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SURFACE LECTIN BINDING CHARACTERISTICS OF DEVELOPING STAGES OF *BRUGIA* IN *ARMIGERES SUBALBATUS*: I *BRUGIA PAHANGI*

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Abstract: Surface characteristics of microfilariae and developing stages of *Brugia pahangi* in its natural vector, *Armigeres subalbatus* were assayed using fluorescein isothiocynate conjugated lectins. The following lectins were used: wheat germ agglutinins, lentil agglutinins, *Helix aspersa* agglutinins, Concanavalin A agglutinins, kidney bean agglutinins, asparagus pea agglutinins and pea nut agglutinins. It was observed that developing stages of *B. pahangi* the mosquito showed a dynamic surface carbohydrate characteristics. The larvae change their surface coat configuration frequently during the 10 day observation period. However, blood dwelling microfilariae and fully matured infective larvae obtained from the mosquito's head showed little or no binding affinity for the lectins tested. It's postulated that the rapid turnover of the surface carbohydrates, while development of the larvae is taking place, is the worm's response to a 'hostile' mosquito environment.

INTRODUCTION

Studies on the structural as well as histochemical nature of filarial worm surface (Mclaren, 1972; Lumsden, 1975; Martinez-Palomo, 1978; Bird, 1980; Cherian *et al.*, 1980; Sayers *et al.*, 1984) suggest that the cuticle is a fluid structure in which surface components are continuosly being shed and replaced (Philipp *et al.*, 1980; Maizels *et al.*, 1984). However, the evidence for turnover of surface components of filarial nematode is scarce (Marshall and Howells, 1986; Howells and Blainley, 1983).

Recently, a number of lectin binding studies on surface characteristics of filarial worms have been reported (Furman and Ash, 1983a, b; Forsyth *et al.*, 1984; Kaushal *et al.*, 1984; Devaney, 1985; Taylor *et al.*, 1986; Nwachukwu *et al.*, 1987; Rao *et al.*, 1987a, b, 1988; Schraemeyer *et al.*, 1987). Histochemical studies have revealed carbohydrate residues on their cuticular surface (Simpson and Laurence, 1972; Laurence and Simpson, 1974; Cherian *et al.*, 1980; Sayers *et al.*, 1984). Though these reports are numerous most were done on either the microfilariae (mf), infective stages (L3) or the adults. Little is known about the composition and properties of the surface coats of developing stages of filarial worms in their

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insect hosts. Ham *et al.* (1988) had observed dynamic changes in surface carbohydrate moieties of larval stages of *Onchocerca lienalis* in *Simulium ornatum.*

The aim of the present study was to determine whether developmental stages of *Brugia pahangi* in its natural vector, *Armigeres subalbatus,* possess different carbohydrate groups on their surface coats. The significance of these changes in relation to the evasion of the mosquitoes immune responses by the parasite is discussed.

Materials and Methods

Ar. subalbatus at ages 7-10 days were fed directly onto an anaesthetized *B. pahangi* infected-cats. At the same time, 0.5 m*l* of the cat blood was withdrawn through jugular puncture, mixed with a drop of EDTA (1 mg per drop) and kept for isolation of mf. The peripheral mf concentration of the cats ranged from 120-200 mf/20 μl.

Isolation of mf:

A half m*l* blood from a cat infected with *B. pahangi* was thoroughly mixed with 9.5 m*l* of distilled water. The mixture was then passed through a 5 μm Nuclepore membrane, followed by two saline washes. The Nuclepore membrane was then removed from the filter-chamber and placed in 2 m*l* PBS and kept at 37°C until used.

Both sheathed and exsheathed mf were used in the lectin-binding studies. Exsheathed mf were obtained from two sources i.e. (i) from the haemocoel of the mosquitoes and (ii) chemically exsheathed mf. Exsheathment of mf was done by exposing the mf to high concentration of calcium ions in medium 199. In the original protocol of Devaney and Howells (1979) phosphate-free HBSS with 10 mM Ca^{++} and above gave an *in vitro* exsheathment rate of more than 80% within an hour. In the present study, the Ca^{++} concentration in medium 199 was increased to 20 mM (normal medium 199 contained approximately 1.8 mM Ca^{++}). Magnesium sulphate was also added to the medium at 0.2 mg/m*l*. Microfilariae begun to exsheath within 60 min at 37°C; most of the worms were half-body length through the sheath. More than 72% of the mf exsheathed within 90 min (Table 1). Many mf were covered with calcium phosphate crystals at the end of the incubation period; two or more washings in normal medium 199 or PBS removed these crystals.

Developing larval stages:

Mosquitoes were dissected every 24 hours post-infection (p.i.) over a period of 10 days.

Table 1 Exsheathment of microfilariae in Medium-199 containing 20 mM calcium chloride

Incubation period (min)	Percentage exsheathment (No. exsheathed/Total mf)			x±s.e.
	Replicate			
	1	2	3	
30	2.9 (3/102)	42.4 (75/177)	0 (0/76)	15.1±13.7
60	35.7 (15/42)	42.9 (48/112)	5.9 (3/51)	28.2±11.3
90	61.2 (60/98)	84.6 (33/39)	70.2 (33/47)	72.0±6.8
120	94.4 (51/54)	88.5 (115/130)	77.4 (48/62)	86.8±4.9
180	90.2 (165/183)	80.7 (50/62)	94.7 (72/76)	88.5±4.1
240	92.0 (138/150)	94.2 (97/103)	100 (43/43)	95.4±2.4

Larvae were removed with the aid of flame-drawn capillary tubes and placed into individual cavity slides containing 25 μl of PBS (pH 7.2).

Though L3 was found in the thorax as early as day 7 p.i., larvae were not assayed until day 10 when the infective stages were found in all parts of the mosquitoes. Thus L3 tested on day 10 were in different stages of maturation.

Lectins:

Fluorescein isothiocynate (FITC)-conjugated lectins (Sigma Co., U.K.) and their complementary sugars used were as listed in Table 2. All lectins were made up to a concentration of 50 $\mu g/ml$ in PBS (pH 7.2) and the inhibitory sugars to a 0.1 M concentration.

Lectin-binding experiments:

The binding and inhibition assays were carried out in glass cavity slides. At least 5 parasites were placed per cavity. The PBS-suspending medium was removed prior to the addition of 50 μl of either lectins or lectin-sugar mixture. The cavity was then topped with a glass coverslip, sealed with vaseline and incubated in a humid chamber at 25°C for 30 min (Rao *et al.*, 1987). PBS and sugar controls were included in each assay.

After incubation, the parasites were washed twice in PBS before being examined. Washed parasites were resuspended in PBS in cavity slides and examined as a wet mount under an incident UV light microscope (Reichert-Jung-Polyvar; Filter system B1).

Fluorescent staining was graded as 0=Negative, no staining; +=Positive, weak staining; ++=Bright fluorescence; +++=Intense fluorescence.

Observations were made in duplicate. Whenever possible, parasites were obtained from the same mosquitoes for each series of lectins.

