoleで，皮膚リーシュマニア症が治癒したという報告（Peter, 1973; James and Stephen, 1975）があるので，アンチモン剤とmetronidazoleのpromastigote型原虫に対する薬効を，*in vitro*で原虫の運動性と増殖状態を示標に，比較検討した。

材料および方法

1）*Leishmania*原虫：

原虫は，サウジアラビアのブレイダ市に1987年6月より同年12月まで滞在し，皮膚リーシュマニア症に感染した51歳の日本人男性の右大腿伸側の皮疹より，1988年3月31日分離し（KN株），NNN培地（Leventhal and Cheadle, 1989）で継代して，使用した。

2）薬効試験：

アンチモン剤は，5価のPentostam®（Wellcome社，England，1 ml中に100 mgのpentavalent antimonyを含む。以下P剤と略記す）を，metronidazoleは，Flagyl®（塩野義製薬株式会社，基礎実験用原末，以下M剤と略記す）を使用した。試験管（内径1 cm×高さ10 cm）内に1.3 mlのNNN培地を斜面に固め，0.1 mlのpromastigote型原虫を含む生理的食塩水（以下原虫生食と略記す）を入れ，さらにP剤またはM剤を溶解した生理的食塩水（以下薬剤生食と略記す）を0.9 ml加え，25°Cで静置培養した。各群は，2本の試験管を使用した。薬剤の投入は，原虫生食と薬剤生食を同日に加えた初日投薬法と，原虫生食のみで3日間培養し，4日目に生虫を確認した後，薬剤生食を投入した4日後投薬法の2方法で行った。薬剤生食を投入後，少なくも14日間，

東京女子医科大学寄生虫学教室（〒162 東京都新宿区河田町8－1）

原虫の運動性と増殖状態を観察した。そして，14日目（または15日目）に，鞭毛または虫体部に自発的な動きが認められる原虫（以下運動性原虫と略記す）が観察されなくなった場合には，培地の液体部を0.1 mℓ採り，0.9 mℓの薬剤を含まない生食を入れた新培地に継代し，さらに少なくも14日間（通算28日間）培養（以下再培養と略記す）観察した。なお，P剤投薬群をP群，M剤投薬群をM群，初日投薬法を用いた実験群を初日投薬群，4日後投薬法を用いた実験群を4日後投薬群と略記した。

3）原虫の運動性と増殖状態の記録：

試験管内の原虫生食を充分撹拌後，スライドに1滴載せ，400倍率の顕微鏡下で5視野観察し，得られた1視野当たりの運動性原虫の最大数を，次の基準で記録した。

＋　　1視野の運動性原虫の最大数が10未満
＋＋　　1視野の運動性原虫の最大数が10以上100未満
＋＋＋　1視野の運動性原虫の最大数が100以上
－　　5視野中，運動性原虫が検出されない

なお，観察14日目までに，運動性原虫が認められなくなり，再培養しても運動性原虫が観察されなかった時には，死滅と推定した（矢後，1990）。

成　績

1）原虫の運動性を阻止するアンチモン剤とMetronidazoleの薬量の比較：

a）初日投薬群における阻止量

原虫の運動性を阻止するP剤とM剤の薬量を比

Table 1　The effects of pentavalent antimony and of metronidazole on the movement and multiplication of promastigotes in initial-day cultures*

mg†	0		1				3				5		7		9	
Drug	none		P‡		M‡		P		M		P	M	P	M	P	M
No.§	1	2	3	4	5	6	7	8	9	10	11‖	12‖	13‖	14‖	15‖	16
Day 0	·¶	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
2	++	+	++	+	+	+	−	−	−	+	−	−	−	−	−	−
3	++	+	+	++	++	++	−	−	+	−	−	−	−	−	−	−
4	+	+	++	+++	++	++	−	−	−	+	−	−	−	−	−	−
5	++	+	+	+++	++	++	−	+	−	−	−	−	−	−	−	−
6	+	+	+	++	+	+	−	+	−	+	−	−	−	−	−	−
7	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
8	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
9	++	++	+	+	++	+	−	+	−	−	−	−	−	−	−	−
10	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−
11	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
12	+	+	+	++	+	+	−	−	−	−	−	−	−	−	−	−
13	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
14	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−

* Promastigotes were cultured with a drug from the initial day.
† The dose was added to each test tube.
‡ Pentavalent antimony (Pentostam®) is abbreviated as P and metronidazole (Flagyl®) as M.
§ The assigned number of the test tube, as referred to in the text.
‖ Each group was examined in duplicates, but the results were the same in each case.
¶ Observations were made in 5 visual fields under a microscope, at a magnification of 400, and the movement and multiplication of promastigotes were recorded as follows: ·, not tested; −, moving promastigotes were not observed; +, maximum number of moving promastigotes per field was 9 or less; ++, the number of moving promastigotes was between 10 to 99; +++, the number was greater than 100.

較するために，各試験管に原虫生食0.1 mℓと，P剤またはM剤を1mg，3mg，5mg，7mgおよび9mgをそれぞれ0.9 mℓの生食に溶解した薬剤生食を同日に加え，原虫の運動性と増殖状態を14日間観察し，表1を得た。

1mgの薬剤を投入した群(以下1mg群と略記す)では，表1の試験管番号3および4(以下試番3-4と略記す)のP群と試番5-6のM群で観察されるように，原虫は薬剤生食投入後14日目まで生存し，対照の0 mg群（試番1-2）との差は，認められなかった。3mg群(試番7-10)でも，P剤を投入した試番7には，運動性原虫は観察期間中検出されなかったが，M剤を投入した試番9でも，運動性原虫は3日目に観察されたのみで，また試番10でも観察9日目以後には運動性原虫は検出されず，P群との著しい差はなかった。5mg，7mgそして9mg群でも，P群（試番11，13および15)，M群（試番12，14および16）ともに，運動性原虫は，観察2日目以後認められなかった。