Table 2 Lectins and their complementary sugar inhibitors

Lectin (Abbrevation)	Sugar specificity
Wheat germ agglutinins, *Triticum vulgaris* (WGA)	N-acetyl-D-glucosamine
Lentil, *Lens culinaris* (LCH)	N-acetyl-D-glucosamine, α-D(+)-glucose, D-mannose, α-methyl-D-mannoside
Helix aspersa (HAA)	N-acetyl-D-glucosamine, N-acetyl-D-galactosamine
Concanavalin A, *Canavalia ensiformis* (Con A)	N-acetyl-D-glucosamine, D(+)-glucose, D(+)-mannose, Methyl-α-D-mannopyranoside
Kidney bean, *Phaseolus vulgaris* (KBA)	N-acetyl-D-glucosamine
Asparagus pea, *Tetragonolobus purpureus* (APA)	N-acetyl-D-glucosamine, L(−)-fucose, D(+)-mannose, Methyl-α-D-mannopyranoside
Pea nut, *Arachis hypogaea* (PNA)	D(+)-galactose

Table 3 FITC-conjugated lectin binding on *Brugia pahangi* microfilariae

Microfilariae	Region affected	PNA	WGA	LCA	APA	HAA	Con A	KBA
Sheathed MFF	Sheath		+					
	Cuticle							
	Anal pore							
	Excretory pore		++					
	Entire worm							
Chemically† exsheathed microfilariae	Cuticle							
	Anal pore							
	Excretory pore							
	Entire worm							
Exsheathed microfilariae from haemocoel	Cuticle							
	Anal pore							
	Excretory pore		++					
	Entire worm							
Melanized microfilariae	Entire worm‡	++	+			++	++	
		1				2, 3		

†: Calcium exsheathed microfilariae.
‡: Binding was onto the melanized capsular material.
Inhibitors: 1=D(+)-galactose, 2=N-acetyl-D-galactosamine, 3=N-acetyl-D-glucosamine
Fluorescence intensity: +++ intense, ++ moderate, + poor

Results

None of the lectins tested except wheat germ agglutinin (WGA) bound onto the sheathed and exsheated mf (Table 3). A + and ++ fluorescence with WGA was observed on the sheath and excretory pore respectively. Haemocoel-exsheathed and Ca^{++} exsheathed mf did not show any significant differences in their cuticular surface lectin binding characteristics; none bound any of the lectins tested. The excretory pore of haemocoel-exsheated mf however bound WGA whereas Ca^{++} exsheathed mf did not. N-acetyl-D-glucosamine (GlcNAc) inhibited neither WGA binding to the sheath nor the excretory pore.

All lectins except lentil lectin (LCA), asparagus pea agglutinin (APA) and kidney bean agglutinin (KBA) bound onto the surface of melanized mf. A ++ fluorescent staining of melanized granular deposits on the melanized mf was observed. The staining by *Helix aspersa* agglutinin (HAA) was blocked by GlcNAc and N-acetyl-D-Galactosamine (GalNAc), and pea nut agglutinin (PNA) by D(+)-galactose (Gal). WGA and Concanavalin A (Con A) binding were not inhibited by any of their complementary sugars.

Fluorescent staining of developmental stages of *B. pahangi* in *Ar. subalbatus* is shown in Table 4. The degree of staining and types of lectins involved varied as differentiation and development of these stages occurred. Con A, HAA, KBA and WGA bound weakly to the worm on day 3 p.i. A strong positive fluorescent staining was not detected on the cuticular surface of larval developing stages till day 4 p.i. when KBA bound onto the entire surface of the worm. Also WGA binding was seen at day 4 p.i. at both the anterior and posterior ends

Table 4 FITC-conjugated lectin binding onto developing stages of *Brugia pahangi*

Day P.I.	Region of larvae	PNA	WGA	LCA	APA	HAA	Con A	KBA
1	Anterior one-third							
	Excretory pore		++				+	
	Middle one-third							
	Anal plug		++			++ 1		
	Posterior one-third							
	Entire worm							
2	Anterior one-third							
	Excretory pore		+					
	Middle one-third							
	Anal plug		++	+++ 8			++ 5,1	
	Posterior one-third							
	Entire worm							
3	Anterior one-third							
	Excretory pore		+				++	
	Middle one-third							
	Anal plug	+++ 6	++	++			+	
	Posterior one-third							
	Entire worm		+			+ 4,1	+ 1	+
4	Anterior one-third		+					
	Excretory pore	+++						+
	Middle one-third							
	Anal plug		+++					+
	Posterior one-third		++					
	Entire worm							++
5	Anterior one-third				+ 5			
	Excretory pore		+				+ 8	
	Middle one-third							
	Anal plug	+++ 6	+++	++	+ 5,8	+++ 1,4	+++ 8	+++
	Posterior one-third				+ 5,7			
	Entire worm	+		++				+++
6	Anterior one-third							
	Excretory pore					++ 1,4		
	Middle one-third							
	Anal plug	+++ 6	+++	+++ 1	+ 1	+++ 1,4	+++	
	Posterior one-third							
	Entire worm	+ 6		+			+	+++

Table 4 (Cont.)

Day P.I.	Region of larvae	PNA	WGA	LCA	APA	HAA	Con A	KBA
7	Anterior one-third							
	Excretory pore	++ 6						
	Middle one-third	+ 6		++ 9				
	Anal plug	+++ 6	+++	+++			+++ 1	+++
	Posterior one-third							
	Entire worm			+		++ 1,4	++ 1	
8	Anterior one-third							
	Excretory pore							
	Middle one-third							
	Anal plug	+++ 6	+++			+++ 1,4	++	++
	Posterior one-third							
	Entire worm	++ 6					+ 5	+
9	Anterior one-third							
	Excretory pore							
	Middle one-third							
	Anal plug	+++ 6	++ 1	++ 1,9		+++ 1,4	++ 1	+ 1
	Pesterior one-third							
	Entire worm							

Inhibitors: 1=N-acetyl-D-glucosamine, 4=N-acetyl-D-galactosamine, 5=Methyl-α-D-mannopyranoside, 6=D(+)-galactose, 7=L(−)-fucose, 8=D(+)-mannose, 9=α-D(+)-glucose

Fluorescence intensity: +++ intence, ++ moderate, + poor

of the larvae. Both GalNAc and GlcNAc inhibited HAA binding to the cuticular surface at day 3 p.i. whereas Con A was inhibited only by GlcNAc (Table 4). Neither WGA nor KBA were inhibited by any of the sugars tested at day 4 p.i. At day 4, the larvae were in their late first-stage (L1).

During the first four days of development of L1, all lectins tested except APA showed positive binding to either the excretory pore or the anal pore. GlcNAc did not interfere with WGA binding, neither to excretory pore nor to the anal pore. D-mannose (Man) and methyl-α-D-mannopyranoside (ManMe) reduced the intensity of LCA and Con A binding to the anal pore respectively at day 2 p.i. GlcNAc did not interfere with Con A binding to the excretory pore at day 1 p.i. HAA and Con A binding to the anal pore at day 1 p.i. and day 2 p.i. respectively were both inhibited by GlcNAc. A different set of lectin receptors for WGA, LCA and Con A appeared at day 3; none of the sugar inhibitors interfered with their binding to either the excretory or anal pore. PNA binding to the anal pore was blocked by Gal.

Con A, HAA and KBA all gave a weak fluorescent staining along the entire length of the worm at day 3 p.i. Both GalNAc and GlcNAc inhibited HAA-binding to the cuticular surface

though Con A was blocked only by GlcNAc.

Only WGA and KBA bound onto larvae isolated on day 4 p.i. Intense binding of WGA to the excretory pore and anus was observed in contrast to a weak binding seen on larvae on day 3. KBA bound weakly along the entire length of the larvae surface. Neither WGA nor KBA binding were inhibited by their complementary sugars.

At day 5 p.i., PNA, LCA and KBA bound along the entire length of the worm whereas APA bound only to the anterior and posterior ends. The anal pore bound all lectins tested.

Though the intensity of PNA binding was + on the cuticular surface and +++ on the anal plug, both were equally blocked by the sugar. WGA, LCA and KBA showed on change in binding when incubated with their complementary inhibitory sugars. ManMe blocked APA binding to the anterior and posterior cuticular surfaces and the anus, whereas Man and L(−)-Fucose (Fuc) inhibited binding to only the anus and the posterior cuticular surface respectively. Both GlcNAc and GalNAc interfered with HAA binding to the anus. Man inhibited Con A binding to both the excretory pore and the anal plug.

At day 6, Con A (in addition to PNA, LCA and KBA) bound to the entire worm. At the same time the anterior and posterior ends lost their receptors for APA. Gal blocked binding of PNA onto the worm; none of the sugar inhibitors of Con A and KBA interfered with lectin binding. The anal plug bound all lectins except KBA at day 6 p.i. HAA, APA and LCA were all inhibited by GlcNAc; GalNAc blocked HAA binding to the anal plug.

At day 7 p.i., further changes to the surface carbohydrates occurred. Whereas APA and HAA lost their receptors on the anal plug, KBA receptors reappeared. Only LCA and Con A, in addition to HAA, bound the entire cuticular surface. LCA binding to the middle-third of the cuticular surface of the worm was blocked by Glc whereas the rest of its surface did not. Though the intensity of Con A binding to anal plug and cuticular surface differed, both were equally blocked by GlcNAc. Man inhibited Con A binding to the middle- and posterior -third of the cuticular surface but not to the anal plug; the anal plug was blocked instead by GlcNAc. Both GalNAc and GlcNAc blocked HAA binding to the worm. PNA binding to the middle-third of the cuticular surface of the worm, the anal plug and excretory pore were inhibited by Gal.

The worms lost their LCA and HAA receptors on day 8 p.i. At the same time, cuticular receptors for KBA and PNA reappeared. PNA binding was inhibited by Gal; GlcNAc did not interfere with KBA binding onto the worm. Whereas ManMe blocked Con A binding onto the worm, Man blocked only Con A binding to the middle-third of the cuticular surface. All lectins except LCA and APA bound onto the anal plug. Both GalNAc and GlcNAc inhibited HAA binding and none of the sugar inhibitors of Con A and WGA blocked their binding onto the anal plug.

A dramatic change in lectin binding was observed at day 9 p.i. The entire cuticular surface seemed to have lost its carbohydrate moieties. All lectins tested except APA bound only to the anal plug. Con A, HAA, KBA, LCA and WGA binding to the anal plug were inhibited by GlcNAc and PNA by Gal. Also, LCA and HAA were inhibited by Glc and GalNAc, respectively. All larvae used in these experiments were in their late second-stage (L2). Some very advanced L2 were seen though none had moulted to L3 at day 9 p.i.

At day 10 p.i., L3 were seen in the head, thorax and abdomen. Early L3 from the thorax gave fluorescent staining of the anus and preanal cuticular surface with WGA (Table 5). None of the mature L3 in the thorax bound any lectins tested. Stage-3 larvae (L3) isolated

Table 5 FITC-conjugated lectin binding onto *Brugia pahangi* infective stage larvae (L3) cuticular surface

Source	Region of larvae	PNA	WGA	LCA	APA	HAA	Con A	KBA
Head	Anterior one-third			++	++			
	Middle one-third							
	Anus and anal cavity							
	Posterior one-third							
	Entire one-third							
	Entire worm							
Thorax	Anterior one-third							
	Middle one-third							
	Anus and anal cavity							
	Posterior one-third							
	Entire worm							
Abdomen	Anterior one-third			++				
	Middle one-third							
	Anus and anal cavity	+++						
	Posterior one-third							
	Entire worm			+				

Fluorescence intensity: +++ intence, ++ moderate, + poor

from the abdomen gave positive fluorescent staining of the anus with WGA, buccal cavity with KBA and APA and the cuticular surface of the anterior end with LCA. An L3 obtained from the abdomen bound weakly with lentil along its entire cuticular surface. Most of the L3 obtained from the head did not bind any of the lectins tested. One L3 however bound LCA onto its anterior end and another bound Con A onto its anus. The worm which bound Con A to its anus was an early L3 stage; probably it had accidentally migrated from the thorax into the head before attaining full maturity. Buccal cavity contents of L3 from the head bound all except PNA and HAA.

None of the sugar inhibitors blocked lectin binding to the buccal cavity (and its contents) or anterior end of L3 isolated from the head of the mosquitoes. However, binding of APA to the buccal cavity of L3 from the abdomen was blocked by Man and GlcNAc; KBA binding to the buccal cavity was not inhibited by GlcNAc. None of the complementary sugar inhibitors blocked WGA and LCA binding to the anus or the anterior-third of the cuticular surface of the worm.

Lectins also bound weakly to the buccal cavity (and its contents) of various larval stages (Table 6). HAA bound to the pharyngeal thread of one- and 3-day olds and to the buccal cavity of late L2. The carbohydrate moieties responsible for HAA binding differed with the stage of developing larvae. PNA bound onto the buccal cavity of 3-day olds and the binding was inhibited by Gal. None of the larvae dissected-out on day 4 and day 5 p.i. bound any of the lectins tested. Six-day olds larvae bound PNA and WGA and 7-day olds with KBA, LCA and WGA; none of these bindings were inhibited by their respective complementary sugars. Eight-day olds bound HAA, KBA and WGA; both KBA and WGA bindings were not inhibited by their specific sugars. Late L2 on day 9 bound all lectins except APA. GlcNAc interfered

with LCA, HAA and Con A binding. PNA binding to the buccal cavity was blocked by Gal; and LCH and Con A were also blocked by Man and α-D(+)-glucose (Glc) respectively. None of the L3 collected from the thorax of the mosquitoes bound any lectins onto their buccal cavity. Those from the abdomen bound only KBA. L3 obtained from the head bound all except HAA and PNA; APA binding to the buccal cavity was inhibited by Man and GlcNAc.

Discussion

Surface lectin binding characteristics of mf and developing stages of *B. pahangi* in *Ar. subalbatus* were assayed using FITC-labeled lectins. Blood derived *B. pahangi* mf bound only WGA onto their sheaths and excretory pore; similar observations were reported by others (Furman and Ash, 1983a,b; Devaney, 1985; Rao *et al.*, 1988). Also Furman and Ash (1983b) and Sayers *et al.* (1984) observed Con A binding onto the sheath. In addition, Sayers *et al.* (1984) noted Con A binding onto the epicuticle of blood-derived *B. pahangi* mf while Furman and Ash (1983a) did not observe binding of any of the lectins tested (*Limulus polyphemus,* pea nut, *Ricinus communis* and soybean lectins) to either the epicuticle of mature or *in utero* derived mf. The sheath of *in utero*-derived mf, however, bound *Limulus polyphemus,* pea nut, *Ricinus communis* and soybean lectins (Furman and Ash, 1983).

Exsheathment of mf, either naturally in the mosquito's haemocoel or chemically-induced,

Table 6 FITC-conjugated lectin binding to the buccal cavity (and its contents) of developing stages of *Brugia pahangi* in *Armigeres subalbatus*

Day P.I.	PNA	WGA	LCA	APA	HAA	Con A	KBA
1					++[a]		
2							
3	+++ 6				++ 1, 4		
4							
5							
6	+	+					
7		+	+				+
8		+			+ 4		+
9	+ 6	+	+ 8, 1		+[c] 1	+ 9, 1	+
10		+[b]	+[b]	+[b] 8, 1		+[b]	+[c]

a: Pharyngeal thread.
b: Infective stage larvae from head.
c: Infective stage larvae from head and abdomen.
Inhibitors:
1=N-acetyl-D-glucosamine, 4=N-acetyl-D-galactosamine,
6=D(+)-galactose, 8=D-mannose, 9=α-D(+)-glucose
Fluorescence intencity: +++ intense, ++ moderate, + poor