すなわち，P剤およびM剤ともに，1mg群では，原虫の自発的な運動性は阻止できないが，運動性を投薬14日目までに阻止するには3mg以上，さらに2日目以後14日目まで阻止するには，5mg以上必要であった。しかし，P剤とM剤による差はなかった。

b）4日後投薬群における阻止量

初日投薬群で，原虫の自発的な動きが，観察期間中，一度も認められなかった5mg，7mgそして9mg群（表1の試番11-16）では，培地や原虫に原因がある可能性を否定できない。また，実際にサンドフライに刺咬された場合，掻痒感や疼痛で虫刺に気づいても，治療は原虫が増殖し，特有の皮疹が形成されてから，開始される場合が多い。そこで，より実状に添うモデル実験として，原虫生食0.1 mℓのみを3日間培養し，4日目に原虫が生存増殖しているのを確認してから薬剤生食を投入し，その後は初日投薬群と同様に観察を行い，表2を得た。

P剤およびM剤を1mg投入した試番3-6では，初日投薬群（表1の試番3-6）と同様に，原虫は

Table 2 The effects of pentavalent antimony and of metronidazole on the movement and multiplication of promastigotes in the 4th-day cultures*

mg†	0		1				3				5				7				9			
Drug	none		P†		M†		P		M		P		M		P		M		P		M	
No.†	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Day 0(4)‡	++†	++	++	+	++	++	++	++	++	+	+	+	++	++	++	++	++	++	++	++	+++	++
1(5)	++	+	+	+	+	+	+	+	+	++	−	−	+	++	+	−	+	+	−	−	+	+
2(6)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
3(7)	+	+	+	+	+	++	−	−	+	++	−	−	+	−	−	−	+	+	−	−	+	+
4(8)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
5(9)	+	++	−	−	−	+	−	−	+	+	−	−	−	−	−	−	−	+	−	−	−	+
6(10)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
7(11)	+	+	−	+	+	+	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−
8(12)	+	++	+	+	+	++	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−
9(13)	+	++	+	−	++	++	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
10(14)	+	+	+	−	+	++	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
11(15)	+	++	+	+	+	++	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
12(16)	+	+	+	+	+	++	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
13(17)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
14(18)	+	+	+	+	+	++	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

* Promastigotes were cultured without drugs for the first 3 days and a drug was added on the 4th day.

† Refer to the explanations of Table 1, ‡ The day in parentheses indicates the total number of days including preincubation for 3 days. Observations on day 0 were carried out just before addition of drugs.

14日目（培養18日目）まで生存した。5 mg，7 mgそして9 mg群（試番11-22）のP剤中では，投薬1日目より3日目以後には，運動性原虫は観察されなかったが，M剤でも，5 mg群の試番13で投薬9日目以後，試番14では3日目以後，7 mg群の試番17では5日目以後，試番18では7日目以後，9 mg群の試番21では5日目以後，試番22では7日目以後，それぞれ14日目まで，運動性原虫は観察されなくなった。

すなわち，1 mg群では，原虫はP剤またはM剤中で，14日目まで生存したが，5 mg，7 mgおよび9 mg群では，両剤中で投薬14日目までには，運動性が認められなくなった。しかし，運動性を停止させるのには，P剤よりM剤の方が日数を要した。

2）治療に使用される薬量の比によるアンチモン剤とMetronidazoleの原虫の運動性への阻止効果の比較：

P剤とM剤とでは，治療に使用する薬量が異なり，一般には1日に成人で，P剤は600 mgを1回筋注し，M剤は750 mgを，分3で経口投与する。しかし，M剤はアメーバ性赤痢や，ランブル鞭毛虫症でも，時には1日に通常の約3倍量の2,000 mgより2,400 mg（Martinez-Palomo, 1986; WHO,1990）を投薬することがある。そこで，P剤とM剤を，同量で比較するのは実際的ではないので，P剤の600 mgを基準にし，1量と表記すると，M剤の通常の使用量である1日750 mg（1錠250 mgを3錠）は1.25量，1,500 mg（6錠）は2.50量，そして2,250 mg（9錠）は3.75量になる。*in vitro*のモデル実験でも，P剤の1量を0.5 mg，1 mg，1.5 mgそして2 mgとした時，それぞれのP剤の薬量に相当するM剤の1量，1.25量，2.50量そして3.75量使用し，初日投薬法と4日後投薬法で，原虫の運動性と増殖状態を観察した。

a）P剤 0.5 mgを1.00量とした場合

P剤の0.5 mgを1.00量とし，M剤を同量の0.5 mg，1.25量の0.625 mg，2.50量の1.25 mgそして3.75量の1.875 mg溶解した薬剤生食を，原虫生食に加えて培養し，表3を得た。

初日投薬群（表3-a）のP剤の0.5 mg（試番3-4）では，原虫は14日間生存した。M剤の1.00量（試番5-6），1.25量（試番7-8）および2.50量（試番9-10）でも，P剤同様，原虫は14日間生存した。しかし，M剤の3.75量の1.875 mg群の試番11では，3日目より14日目まで，さらに再培養した28日目まで，運動性原虫は検出されなかった。

4日後投薬群（表3-b）でも，初日投薬群と同様にP剤の1.00量（試番15-16），M剤の1.00量（試番17-18），1.25量（試番19-20），2.50量（試番21-22）では，原虫は14日間生存した。しかし，M剤の3.75量の1.875 mg群の試番23では，11日目｛培養開始（以下培養と略記す）15日目｝以後，試番24では10日目（培養14日目）以後より，再培養した21日目（培養25日目）までは，運動性原虫は，検出されなくなった。

すなわち，P剤の0.5 mgを1.00量とした場合には，P剤の1.00量およびM剤の1.00量，1.25量および2.50量では，初日投薬法でも4日後投薬法でも，原虫の生存は阻止できなかったが，3.75量の1.875 mgでは，両法で運動性原虫の出現を一時的に阻止し得た。

b）P剤1 mgを1.00量とした場合

P剤の1.00量を前実験（表3）の倍量の1 mgとし，M剤をP剤と同量の1 mg，1.25量の1.25 mg，2.5量の2.5 mgそして3.75量の3.75 mg使用して，同様の実験を行い表4を得た。

初日投薬群（表4-a）では，P剤（1.00量）の試番3で，原虫は再培養した28日目まで生存した。しかし，M剤の1.25量の試番8では，5日目以後，2.50量の試番9では2日目以後，試番10では4日目以後，さらに3.75量の試番11および12では観察1日目より，それぞれ再培養した28日目まで，運動性原虫は検出されなかった。

4日後投薬群（表4-b）でも，P剤（1.00量）の試番15-16では，14日目まで原虫は生存したが，M剤の1.25量（試番19-20）と，2.50量（試番21-22）では，14日目までには運動性が消失した。なかでも，試番22では，再培養した21日目（培養25日目）まで，運動性原虫が観察されなかった。そして，3.75量の試番24では，再培養した28日目（培養32日目）まで，運動性原虫が観察されなかった。

すなわち，P剤の1.00量を1 mgとした場合，初日投薬法でも4日後投薬法でも，必ずしも原虫の

Table 3 The effects on the movement and multiplication of promastigotes of 1, 1.25, 2.50 and 3.75 units of metronidazole when pentavalent antimony at 0.5 mg is regarded as 1 unit

(a) Initial-day cultures*

Drug	none		P*		M*							
mg*	0		0.5		0.5		0.625		1.25		1.875	
Ratio	0		1.00		1.00		1.25		2.50		3.75	
No. *	1	2	3	4	5	6	7	8	9	10	11	12
Day 0	•*	•	•	•	•	•	•	•	•	•	•	•
1	++	+	+	+	+	+	+	+	++	++	+	−
2	+	++	+	+	+	+	+	+	+	+	+	+
3	++	++	++	+	+	++	+	+	++	+	−	−
4	•	•	•	•	•	•	•	•	•	•	•	•
5	++	+	++	++	++	++	++	++	+	+	−	−
6	•	•	•	•	•	•	•	•	•	•	•	•
7	++	+	++	++	++	++	++	++	++	++	−	+
8	++	++	+	++	++	++	+	+	+	+	−	+
9	++	++	++	++	+	+	++	++	++	+	−	+
10	++	++	+	+	++	++	++	++	++	++	−	+
11	•	•	•	•	•	•	•	•	•	•	•	•
12	++	++	+	+	+	++	++	++	+	++	−	++
13	++	+	+	+	+	++	+	+	+	++	−	++
14	++	+	+	+	++	++	++	++	+	++	−	+
21	•	•	•	•	•	•	•	•	•	•	−	•
28	•	•	•	•	•	•	•	•	•	•	−	•

(b) 4th-day cultures†

Drug	none		P		M							
mg	0		0.5		0.5		0.625		1.25		1.875	
Ratio	0		1.00		1.00		1.25		2.50		3.75	
No.	13	14	15	16	17	18	19	20	21	22	23	24
Day 0 (4) †	++	++	+++	++	++	++	++	++	++	++	++	++
1 (5)	•	•	•	•	•	•	•	•	•	•	•	•
2 (6)	++	++	+	++	++	++	+	++	+	+	+	+
3 (7)	++	++	+	+	++	+	+	+	+	+	+	+
4 (8)	++	++	+	+	++	++	++	++	+	++	+	+
5 (9)	++	+	+	+	+	++	+	++	+	+	−	+
6 (10)	+	++	+	+	+	++	++	++	+	++	−	+
7 (11)	•	•	•	•	•	•	•	•	•	•	•	•
8 (12)	•	•	•	•	•	•	•	•	•	•	•	•
9 (13)	++	++	+	+	+	+	+	+	+	+	+	+
10(14)	++	++	+	++	+	++	+	++	+	−	+	−
11(15)	+	+	++	++	+	+	++	++	+	+	−	−
12(16)	+	++	+	+	+	+	++	+	+	+	−	−
13(17)	•	•	•	•	•	•	•	•	•	•	•	•
14(18)	++	++	+	+	+	++	++	+	+	+	−	−
21(25)	•	•	•	•	•	•	•	•	•	•	−	−
28(32)	•	•	•	•	•	•	•	•	•	•	+	++

* Refer to the explanations in Table 1.

† Refer to the explanations in Table 2.

Table 4 The effects on the movement and multiplication of promastigotes of 1, 1.25, 2.50 and 3.75 units of metronidazole when pentavalent antimony at 1 mg regarded as 1 unit

(a) Initial day cultures*

Drug	none		P*		M*							
mg	0		1.0		1.0		1.25		2.5		3.75	
Ratio	0		1.00		1.00		1.25		2.50		3.75	
No. *	1	2	3	4	5	6	7	8	9	10	11	12
Day 0	•*	•	•	•	•	•	•	•	•	•	•	•
1	+	+	+	+	+	−	+	+	+	+	−	−
2	++	+	+	−	+	+	+	+	−	+	−	−
3	•	•	•	•	•	•	•	•	•	•	•	•
4	+++	+	−	−	++	+	+	+	−	−	−	−
5	++	++	+	+	++	++	++	−	−	−	−	−
6	++	++	+	−	++	++	++	−	−	−	−	−
7	++	++	+	−	++	++	+	−	−	−	−	−
8	++	+	+	−	++	++	++	−	−	−	−	−
9	•	•	•	•	•	•	•	•	•	•	•	•
10	•	•	•	•	•	•	•	•	•	•	•	•
11	++	++	+	−	+	++	++	−	−	−	−	−
12	++	+	+	+	++	++	++	−	−	−	−	−
13	++	++	+	−	++	++	++	−	−	−	−	−
14	++	++	+	−	++	+	+	−	−	−	−	−
21	•	•	+	−	•	•	•	−	−	−	−	−
28	•	•	++	−	•	•	•	−	−	−	−	−