did not cause any significant changes in the surface characteristics. None of the lectins tested bound onto the cuticle of exsheathed mf. Similar results have been reported in *B. pahangi* by others (Furman and Ash, 1983a,b; Devaney, 1985; Rao *et al.*, 1988) and in *Wuchereria bancrofti* and *Litomosoides carinii* (Rao *et al.*, 1987a). However, melanized mf of *B. pahangi* bound PNA, WGA, HAA and Con A; the fluorescent staining was most intense on the capsular material rather than on the surface of the worm itself. Encapsulation and melanization of mf involve both the humoral and cellular components of the mosquito defence system (Chen and Laurence, 1985; Forton *et al.*, 1985; Christensen and Forton, 1986). Recently, Nappi and Christensen (1986) had demonstrated transformation of mosquito blood cells or haemocytes in response to a parasitic invasion. They observed an increase in the number of haemocytes exhibiting WGA binding in mosquitoes eliciting a melanization response against inoculated mf. Surface carbohydrates of melanized mf identified by Con A, HAA, PNA and WGA in the present study may well represent aggregations of specific haemocyte population involved in encapsulation and melanization or a byproduct of such a response. Futher, Chen and Laurence (1987) had shown that the material around an encapsulated mf is a protein-carbohydrate complex; our sugar inhibition studies had indicated that several carbohydrate molecules or haemocyte types were present on the surface of an encapsulated and melanized mf. The reasons why most of the mf of *B. pahangi* are not encapsulated are largely unknown. It has been suggested that mf acquire midgut antigens on their surface during migration through the midgut wall (Sutherland *et al.*, 1984; LaFond *et al.*, 1985). Christensen *et al.* (1987), however, failed to detect midgut antigens on the surface of midgut-penetrated mf though a significant loss of microfilarial surface electronegativity was observed.

Table 4 shows that surface carbohydrate moieties associated with Con A, HAA, PNA and WGA dissappeared and reappeared at different times during the developmental period. Certain parts of the larvae *e.g.* the anal pore or plug did not change lectin binding specificities throughout the period; Con A, PNA and WGA associated moieties were the most frequently encountered. Similar results were reported with developmental stages of *O. lienalis* in *Simulium* (Ham *et al.*, 1988).

Whereas the carbohydrate moieties associated with most of the lectins tested could be identified, those for WGA were beyond the range of sugars used in the present study. WGA specifically recognizes GlcNAc and binds to a sequence of three β-(1-4)-linked residues of the sugar (Allen *et al.*, 1973; Goldstein *et al.*, 1975). However, GlcNAc itself has a lower affinity for WGA than oligomers of the sugar, thus WGA will not be completely inhibited by GlcNAc (Peters and Latka, 1986). Kaushal *et al.* (1984) showed that GlcNAc could reduced WGA-binding by only 50-70% though Devaney (1985) reported complete inhibition by GlcNAc. The surface molecules of developing stages of *B. pahangi* may bear several sugar residues or these molecules may exist as oligomers of lectins with differing carbohydrate binding specificities. Also, WGA is known also to interact with sialic acid residues (Bhavanandan and Katlic, 1979). However, Furman and Ash (1983a), Kaushal *et al.* (1984) and Rao *et al.* (1988) failed to detect sialic acid residues on blood-derived mf. Smail (1987) reported inhibition of WGA binding onto L1 of *O. lienalis* by neuraminic acid though removal of sialic acid residues using neuraminidase failed to produce such inhibition. The results implied that WGA bound onto oligomers of GlcNAc or onto a receptor site resembling neuraminic acid; it explains also the failure of some sugars to inhibit their complementary lectins.

The fluidity of the surface carbohydrates may be a consequence of the mosquito's haemocyte responses to the developing larvae. Interaction between mosquito haemocytes and the worm's surface carbohydrates may determine its successful development. The fact that Con A, HAA, PNA and WGA-associated moieties were the most commonly encountered on both melanized mf and developing larval stages suggest that the larvae may be evading host responses through molecular mimicry. However, there is a possibility that the observed differences in surface lectin binding characteristics were due to larval age differences. In the present study, the larvae were assayed every 24 hours and due care was taken such that dissection for the larvae was performed at a precise hour everyday; larvae from a single mosquito were used for each series of lectin binding assay. Nevertheless the possibility of some larvae being more mature than others in the series could not be ruled out.

It is interesting also to note that lectin binding was observed on the pharyngeal thread of early developmental stages of *B. pahangi*. A wider range of lectins bound onto the buccal cavity and the pharynx as the larvae approach maturity. Lectin bindings were most likely to the contents of the tract. The pharyngeal thread consists largely of a protein-mucosubstance complex (Simpson and Laurence, 1972) and these were probably stored waste products. Waste materials are not being expelled until the rectal plug is excreted during the last moult.

In conclusion, developing stages of *B. pahangi* in *Ar. subalbatus* showed very dynamic surface carbohydrate characteristics. The larvae change their surface coat configuration frequently over the 10-day observation period. On the other hand, blood-dwelling mf and fully matured infective larvae showed little or no binding affinity for the lectins tested; apparently the worms response differently in their vertebrate host. Infective stages of *O. lienalis* obtained from the head also bound less lectins than those from the thorax (Ham *et al.*, 1988). Rao *et al.* (1987c), however, reported that the cuticular surface of L3 of *W. bancrofti* bound WGA.

These observations suggest that the rapid turnover of the surface carbohydrates, while development of the larvae is taking place, is the worms' response to a 'hostile' host environment. Encapsulation and melanization of invading mf always occur in the haemocoel and have never been reported in the thorax of a vector mosquito. Rapidly changing surface carbohydrates may assist the larvae in evading the mosquito's immune system. On the other hand an active mosquito lectin system (*e.g.* in the form of opsonins or agglutinins) acting in concert with haemocytes may result in the successful elimination or isolation of the parasites.

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SURFACE LECTIN BINDING CHARACTERISTICS OF DEVELOPING STAGES OF *BRUGIA* IN *ARMIGERES SUBALBATUS*: II *BRUGIA MALAYI*

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Abstract: Surface lectin binding characteristics of microfilariae and developing stages of *Brugia malayi* in *Armigeres subalbatus* are presented. *Ar. subalbatus* is not a vector of *B. malayi*. The following lectins were used: wheat germ agglutinins, lentil agglutinins, *Helix aspersa* agglutinins, Concanavalin A agglutinins, kidney bean agglutinins, asparagus pea agglutinins and pea nut agglutinins. Frequent change in surface carbohydrate moieties on developing stages of *B. malayi* was observed. However, blood dwelling microfilariae and mature infective larvae showed little binding affinity for the lectins tested. It is postulated that *B. malayi* larvae evaded the mosquito immune system by continuously changing their surface carbohydrate moieties.

INTRODUCTION

In a previous communication, we have reported lectin binding characteristics of the cuticular surfaces of *Brugia pahangi* in *Armigeres subalbatus*. Larval stages of *B. pahangi* showed dynamic surface characteristics; frequent change in cuticular carbohydrate configuration was seen during their development in the mosquito. Similar observations were made with larval stages of *Onchocerca lienalis* in *Simulium ornatum* (Ham *et al.*, 1988).

The present paper describes the surface characteristics of developing stages of *B. malayi* in *B. malayi*-susceptible *Ar. subalbatus*. The aim of this study was to elucidate and to compare these characteristics with the larval stages of *B. pahangi* in *Ar. subalbatus* (previous communication); *Ar. subalbatus* is not a natural vector of *B. malayi*.

MATERIALS AND METHODS

B. malayi-susceptible *Ar. subalbatus* at ages 9 to 10 days were fed directly onto an anaesthetized *B. malayi*-infected cats. The peripheral microfilariae (mf) concentration of the cats ranged from 80-120 mf/20 μl blood.

The methods of isolation of *B. malayi* larvae, lectin binding experiments and types of lectins used were as described previously for studies with *B. pahangi* (Zahedi *et al.* 1990).

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Observations were made in duplicate and whenever possible parasites were obtained from the same mosquitoes for each series of lectins.