(b) 4th-day cultures†

Drug	none		P		M							
mg	0		1.0		1.0		1.25		2.5		3.75	
Ratio	0		1.00		1.00		1.25		2.50		3.75	
No	13	14	15	16	17	18	19	20	21	22	23	24
Day 0 †(4)	++	+	++	+	+	+	+	+	++	++	++	+
1 (5)	++	+	++	++	+	+	+	++	++	+	++	+
2 (6)	+	++	+	+	+	++	++	+	++	+	+	+
3 (7)	•	•	•	•	•	•	•	•	•	•	•	•
4 (8)	+	+	+	+	++	+	+	+	++	++	+	+
5 (9)	++	++	+	+	+	+	+	+	+	+	+	+
6 (10)	•	•	•	•	•	•	•	•	•	•	•	•
7 (11)	+	+	+	+	+	+	+	+	−	+	−	−
8 (12)	+	+	+	+	+	+	+	+	+	+	+	+
9 (13)	+	+	+	+	−	+	+	+	−	+	−	−
10 (14)	+	+	+	+	+	+	+	+	−	−	−	−
11 (15)	+	+	+	+	+	++	−	+	−	+	−	−
12 (16)	•	•	•	•	•	•	•	•	•	•	•	•
13 (17)	•	•	•	•	•	•	•	•	•	•	•	•
14 (18)	+	+	+	+	++	+	−	−	−	−	−	−
21 (25)	•	•	•	•	•	•	++	++	++	−	−	−
28 (32)	•	•	•	•	•	•	++	+	+++	+++	+	−

* Refer to the explanations in Table 1.

† Refer to the explanation in Table 2.

運動性は阻止できないが，M剤の2.50量または3.75量では，初日投薬法では，投薬後少なくも4日目から再培養した28日目まで，4日後投薬法では，投薬後14日目までには，原虫の運動性を停止させ得た。

c）P剤1.5 mgを1.00量とした場合

P剤の1.00量を，表4の実験よりさらに0.5 mg増量し，1.5 mgとし，M剤をP剤と同量の1.5 mg，1.25量の1.875 mg，2.50量の3.75 mg，3.75量の5.625 mg使用し，同様な実験を行い，表5を得た。

初日投薬群（表5-a）では，P剤の1.00量を投薬した試番3で，培養6日目から15日目まで，また試番4では5日目から8日目，および再培養で，運動性原虫が観察されたが，M剤の2.50量（試番9-10）および3.75量（試番11-12）では，試番9の観察2日目に，運動性原虫が観察されたのみで，その後再培養しても，運動性原虫は観察されなかった。

4日後投薬群(表5-b)でも，P剤の1.00量(試番15-16)では，14日目まで運動性原虫が観察されたが，M剤では，1.25量（試番19-20）で13日目と14日目には，運動性原虫が観察されなくなり，さらに2.50量（試番21-22）および3.75量（試番23-24）では，投薬3日目（試番24）より10日目試番21）以後には，再培養しても，運動性原虫は検出されなかった。

すなわち，初日投薬群でも，4日後投薬群でも，P剤の1.00量（1.5 mg）では，原虫の運動性を阻止できないが，M剤の2.50量（3.75 mg）および3.75量（5.625 mg）では，原虫の運動性は，14日目までには消失し，再培養しても，運動性原虫は観察されなかった。

d）P剤2 mgを1.00量とした場合

P剤の1.00量を,表5の実験より,さらに0.5 mg増量し2 mgとし，同様な実験を行い，表6を得た。

初日投薬群（表6-a）では，P剤の1.00量（2 mg)の試番3-4では，観察1日目より，再培養した28日目まで，運動性原虫は認められなかった。しかし，M剤でも，1.00量の試番6では7日目以後，1.25量の試番7では5日目以後より，再培養したそれぞれ28日目まで，運動性原虫は観察されなかった。さらに，2.50量（試番9-10），3.75量（試番11-12）では，1日目または2日目以後より，再培養した28日目まで，運動性原虫は観察されなかった。

4日後投薬群(表6-b)でも，P剤の1.00量(試番15-16)では，投薬1日目より再培養した28日目まで，運動性原虫は検出されなかった。しかし，M剤でも1.00量(試番17-18)より3.75量(試番23-24)まで，少なくも投薬11日目以後より，再培養した28日目まで，運動性原虫は観察されなかった。

すなわち，1.00量を2 mgとした場合，P剤の1.00量中では，両投薬法で1日目以後，運動性原虫は観察されないが，M剤でも初日投薬群の2.50量および3.75量，4日後投薬群の1.00量，1.25量，2.50量および3.75量中では，再培養しても運動性原虫は観察されなかった。

考　察

皮膚リーシュマニア症の治療には，一般にpentavalent antimony（P剤）が使用されるが，metronidazole（M剤）により治癒したと考えられる症例報告がある。Peter (1973) は，パナマで感染した24歳の白人男性に，M剤を1日750 mg分3で，10日間投薬後，10日間の休薬期間を置いて1クールとし，2クールで治癒した例を，James and Stephen (1975) は，アフガニスタンで感染した24歳の白人男性に，同方法で3クール投与し，完治した例を報告している。また，サウジアラビアで感染し，局所凍結療法を2回施行し，さらにP剤を合計5,700 mg，10日間にわたって筋注したが，治癒しなかった51歳の日本人男性に，M剤を同方法で3クール投与し治癒した自験例（矢後，1991）などがある。ところが，M剤を無効または必ずしも有効とは認められないという報告もある。例えば，Griffiths and Sodeify (1976) は，南イランの24例に，投薬量や投薬方法の詳細は不明であるが，2重盲検法でM剤による治療を行い，何らかの効果が認められたのは6例のみで，10例は無効，6例は来院しなかったので結果は不明，2例は12カ月より21カ月の間に完治したが，自然治

Table 5 The effects on the movement and multiplication of promastigotes of 1.00, 1.50, 2.50 and 3.75 unit of metronidazole when pentavalent antimony of 1.5 mg is regarded as 1 unit