RESULTS

Sheathed and Ca^{++} exsheathed *B. malayi* mf bound KBA, LCA and WGA (Table 1). Faint fluorescent staining of cast sheaths by these lectins were also detected. Haemocoel-derived exsheathed mf bound KBA and LCA, while WGA bound only onto the excretory pores. Melanized mf bound Con A, HAA and PNA. It was noted that lectin-binding was onto the capsular envelope rather than the exsheathed mf itself. A weak binding of WGA onto the melanized capsule was also observed. The lectin-bindings could not be blocked by any of their complementary sugars.

Table 2 shows lectin binding characteristics of developing larval stages of *B. malayi* in *Ar. subalbatus*. All lectins tested bound weakly onto the cuticular surface of 24-hr old larvae. In addition to the worm's surface, Con A and PNA also bound onto both anal and excretory pores of the larvae. HAA, KBA and PNA binding onto the worm were completely blocked by their complementary sugars (in this issue, p. 273). Both Methyl-α-D-mannopyranoside (ManMe) and α-D(+)-glucose (Glc) inhibited Con A binding onto the cuticle, anal and excretory pores of the worm. Also Con A binding to the cuticle and excretory pores was blocked by N-acetyl-D-glucosamine (GlcNAc) and D-mannose (Man), respectively. None of the complementary sugars of APA, LCA and WGA interfered lectin binding onto the worm's cuticular surface.

The larvae lost all except APA and LCA binding onto their cuticular surface on day 2

Table 1 FITC-conjugated lectin binding on *Brugia malayi* microfilariae

Microfilariae	Region affected	PNA	WGA	LCA	APA	HAA	Con A	KBA
Sheathed MFF	Sheath							
	Cuticle							
	Anal pore							
	Excretory pore							
	Entire worm		+	+				+
Chemically† exsheathed microfilariae	Cuticle							
	Anal pore							
	Excretory pore							
	Entire worm		+	+				+
Exsheathed microfilariae from haemocoel	Cuticle							
	Anal pore							
	Excretory pore		++	++				
	Entire worm			+				+
Melanized microfilariae	Entire worm‡	++	+			++	++	

†: Calcium exsheathed microfilariae.
‡: Binding was onto the melanized capsular material.
Fluorescence intensity: +++ intense, ++ moderate, ± poor

Table 2 FITC-conjugated lectin binding onto developing stages of *Brugia malayi*

Day P.I.	Region of larvae	PNA	WGA	LCA	APA	HAA	Con A	KBA
1	Anterior one-third							
	Excretory pore	++ 6					+ 5, 8, 9	
	Middle one-third							
	Anal plug	+++ 6				++ 1, 4	++ 5, 9	
	Posterior one-third							
	Entire worm	+ 6	+	+	+	+ 1, 4	+ 1, 5, 9	++ 1
2	Anterior one-third							
	Excretory pore		+	++ 8, 9			+++ 1, 5, 8, 9	
	Middle one-third							
	Anal plug	++ 6	++	++ 8	+ 1, 5, 8	+++ 1, 4	+++	++ 1
	Posterior one third	+ 6					+ 1, 5, 8, 9	
	Entire worm			+ 1, 8, 9	+ 1, 8			
3	Anterior one-third							
	Excretory pore		++			++ 1, 4	++ 5, 8, 9	
	Middle one-third							
	Anal plug	+++ 6	++	+	++ 7	+++ 1, 4	+++	++
	Posterior one-third							
	Entire worm	+ 6	+	+	+ 7	+ 1, 4	+ 5, 8, 9	++
4	Anterior one-third							
	Excretory pore		+	+ 8, 9			+ 1, 5, 8, 9	
	Middle one-third							
	Anal plug	+++ 6	++	+++		+++ 4, 1	++	
	Posterior one-third							
	Entire worm	+ 6		+ 9	+ 1, 5	+ 1, 4	+ 5	++
5	Anterior one-third							
	Excretory pore		+				+ 5, 8	++
	Middle one-third							
	Anal plug	+++ 6	++	+++	+ 1, 7, 8	+++ 1, 4	+++	++
	Posterior one-third							
	Entire worm			+ 8	+ 1, 7, 8	++ 1, 4	++	++
6	Anterior one-third							
	Excretory pore		+				+ 5, 8	++
	Middle one-third							
	Anal plug	+++ 6	++	+++	+ 1, 7, 8	+++ 1, 4	+++	++
	Posterior one-third							
	Entire form			+ 8	+ 1, 7, 8	++ 1, 4	++	++
7	Anterior one-third							
	Excretory pore							
	Middle one-third							
	Anal plug	+++ 6	+	++		+++ 1, 4	+++ 1	
	Posterior one-third					++ 1, 4		
	Entire worm	+ 6	+	++			++ 1, 5	+++

Table 2 (Cont.)

Day P.I.	Region of larvae	PNA	WGA	LCA	APA	HAA	Con A	KBA
8	Anterior one-third							
	Excretory pore							
	Middle one-third							
	Anal plug	+++ 6	++	++		+++ 1,4	++	
	Posterior one-third							
	Entire worm			+ 1,9			+ 1,8,9	
9	Anterior one-third							
	Excretory pore							
	Middle one-third	+ 6						
	Anal plug	+++ 6	++	++			+++	+ 1
	Pesterior one-third							+
	Entire worm			+ 1,8,9				

Inhibitors: 1=N-acetyl-D-glucosamine, 4=N-acetyl-D-galactosamine, 5=Methyl-α-D-mannopyranoside, 6=D(+)-galactose, 7=L(−)-fucose, 8=D(+)-mannose, 9=α-D(+)-glucose

Fluorescence intensity: +++ intence, ++ moderate, + poor

p.i. The binding could be blocked by both Man and GlcNAc; in addition Glc too blocked LCA binding onto the cuticle. A very weak fluorescent staining with Con A and PNA was also detected at the posterior third of the cuticular surface of the larvae. PNA binding to the surface could be inhibited by D(+)-galactose (Gal) and Con A binding by ManMe, Man, Glc and GlcNAc. All lectins tested bound onto the anal pore and only Con A, LCA and WGA onto the excretory pore. LCA and PNA binding was inhibited by Gal and Man respectively. APA binding was inhibited by ManMe, Man and GlcNAc and HAA binding was inhibited by N-acetyl-D-galactosamine (GalNAc) and GlcNAc. Further, GlcNAc caused a reduction in the intensity of KBA fluorescent staining. Con A binding to the excretory pore could be blocked by all its complementary sugars and LCA binding only by Man and Glc.

Larvae obtained on day 3 p.i. bound all lectins tested onto their cuticular surfaces. Gal and L-(−)-Fucose (Fuc) inhibited APA and PNA binding, respectively, and HAA was blocked by both GalNAc and GlcNAc. APA binding onto the anterior-half of the larvae could be blocked by GlcNAc. Similarly, GlcNAc also blocked KBA binding to the middle-third of the worm. Glc, Man and ManMe inhibited Con A binding onto the worm's cuticle. None of the complementary sugars of LCA and WGA interfered with the binding. As was the case with larvae dissected on day 2, 72-hr old larvae bound all the lectins tested onto their anal pore. PNA binding could be blocked by Gal; APA by Fuc and HAA by both GalNAc and GlcNAc. None of the other lectin bindings could be inhibited by any of the sugars tested. Fluorescent staining of the excretory pore was observed with Con A, HAA and WGA. HAA binding was inhibited by GalNAc and GlcNAc and Con A by Glc, Man and ManMe.

Late first-stage larvae (L1) dissected on day 4 p.i. bound all lectins except WGA onto its cuticular surface. Con A, LCA and PNA binding were inhibited by Gal, Glc and ManMe respectively. Also Con A binding to the anterior half of the larvae could be inhibited by GlcNAc; the posterior-half was inhibited by Glc. APA binding was inhibited by ManMe and

GlcNAc and HAA binding by GalNAc and GlcNAc. Moderate to intense fluorescent staining of the anal pore was obtained with all lectins except APA and KBA. Whereas PNA and HAA binding could be inhibited by Gal and GalNAc, respectively, none of the other lectin bindings could be blocked by their complementary sugars. The excretory pore bound Con A, LCA and WGA. Man and Glc inhibited LCA binding; Con A could be blocked by all of its complementary sugars.