(a) Initial-day cultures*

Drug	none		P*		M*							
mg*	0		1.5		1.5		1.875		3.75		5.625	
Ratio	0		1.00		1.00		1.25		2.50		3.75	
No. *	1	2	3	4	5	6	7	8	9	10	11	12
Day 0	•*	•	•	•	•	•	•	•	•	•	•	•
1	•	•	•	•	•	•	•	•	•	•	•	•
2	++	+	−	−	+	+	−	−	+	−	−	−
3	•	•	•	•	•	•	•	•	•	•	•	•
4	+	++	−	−	+	+	−	+	−	−	−	−
5	++	+	−	+++	+	+	−	+	−	−	−	−
6	++	+	+	++	+	++	++	−	−	−	−	−
7	++	+	+	+	++	+	−	+	−	−	−	−
8	++	++	+	+	+	+	−	+	−	−	−	−
9	•	•	•	•	•	•	•	•	•	•	•	•
10	+	+	+	−	+	+	+	++	−	−	−	−
11	•	•	•	•	•	•	•	•	•	•	•	•
12	+	+	+	−	+	+	++	+	−	−	−	−
13	+	+	+	−	+	+	+	+	−	−	−	−
14	•	•	•	•	•	•	•	•	•	•	•	•
15	+	+	+	−	+	+	+	+	−	−	−	−
22	•	•	•	+	•	•	•	•	−	−	−	−
29	•	•	•	+	•	•	•	•	−	−	−	−

(b) 4th-day cultures†

Drug	none		P		M							
mg	0		1.5		1.5		1.875		3.75		5.625	
Ratio	0		1.00		1.00		1.25		2.50		3.75	
No.	13	14	15	16	17	18	19	20	21	22	23	24
Day 0 (4)†	++	++	++	++	+++	++	+	++	++	+++	++	++
1 (5)	+	++	++	++	++	+	+	++	+	+++	+	++
2 (6)	•	•	•	•	•	•	•	•	•	•	•	•
3 (7)	+	++	+	+	+	+	+	+	++	+	+	−
4 (8)	•	•	•	•	•	•	•	•	•	•	•	•
5 (9)	++	+	+	+	+	+	+	+	−	−	+	−
6 (10)	+	+	+	−	++	+	+	+	+	+	+	−
7 (11)	+	+	+	+	−	+	+	+	+	+	+	−
8 (12)	+	+	−	+	+	+	+	+	+	−	−	−
9 (13)	+	+	+	+	+	+	+	+	+	−	−	−
10 (14)	+	+	+	+	+	+	+	+	−	−	−	−
11 (15)	•	•	•	•	•	•	•	•	•	•	•	•
12 (16)	•	•	•	•	•	•	•	•	•	•	•	•
13 (17)	+	+	+	+	+	−	−	−	−	−	−	−
14 (18)	+	+	+	+	+	+	−	−	−	−	−	−
15 (19)	•	•	•	•	•	•	•	•	•	•	•	•
21 (25)	++	+	•	•	•	•	++	++	−	−	−	−
28 (32)	+	+	•	•	•	•	+	++	−	−	−	−

* Refer to the explanations in Table 1. † Refer to the explanation in Table 2.

Table 6 The effects on the movement and multiplication of promastigotes of 1.00, 1.25, 2.50 and 3.75 units of metronidazole when pentavalent antimony of 2 mg is regarded as 1 unit

(a) Initial-day cultures*

Drug	none		P*		M*							
mg*	0		2.0		2.0		2.5		5.0		7.5	
Ratio	0		1.00		1.00		1.25		2.50		3.75	
No. *	1	2	3	4	5	6	7	8	9	10	11	12
Day 0	•*	•	•	•	•	•	•	•	•	•	•	•
1	+	+	−	−	+	+	−	+	+	+	+	−
2	+	+	−	−	+	+	+	+	−	−	−	−
3	+	+	−	−	−	+	+	+	−	−	−	−
4	•	•	•	•	•	•	•	•	•	•	•	•
5	++	++	−	−	−	+	−	−	−	−	−	−
6	•	•	•	•	•	•	•	•	•	•	•	•
7	++	++	−	−	−	−	−	−	−	−	−	−
8	+	++	−	−	−	−	−	−	−	−	−	−
9	++	++	−	−	−	−	−	−	−	−	−	−
10	+	++	−	−	+	−	−	−	−	−	−	−
11	•	•	•	•	•	•	•	•	•	•	•	•
12	++	++	−	−	−	−	−	−	−	−	−	−
13	++	++	−	−	+	−	−	−	−	−	−	−
14	++	++	−	−	+	−	−	−	−	−	−	−
21	•	•	−	−	+	−	−	+	−	−	−	−
28	•	•	−	−	++	−	−	++	−	−	−	−

(b) 4th-day cultures†

Drug	none		P		M							
mg	0		2.0		2.0		2.5		5.0		7.5	
Ratio	0		1.00		1.00		1.25		2.50		3.75	
No.	13	14	15	16	17	18	19	20	21	22	23	24
Day 0 (4)†	++	+	++	+	++	++	++	++	++	++	+++	++
1 (5)	+	++	−	−	+	++	++	+	+	++	+	+
2 (6)	•	•	•	•	•	•	•	•	•	•	•	•
3 (7)	+	+	−	−	+	+	++	+	+	+	+	+
4 (8)	•	•	•	•	•	•	•	•	•	•	•	•
5 (9)	++	++	−	−	+	+	+	+	+	+	+	−
6 (10)	+	++	−	−	+	+	−	+	−	+	+	+
7 (11)	++	++	−	−	+	+	−	+	+	+	−	−
8 (12)	++	++	−	−	+	+	−	+	−	+	−	−
9 (13)	++	+	−	−	−	+	−	+	−	−	−	−
10(14)	•	•	•	•	•	•	•	•	•	•	•	•
11(15)	++	++	−	−	−	−	−	−	−	−	−	−
12(16)	•	•	•	•	•	•	•	•	•	•	•	•
13(17)	+	+	−	−	−	−	−	−	−	−	−	−
14(18)	++	++	−	−	−	−	−	−	−	−	−	−
21(25)	•	•	−	−	−	−	−	−	−	−	−	−
28(32)	•	•	−	−	−	−	−	−	−	−	−	−

* Refer to the explanations in Table 1.
† Refer to the explanation in Table 2.

癒の可能性もあると報告している。Walton *et al.* (1974)は，パナマ地峡で感染した14歳から52歳の6例に，M剤の投与を試みたが，6例中5例は無効であったと報告し，有効であった1例には，1日1,500 mgを分3で，10日間投薬後，10日間休薬し，再び20日間投薬した。このように，M剤が無効または必ずしも有効とは認められないという報告がある。症例数も少なく，投与法も一定していない上に，本症には自然治癒があり得るので，臨床例からM剤の有効性の有無に結論を出すのは難しい。そこで，原虫（KN株，promastigote型）に対する効果を，P剤とM剤を用いて，*in vitro* で，運動性と増殖状態を示標に実験した。まず，P剤とM剤を同量使用した初日投薬群（表1）では，薬量による両剤の効果に著しい差は認められないが，4日後投薬群（表2）では，M剤の方がP剤より，原虫の運動性を停止するのに日数を要した。例えば，5 mgのP群（表2の試番11-12）では，薬剤投入後1日目より，原虫の運動性が観察されなくなったが，M群の試番13では，運動性原虫が消失したのは9日目以後であった。7 mgでも，P群の試番15-16に比較すると，M群の試番17-18では，より長い日数原虫の動きが観察され，9 mgでも，同様であった。他にも例えば，表6-bのP群の2 mgの試番15-16では，投薬翌日より28日目まで運動性は停止したが，M群の試番17-18では，投薬9日目または11日目以後，運動性が停止した。このように，M剤の方がP剤より，原虫の運動性を停止するのに日数を要している。一般に，P剤の生体に対する薬理作用は，SH基と容易に反応することによると考えられている（American Society of Hospital Pharmacists, 1990)。しかし，リーシュマニア症に対する作用機序は，不明である(グッドマン，ギルマン，1988)。酵素などのSH基を阻害すると推定されるが確証はなく，また直接的な殺原虫作用も，組織との接触により活性化されることもないと考えられている。宿主であるヒトにも，SH基を持つ酵素などは存在するが，P剤がヒトより原虫に強い障害を与える理由として，グッドマン，ギルマン（1975）は，次のような可能性を考察している。(1)P剤がヒトの細胞より原虫に，より速やかに浸透する，(2)原虫がもつSH基の方が，ヒトのSH基よりP剤の作用を受けやすい，(3)ヒトより原虫の代謝経路の方が単純なので，SH基が障害を受け，代謝経路の一部が阻害された場合には，生体全体に及ぼす影響は，原虫の方に強い，(4)ヒトの方が原虫より代謝速度が速いので，P剤を速やかに他の無害な物質に変え得るなどである。また，きわめて広い抗原虫作用や，抗菌性のスペクトルを持つM剤の作用機序の詳細も，明らかではない。M剤は，生理的なpHではイオン化せず，容易に嫌気性菌や細胞内に取り込まれ，最終的にはDNAの分裂や合成を阻害し，殺菌作用や細胞障害を生ずると考えられている（American Society of Hospital Pharmacists, 1990)。P剤が，M剤より早期に原虫の運動性を停止するのは，P剤が原虫のSH基を阻害すると考えれば，核酸の合成を阻害すると考えられるM剤より，その効果は速やかに発現すると推定される。また，初日投薬法と4日後投薬法による差を，同量のM剤で比較すると，例えば，表1の初日投薬群（試番9，10，12，14および16）と，表2の4日後投薬群（試番9，10，13，14，17，18，21および22）では，4日後投薬群の方が，原虫の運動性を阻止するのに，より日数を要している。この現象も，4日後投薬群では，原虫の分裂増殖，すなわち核酸の合成が，初日投薬群よりある程度進行してから，M剤が投入されるので，薬剤の作用を受ける時期が遅延する原虫も生じ，また，原虫数も増加しているので，1原虫に対する薬量も少なくなり，運動性を停止するまでに，日数を要すると推定している。同様の現象は，表4，表5および表6の，それぞれのa表の試番5-12と，b表の試番17-24にも観察される。

薬物の効果を判定するのには，動物の半数を殺す50%致死量（LD_{50}値）や，目的とする薬物効果を動物の半数に発現させる50%有効量（median effective dose: ED_{50}値）などが用いられる。成績の項で得られた結果からは，分裂増殖中の原虫を，100%生存させる薬量の算出は困難なので，LD_{50}値に代わる値を次のように定義し，L値と名づけた。すなわち，投薬14日目（または15日目）または再培養で，試みた2試験管中に，ともに運動性原虫が認められる薬量の最高値を生存最高薬量，

また再培養した28日目までに，2試験管中にともに運動性原虫が観察されない薬量の最低値を死滅最低薬量とし，(生存最高薬量＋死滅最低薬量)÷2を求め，L値とした。まず，P剤の初日投薬群のL値を算出すると，生存最高薬量は表5-aの試番3-4の1.5 mgで，死滅最低薬量は，表6-aの試番3-4の2 mgである。そこで，L値は(1.5+2)÷2＝1.75 mgになる(L表参照)。同様に，P剤の4日後投薬群の生存最高薬量は，表5-bの試番15-16の1.5 mgで，死滅最低薬量は，表6-bの試番15-16の2 mg，したがって，L値は初日投薬群と同じ1.75 mgとなり，投薬法による差はなかった。同様に，M剤の初日投薬群の生存最高薬量は，表5-aの試番7-8の1.875 mgで，死滅最低薬量