Some minor changes occurred on the cuticular surfaces of larvae dissected on day 5 p. i. Binding sites for WGA resurfaced while those for PNA and HAA dissappeared. Some larvae, however, bound PNA onto their posterior-third of the cuticular surface. LCA binding could be inhibited by GlcNAc while Con A by both GlcNAc and Man. Fuc and Man reduced the intensity of APA binding onto the cuticular surface. An intense fluorescent staining of the anal plug was observed with all lectins except APA. GlcNAc reduced the staining intensity of both Con A and LCA binding and that of PNA could be reduced by Gal. Con A could also be completely inhibited by Glc. GalNAc and GlcNAc blocked HAA binding onto the anal plug. The excretory pore bound APA, HAA and WGA. HAA binding onto the excretory pore was blocked by GalNAc and GlcNAc. APA staining intensity was reduced by both Fuc and Man.

Binding sites for HAA reappeared while those for WGA dissappeared from the cuticular surfaces of larvae dissected on day 6 p.i. There was no cuticular surface binding with PNA. HAA binding could be inhibited by GalNAc and GlcNAc. APA, Con A, KBA and LCA, all bound onto the worm's cuticle. The anal plug bound all lectins tested. Man blocked APA binding on both anal pore and cuticular surface. APA could also be completely blocked by GlcNAc and Fuc. Man inhibited LCA binding onto the cuticle. Con A and KBA bound onto the excretory pore; both ManMe and Man blocked Con A binding to the excretory pore.

All lectins tested except APA and HAA bound onto the cuticular surfaces of second-stage larvae (L2) obtained on day 7 p.i. While binding sites for APA and HAA dissappeared, sites for PNA and WGA reappeared on the worm's cuticle. Some larvae, however, bound HAA onto their posterior-third of the cuticular surface; the binding could be inhibited by GalNAc and GlcNAc. Gal inhibited PNA binding while ManMe blocked Con A. GlcNAc could reduce the fluorescent staining intensity of Con A. Glc blocked Con A binding to the posterior-half of the worm. Binding to the anal plug was observed with all lectins except APA and KBA. PNA binding could be inhibited by Gal while HAA by GalNAc and GlcNAc. A reduction in fluorescent staining intensity of Con A was observed with GlcNAc.

Late L2 on day 8 bound only Con A and LCA onto their cuticular surfaces. Both Con A and LCA binding was inhibited by Glc and GlcNAc. In addition Con A could also be blocked by Man. There was no change in the anal plug's lectin binding pattern between larvae obtained on day 7 and day 8.

Further changes occurred on the cuticular surfaces of the late L2 on day 9 p.i. None of the lectins tested except LCA bound onto the cuticle. Some larvae, however, bound KBA and PNA onto the middle and posterior-third of their cuticle respectively. LCA binding could be inhibited by Glc, GlcNAc and Man. PNA binding was inhibited by Gal. The anal plug bound all except APA and HAA *i.e.* it showed the reappearance of KBA and the dissappearance of HAA. Fluorescent staining of the anal cavity was observed with KBA, LCA and WGA. PNA-binding was inhibited by Gal and KBA by GlcNAc.

Early third-stage larvae (L3) dissected from the thorax on day 9 p.i. showed significant

Table 3 FITC-conjugated lectin binding onto *Brugia malayi* infective stage larvae (L3) cuticular surface

Source	Region of larvae	PNA	WGA	LCA	APA	HAA	Con A	KBA
Head	Anterior one-third							
	Middle one-third							
	Anus and anal cavity							
	Posterior one-third							
	Entire one-third							
	Entire worm							
Thorax	Anterior one-third							
	Middle one-third							
	Anus and anal cavity			++ 1,9		++ 1,4		
	Posterior one-third							
	Entire worm							
Abdomen	Anterior one-third							
	Middle one-third							
	Anus and anal cavity							
	Posterior one-third							
	Entire worm							
Early L3 in thorax	Buccal cavity		++	++		+ 4	+ 5,9	+
	Anterior one-third							
	Middle one-third							
	Anus and anal cavity	+	++	++		++ 1,4	++	+ 1

Inhibitors: 1=N-acetyl-D-glucosamine, 4=N-acetyl-D-galactosamine, 5=Metyl-α-D-mannopyranoside, 9=α-D(+)-glucose
Fluorescence intensity: +++ intence, ++ moderate, + poor

changes in surface binding characteristics compared with late L2 larvae obtained on the same day (Table 3). The cuticular surface of early L3 did not bind any of the lectins tested. Fluorescent staining of anal cavity and anal plug remnants were observed with all lectins except APA. HAA binding was inhibited by GlcNAc and GalNAc and KBA by GlcNAc. None of the lectins tested bound onto the cuticular surface of L3 dissected on day 10 p.i. Some larvae obtained from the thorax, however, still bound HAA and LCA onto their anus and anal cavity; these may be young immature L3. LCA binding was inhibited by GlcNAc and Glc and HAA by GlcNAc and GalNAc.

As was the case with *B. pahangi,* lectins also bound weakly to the buccal cavity (and its contents) of larval stages of *B. malayi* (Table 4). PNA bound onto the buccal cavity of L1 dissected on day 3 and day 4 p.i., of L2 on day 6 p.i. and of infective larvae obtained from the head. The alimentary tract of L1 on day 3 and day 4 p.i. was also stained with PNA. Gal inhibited PNA binding onto all larval stages. WGA bound onto the buccal cavities of late L1 (day 4 p.i.), early L2 (day 5, 6 and 7 p.i.) and of infective larvae obtained from the head. LCA bound only onto late larval developmental stages. An intense fluorescent staining of both the alimentary tract and buccal cavity of early L2 (day 5) was observed with LCA. LCA staining was also observed with L2 dissected on day 6 and day 8 p.i. Buccal cavities of infective larvae

obtained from the head and the thorax were also stained. GlcNAc blockec LCA binding for all larval stages. Man inhibited LCA binding on day 8 p.i. and also onto buccal cavities of L3 obtained from the thorax. Glc blocked LCA binding onto the buccal cavities of L3 from the head. A moderate to intense fluorescent staining of the buccal cavities was observed with HAA. HAA staining of the buccal cavity or alimentary tract of late L2 (day 3 and 4 p.i.) and infective larvae could be blocked by both GalNAc and GlcNAc. HAA staining of the buccal cavity and alimentary tract of early L2 (day 5 p.i.) could only be blocked by GalNAc. Con A bound onto the buccal cavities of larvae dissected on day 3 and day 5. Con A binding onto day 3 larvae could be inhibited by Glc, Man and ManMe and those on day 5 p.i. by GlcNAc. APA and KBA did not bind onto buccal cavities of any larval stages.