L表

薬剤	投薬法	生存最高薬量	死滅最低薬量	L値
P	初日投薬群	1.5	2.0	1.75
	4日後投薬群	1.5	2.0	1.75
M	初日投薬群	1.88	2.5	2.19
	4日後投薬群	2.5	2.0	2.25

は，表4-aの試番9-10の2.5 mg，したがって，L値は(1.875+2.5)÷2≒2.19 mgになる。同値は，P剤の初日投薬群のL値の1.75 mgより，約0.44 mg高値である。同様に，M剤の4日後投薬群の生存最高薬量は，表4-bの試番21-22の2.5 mgで，死滅最低薬量は表6-bの試番17-18の2 mgで，生存最高薬量の方が，死滅最低薬量より高値で，前3者とは異なるが，型通り計算すると(2.5+2)÷2＝2.25 mgで，P剤の4日後投薬群の1.75 mgより高値である。これらのことから，M剤のL値は，P剤のL値より高値で，すなわち，P剤と同じ効果を生ずるには，P剤より多量の投薬が必要と推定される。

実験に使用した薬量と，ヒトの治療量を比較してみると，1試験管に投入したP剤またはM剤の薬量は，0.5 mg(表3)より9 mg(表1および2)である。薬剤が，培地のウサギ血液や寒天などに吸着されず，また変性などもなく，均等に溶解分布したと仮定すると，薬剤の最高濃度は，薬剤が原虫生食0.1 mlと薬剤生食0.9 mlの合計1 mlにのみ分布した場合で，最低濃度は1 mlの生食と1.3 mlのNNN培地の合計2.3 mlに均等に分布した場合である。原虫を死滅させ得るM剤の最低濃度を，L表より求めると，4日後投薬群で，P剤と同じ2 mgである。そこで，2 mgが培地の合計2.3 mlに均等に分布した場合，すなわち2 mg/2.3 ml≒0.87 mg/mlが，原虫を死滅させ得るM剤の最低濃度になる。同値は，他の原虫，例えばM剤が *in vitro* で，*Giardia lamblia* のほとんどの種に対して，発育を阻止する濃度の0.0008−0.032 mg/mlや，*Entamoeba histolytica* や *Trichomonas vaginalis* の0.003 mg/ml以下(American Society of Hospital Pharmacists, 1990)に比較すると，高濃度である。青河ら(1971)によれば，健康婦人5例に，M剤250 mgを，1回経口投与した時の血中濃度は，2時間値が最大で，比色法で0.02 mg/mlである。実験で得られた，最も低濃度である0.87 mg/mlは，青河らのヒトで得られた血中濃度の約44倍になり，*in vitro* で *Giardia lamblia* の発育を阻止する有効濃度の約27倍である。Mattock and Peters (1975)は，*in vitro* で，cell line中の *L. mexicana* のamastigote型原虫が，アンチモン剤のsodium stibogluconateにより変性を生ずるには，5-10 mg/mlという高濃度が必要であると報告し，実験で使用した種と実際の感染例の種の差を考察している。Beveridge (1963)は，ヒトやハムスターのリーシュマニア症に対して有効な5価のアンチモン剤が，promastigote型原虫には効きにくく，*in vitro* では生体内より高濃度が必要であると報告している。そして，原虫に5価のアンチモン剤が接触する前に，生体内では，代謝などにより3価になる必要性なども考察している。このように，*in vitro* と *in vivo*，または実験条件により，有効濃度に差が生ずる原因には，原虫の種の差やamastigote型とpromastigote型の差，発育や分裂過程の差や，生体内では，薬剤が代謝され他の物質に変化したり，他の物質との相乗作用などもあり，特にM剤は，クロラムフェニコールなどにも認められるようにpHに依存し，低酸や無酸ではバイオアベイラビリティの低下(Ogata *et al.*, 1985)が生ずるので，経口投与による胃酸との接触が必要であり，

また体内では光による分解などもないなどの，多くの条件の差によるのであろうと推察している。

5価のアンチモン剤が，一般的には内蔵型に有効と考えられているのに，皮膚型には著しい効果が認められないとする報告（Convit and Kerdel-Vegas, 1965; Rahim and Tartar, 1966; 土田ら，1989；矢後，1991）があるのは，アンチモン剤の体内での分布の差にも，原因があると考えられる。5価のアンチモン剤は，肝臓や脾臓により高濃度に分布するので，皮膚に寄生する，例えば *L. tropica* より，肝臓や脾臓に寄生する *L. donovani* の方に高濃度で作用し，結果的に内蔵型に有効になる可能性がある。一方，M剤は全身に比較的均等に分布し，組織内の濃度は，多くの場合血清と同じである（Ralph, 1983）。したがって，P剤とM剤を同量投与しても，肝臓や脾臓に高濃度に分布するP剤の皮膚の濃度より，体内に均等に分布し，蓄積作用もあるM剤（Ralph, 1983）の皮膚の濃度のほうが高くなり，M剤は皮膚リーシュマニア症に，より有効なのであろうと推察している。