Discussion

Some differences in the ability of mf of *B. pahangi* and *B. malayi* to bind onto the test-lectins were detected. *B. pahangi* mf bound only WGA onto its sheath and excretory pore while *B. malayi* bound LCA and KBA in addition to WGA along its entire body length. The fluorescent staining of *B. malayi* mf were, however, weak. It is possible that the observed binding of the subcuticular carbohydrate moieties was an experimental artifact or may be

Table 4 FITC-conjugated lectin binding to the buccal cavity (and its contents) of developing stages of *Brugia malayi* in *Armigeres subalbatus*

Day P.I.	PNA	WGA	LCA	APA	HAA	Con A	KBA
1							
2							
3	+++[a] 6				++ 1, 4	++ 5, 8, 9	
4	++[a] 6	+			+++[d] 1, 4		
5		++	+++[d] 1		+++[d] 4	++ 1	
6	++ 6	+	+ 1				
7		++					
8			+ 1, 8				
9							
10	+	+	++ 1, 8, 9		++ 1, 4		

a=Entire length of the alimentary tract.
d=Anterior two-third of the alimentary tract.
Inhibitors:
1=N-acetyl-D-glucosamine, 4=N-acetyl-D-galactosamine,
6=D(+)-galactose, 8=D-mannose, 9=α-D(+)-glucose
Fluorescence intencity: +++ intense, ++ moderate, + poor

lectin-induced alteration of the cuticular surface configuration. Similarly, Kaushal *et al.* (1984) and Rao *et al.* (1988) observed WGA binding onto sheaths of blood-derived *B. malayi* mf; however, *in vitro*-derived mf did not bind WGA but bound Con A (Kaushal *et al.*, 1984). Kaushal *et al.* (1984) suggested that failure of Con A to bind onto blood-derived mf indicated that there was masking or loss of parasite surface molecules as mf matures *in vitro*. Furman *et al.* (1987) had detected immunochemical changes in mf as they assumed functional maturity.

Ca^{++}-exsheathed *B. malayi* mf bound WGA whereas haemocoel derived exsheathed mf did not; none of the Ca^{++}-exsheathed or haemocoel-derived exsheathed *B. pahangi* mf bound any of the lectins tested. It is possible that the surface changes observed in the present study was Ca^{++}-induced involving one or more cuticular enzymes. Calcium is said to activate an endogenous protease in mf which allowed them to escape from their sheaths (Devaney and Howells, 1979). Sayers *et al.* (1984) had demonstrated the presence of enzymes in the cortex of the cuticle of mf. Microfilariae treated with proteolytic enzymes showed more intense fluorescent staining possibly due to the exposure of more carbohydrate residues (Rao *et al.*, 1987; Devaney, 1985).

As it was the case with *B. pahangi,* melanized mf of *B. malayi* bound Con A, HAA, PNA and WGA and the fluorescent staining was most intense on the capsular material rather than on the surface of the worm. While Gal inhibited PNA binding onto melanized *B. pahangi* microfilariae, the sugar could not block PNA binding onto a melanized *B. malayi* mf. Similarly, while GalNAc and GlcNAc blocked HAA binding onto *B. pahangi* mf, they did not interfere with the lectin binding onto *B. malayi.* Con A and WGA could not be blocked by any of the sugars tested. Thus, cuticular carbohydrate moieties of *B. malayi* were different but structurally related to *B. pahangi.*

Lectin binding onto the pharyngeal thread of developmental stages of *B. malayi* was also observed. More lectin types bound onto the buccal cavity and alimentary tract of *B. malayi* than *B. pahangi.* None of the *B. malayi* larvae bound APA or KBA onto their buccal cavities and alimentary tracts; a weak binding of APA and KBA with late developmental stages of *B. pahangi* was observed.

Significant changes in cuticular surface binding characteristics of developmental stages of *B. malayi* was noted during the 10-day observation period. Early L3 from the thorax expressed more carbohydrate moieties on their anus and anal cavities compared with late L2 obtained on the same day. The cuticular surface of both early L3 from the thorax and late L3 from the head did not bind any of the lectins tested. In addition to Con A, PNA and WGA, HAA and LCA-associated moieties were the most frequently encountered on *B. malayi.*

As it was with *B. pahangi,* the present study too suggests that *B. malayi* larvae evaded the mosquito immune system by continuously changing their surface carbohydrate moieties. Recently, Ferro and Theis (1984) and Lastre *et al.* (1988) demonstrated antigenic sharing between *B. pahangi* and *Aedes aegypti.* They postulated that antigenic similarities between *B. pahangi* and *Ae. aegypti* would allow the parasites to avoid destruction in the insect host. It will be interesting to investigate further the role of the mosquito immune system in regulating field transmission of filariasis.

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RATS AND MICE SERVED AS EXPERIMENTAL PARATENIC HOSTS OF THAI *PARAGONIMUS HETEROTREMUS*

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Abstract: The host-parasite relationship between Thai lung fluke, *Paragonimus heterotremus,* and laboratory animals was investigated by using rats, *Rattus norvegicus,* and mice, *Mus musculus,* inoculated with the metacercariae. It was found that both animals played roles as paratenic hosts of the fluke, but rats seemed more susceptible to the fluke than mice. In addition, juvenile flukes recovered from the skeletal muscles of the animals were able to develop into adults when transferred orally to cats.

INTRODUCTION

Paragonimus heterotremus Chen and Hsia, 1964 was an important parasite which caused an endemic public health problem in Thailand (Vanijanonta *et al.*, 1984). This lung fluke has been confirmed to affect man (Miyazaki and Harinasuta, 1966; Vanijanonta *et al.*, 1981). Dogs and cats also served as definitive hosts under both natural and laboratory conditions (Setasubun *et al.*, 1966; Vajrasthira and Radomyos, 1966).

During August to September 1987, members of the authors had made an extensive field survey at Jet Cod Waterfall in Saraburi Province, Thailand and verified that freshwater crabs, *Larnaudia larnaudii,* collected there harbored quite a number of *P. heterotremus* metacercariae (Kawashima *et al.*, 1988). The availability of the metacercariae spurred us to investigate the susceptibility of laboratory rats, *Rattus norvegicus,* to the infection with Thai *P. heterotremus.* None of the flukes has been detected from wild rats of the genus *Rattus* and *Bandicota* captured in an endemic area of this lung fluke in Thailand (Setasubun *et al.*, 1966),

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while Chinese *P. heterotremus* has been proved to mature in naturally infected hoary bamboo rats, *Rhyzomys pruinosus,* and experimentally infected laboratory rats, *R. norvegicus* (Liu and Yao, 1985).

In the present study, the development of Thai *P. heterotremus* in laboratory rats and mice was studied. In addition, the role of the animals was examined in maintaining the life cycle of this lung fluke by transferring the immature *P. heterotremus* from the rats or mice to cats.

Materials and Methods

Parasite and experimental infection of rats and mice

P. heterotremus metacercariae were harvested from freshwater crabs, *L. larnaudii,* collected at Jet Cod Waterfall in Saraburi Province, Thailand (Kawashima *et al.*, 1988). Sixteen laboratory rats, *R. norvegicus,* of Wistar strain and about 8-week-old, and 3 laboratory mice, *Mus musculus,* of BALB/c and about 10-week-old, were inoculated with the metacercariae. Of them, all mice and 13 rats were inoculated orally with 15 to 20 metacercariae and the remaining 3 rats intraperitoneally with 20 metacercariae. The animals were bled to death under anesthesia 78 or 253 days after inoculation and flukes were recovered by the incubation method (Habe, 1983). In brief, the visceral organs and cavities were flushed with a Ringer's solution to recover flukes which adhered to the serosal surface. Then, the lungs, liver, and skeletal muscles were cut into 2- to 4-mm thick and kept in a Ringer's solution at 37°C for 6 to 12 hrs to release flukes from the tissues. The solution was poured into a petri dish and examined for flukes under a dissecting microscope. All flukes recovered from the pleural cavity and some from the skeletal muscles were compressed between 2 slide glasses, fixed with 70% alcohol, stained with carmine, and mounted with Canada balsam for morphological observation. Flukes were then classified into 4 developmental types: adult, preadult, immature, and juvenile, according to the criteria of Shibahara (1984).