本実験は閉鎖系なので，薬剤の投与は1回のみで実験を行ったが，M剤の服薬を繰り返すと，ヒトの血中や組織内の濃度は増強する。Ralph（1983）によれば，M剤500 mgを1回経口投与または静注した時の平均血中濃度は，約0.01 mg/mℓであるが，500 mgを8時間毎に静注すると，血中濃度は0.015-0.025 mg/mℓになる。自験例（矢後，1991）では，血中濃度は測定してないが，臨床症状から蓄積作用と考えられる所見が得られた。すなわち，M剤を1日750 mg，分3で10日間投薬後，10日間の休薬期間を1クールとして，3クール投薬すると，1クール目では特別な副作用は認められなかったが，2クール目では，服薬開始5日目より1日1-3回の下痢が始まり，口中に金属様の酸味感を生じた。3クール目では，下痢の始まりも服薬開始4日目と早くなり，回数も1日2-3回と増加し，服薬終了後も継続し，口中の金属様の酸味感も，服薬13日目に少し軽減したにすぎなかった。一方，P剤には蓄積作用はなく，腎からの排泄は速やかで，筋注後約6時間以内に，投与した80%以上が尿中に出現する（グッドマン，ギルマン，1988）。通常，P剤は成人1日1回600mg（または10 mg/kg）を，6日間から10日間筋注し，10日間休薬する（Peters and Killick-Kendrick, 1987; Reynolds, 1989）。ところが，近年，特に内蔵リーシュマニア症には，休薬期間は不要で，より大量をより長期に，例えば1日850 mgを，少なくも20日間投与する治療法（WHO, 1984）がある。しかし，P剤の副作用には，関節痛，頭痛，腹痛，顔面浮腫，嘔吐を伴う激しい咳，肝炎，徐脈，呼吸困難，ショック症状などが知られているので，皮膚リーシュマニア症のように，感染部位が皮膚に限局し，生命を脅かさない疾患の治療に，重篤な副作用を持つ薬剤を，大量に長期間投薬するのは控えたい。一方，M剤はランブル鞭毛虫症やトリコモナス症などの治療には，1日750 mgを分3で，5日間より10日間投薬するが，重症の嫌気性細菌感染症では，1日4 gを3週間使用することがある（American Society of Hospital Pharmacists, 1990）。このように，M剤は，P剤より大量を長期間経口投与でき，その副作用も末梢神経障害の他は軽微で，同障害も服薬を中止すると治癒し（Bradley *et al.*, 1977），原虫に対する薬効も，*in vitro* の実験結果からは，P剤と著しい差がないので，現時点では試みる治療剤の1つと考えられる。

結　論

皮膚リーシュマニア promastigote 型原虫に対するアンチモン剤（pentavalent antimony, Pentostam® 使用，P剤）と metronidazole（Flagyl® 使用，M剤）の薬効の差を，*in vitro* で原虫の運動性と増殖状態を示標に比較した。試験管内に斜面につくった1.3 mℓのNNN培地に，原虫を含む0.1 mℓの生理的食塩水（原虫生食）と，薬剤を溶解した0.9 mℓの生理的食塩水（薬剤生食）を加え，少なくも14日間観察した。薬剤の投入は，薬剤生食と原虫生食を同日に加えた初日投薬法と，原虫生食のみで3日間培養し，4日目に薬剤生食を加えた4日後投薬法の2方法で行い，次の結論を得た。

(1) 原虫生食に，P剤またはM剤を，それぞれ

1mg, 3mg, 5mg, 7mgおよび9mg溶解した薬剤生食を加えると，1mg中では，原虫は両剤の両投薬法で，14日間生存した。5mg, 7mgおよび9mg中では，両剤の両投薬法で，14日後までには，自発的な運動性を示す原虫（運動性原虫）は，観察されなくなった。

(2) P剤の0.5 mg, 1 mg, 1.5 mgおよび2 mgをそれぞれ1.00量とし，M剤をP剤の1.00量，1.25量，2.50量および3.75量になるように原虫生食に加えると，P剤の1 mgを1.00量とした時の，M剤の初日投薬法の2.50量，3.75量，およびP剤の1.5 mgを1.00量とした時の，M剤の初日投薬法および4日後投薬法の2.50量および3.75量中では，P剤では原虫は生存できても，M剤中では14日後までには運動性は消失し，再培養しても運動性原虫は，観察されなかった。

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TREATMENT FOR CUTANEOUS LEISHMANIASIS —A COMPARISON BETWEEN THE EFFECTS OF METRONIDAZOLE AND PENTAVALENT ANTIMONY ON THE MOVEMENT AND MULTIPLICATION OF *LEISHMANIA* PROMASTIGOTES—

AYAKO YAGO

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The effects of metronidazole and of pentavalent antimony (P) on the movement and multiplication of *Leishmania* promastigotes were compared. *Leishmania* was isolated from the skin of a Japanese man who had spent time in Saudi Arabia. The promastigotes were cultured at 25°C in test tubes that contained 1.3 m*l* of NNN medium and 1.0 m*l* of 0.85w/v% NaCl. Metronidazole or P was added to cultures by one of two protocols. Either promastigotes were cultured with the drug of choice from the initial day and these cultures were called initial-day cultures. Alternatively, promastigotes were cultured for 3 days initially without drugs and then a drug was included from the 4th day. These cultures were called 4th-day cultures. The movement and multiplication of promastigotes were observed for at least 14 days after initial exposure to either drug.

(1) Promastigotes were alive in the tubes that contained 1 mg of metronidazole or P until the 14th day of exposure to either drug in both initial-day cultures and 4th-day cultures, but their movements were not observed in the tubes that contained 5 mg, 7 mg or 9 mg of metronidazole or P after a 14-day exposure to either drug in both cultures.

(2) In a clinical setting, P is injected as 600 mg (defind here as 1 arbitrary unit) per adult per day and metronidazole is given at a dose of 750 mg (1.25 units), 1,500 mg (2.50 units) or 22,500 mg (3.75 units). P at 0.5 mg, 1 mg, 1.5 mg and 2 mg was regarded in separate tests as equivalent to 1 unit and metronidazole at 1.00, 1.25, 2.50 and 3.75 times each dose of P was added to promastigotes. As shown in tube No. 3 in Table 4 and tube No. 3 and No. 4 in Table 5, the parasites survived in the presence of 1 mg or 1.5 mg (1 unit) of P, but their movements were not observed in the presence of 2.5 mg or 3.75 mg (2.5 units) and 3.75 or 5.625 mg (3.75 units) of metronidazole in initial-day cultures. In 4th-day cultures, as shown in Table 5, the parasites also survived with 1.5 mg (1 unit) of P, but their movements were not observed with 3.75 mg (2.5 units) and 5.625 mg (3.75 units) of metronidazole. In these conditions, the effects of metronidazole were superior to the effects of pentavalent antimony.

Department of Parasitology, Tokyo Women's Medical College, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162, Japan

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