Transfer of flukes to cats

Certain numbers of the juvenile flukes recovered from the skeletal muscles of the laboratory animals were transferred orally to 2 adult domestic cats. The cat No. 1, weighing 2.0 kg, was inoculated with 20 flukes from the rats (Nos. 1-6), and the cat No. 2, weighing 2.1 kg, with 5 flukes from the mice (Nos. 1-3), respectively. Fecal examinations were made every 3 to 5 days by the sedimentation technique (Dubey *et al.*, 1978). The cats were bled to death under anesthesia 134 days after inoculation. Flukes were recovered from the pleural cavity and lungs, and their developmental state was examined.

Results

Experimental infection of rats and mice with the metacercariae

Days from inoculation to necropsy, the location, number and recovery rate of the flukes are given in Table 1. In 3 rats (Nos. 1-3) inoculated intraperitoneally and bled to death 78 days after inoculation, the recovery rate of the flukes averaged 55% of the inoculated metacercariae. All 31 flukes recovered from the skeletal muscles were classified into juveniles. As for 2 flukes recovered from the pleural cavity, one was identified as a preadult and the other as an adult with only a few eggs in the uterus. In 3 rats (Nos. 4-6) inoculated orally

Table 1 Experimental infection of rats and mice with Thai *Paragonimus heterotremus* metacercariae (mc)

Host No.	No. of mc inoculated	Route of inoculation	Days from inoculation to necropsy	Recovery rate of flukes (%)	No. of flukes recovered			
					Total	Skeletal muscles	Pleural cavity	Others*
Rat 1	20	ip	78	50.0	10	9[j]	1[p]	0
Rat 2	20	ip	78	45.0	9	9[j]	0	0
Rat 3	20	ip	78	70.0	14	13[j]	1[a]	0
Rat 4	20	po	78	20.0	4	4[j]	0	0
Rat 5	20	po	78	25.0	5	5[j]	0	0
Rat 6	20	po	78	35.0	7	7[j]	0	0
Subtotal	120			40.8	49	47	2	0
Rat 7	15	po	253	13.3	2	1[j]	1[p]	0
Rat 8	15	po	253	6.7	1	0	1[p]	0
Rat 9	15	po	253	0	0	0	0	0
Rat 10	15	po	253	6.7	1	1[j]	0	0
Rat 11	15	po	253	0	0	0	0	0
Rat 12	15	po	253	6.7	1	0	1[p]	0
Rat 13	15	po	253	13.3	2	1[j]	1[p]	0
Rat 14	15	po	253	6.7	1	1[j]	0	0
Rat 15	15	po	253	0	0	0	0	0
Rat 16	15	po	253	0	0	0	0	0
Subtotal	150			5.3	8	4	4	0
Mouse 1	19	po	78	31.6	6	5[j]	1[j]	0
Mouse 2	20	po	78	35.0	7	6[j]	1[j]	0
Mouse 3	20	po	78	55.0	11	10[j]	1[j]	0
Subtotal	59			40.7	24	21	3	0

ip: intraperitoneal inoculation, po: oral inoculation
j: juvenile, p: preadult, a: adult
*: Lungs, liver and peritoneal cavity

Table 2 Experimental transfer of 78-day-old juveniles of Thai *Paragonimus heterotremus* recovered from the skeletal muscles of rats and mice to cats

Cat			No. of juvenile flukes transferred	Donor animal	Days from transfer to		Recovery rate (%)	No. of flukes recovered		
No.	Body weight at necropsy (kg)	Sex			onset of eggs in feces	necropsy		Total	Pleural cavity	Lungs
1	2.0	Female	20	rat	49	134	95.0	19	0	19
2	2.1	Male	5	mouse	49	134	100	5	0	5

and bled to death 78 days after inoculation, the recovery rate of the flukes averaged 26.7%. All flukes were recovered from the skeletal muscles and identified as juveniles. In 10 rats (Nos. 7-16) inoculated orally and bled to death 253 days after inoculation, the recovery rate of the flukes ranged from 0 to 13.3%, with an average of 5.3%. Of a total of 8 flukes recovered, 4 were recovered from the skeletal muscles and identified as juveniles, and the other 4 were recovered from the pleural cavity and identified as preadults.

In 3 mice, which were inoculated orally and bled to death 78 days after inoculation, the recovery rate of the flukes averaged 40.7%. Though the flukes were recovered from both the skeletal muscles and pleural cavity, all of them were identified as juveniles.

Transfer of juvenile flukes from the rats or mice to cats

Days from transfer to onset of *Paragonimus* eggs in the feces, days from transfer to necropsy and the location, number and recovery rate of flukes are given in Table 2. The eggs appeared in the feces for the first time at 49 days after infection in both cases. All flukes were recovered from the lungs and identified as adults or preadults, with a recovery rate of 96% on an average.

Discussion

P. heterotremus has been recorded in China, Thailand and Laos (Miyazaki, 1974). In China, this lung fluke was able to develop into adult stage in laboratory rats. Also natural infection with the adult flukes was observed in hoary bamboo rats (Liu and Yao, 1985). Though experimental infection studies under the same conditions may be essential to elucidate the nature of the host-parasite relationship, the high susceptibility of the rodents to the infection with Chinese *P. heterotremus* was clearly seen from their studies.

In this study, the host-parasite relationship between Thai *P. heterotremus* and laboratory rats or mice was investigated. Of a total of 57 flukes recovered from the rats, only one was identified as adult and all the rest of them as juveniles. From this results, it was concluded that laboratory rats were slightly susceptible to the infection with Thai *P. heterotremus*. However, the susceptibility of laboratory rats seemed to be higher than that of laboratory mice because one fluke recovered was adult.

The juvenile flukes, which were recovered from the skeletal muscles of the rats or mice and then transferred to cats, developed into adults and discharged eggs in the feces of the recipient cats. Therefore both rats and mice could serve as experimental paratenic hosts of *P. heterotremus* which occurs in Thailand. It has already been revealed that rats served as paratenic hosts of *P. miyazakii, P. mexicanus* and both diploid and triploid types of *P. westermani* under laboratory conditions (Habe, 1978, 1983; Shibahara, 1984). The diploid and triploid types of *P. westermani* also took mice as their experimental paratenic hosts (Habe, 1978; Shibahara, 1981).

In Thailand, wild rodents may play an important role mainly as paratenic hosts to complete the life cycle of *P. heterotremus* in nature. To elucidate the role of wild rodents, accurate detection of the flukes will be necessary by examination of the skeletal muscles as well as the lungs and pleural cavity of wild rodents captured in an endemic area of this lung fluke.

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タイ産ヒロクチ肺吸虫 *Paragonimus heterotremus* のラットおよびマウスへの感染試験

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タイ産ヒロクチ肺吸虫のラットおよびマウスへの感染試験を行い，宿主適合性を調べた。ラット16頭およびマウス3頭に本虫のメタセルカリアを15—20個ずつ経口投与し，投与後78日あるいは253日に剖検したところ，虫体の回収率はラットでは平均40.8%（投与後78日に剖検）あるいは5.3%（同253日に剖検），マウスでは平均40.7%（同78日に剖検）であった。ラットから回収した虫体の約89%は幼若虫であり，いずれも骨格筋から検出され，成虫は胸腔から検出された1隻（回収虫体の約2%）に過ぎなかった。一方マウスから回収した虫体は総て幼若虫であり，その約88%が骨格筋から検出された。試験ラットあるいはマウスの骨格筋由来の幼若虫を，本虫の好適終宿主であるネコに経口投与したところ，幼若虫の95%（ラット由来の幼若虫）あるいは100%（マウス由来の幼若虫）が感染して肺に虫嚢を形成し，投与後49日に糞便内排卵を開始した。以上の成績から，タイ産ヒロクチ肺吸虫のラットおよびマウスに対する宿主適合性は低いものの，齧歯類は待機宿主として本虫の生活環の維持に主要な役割を果たし得ると思われた。また，齧歯類を対象とした本虫感染状況の野外調査では，骨格筋からも虫体検出を試みる必要があると考えられた。

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BASIC STUDIES ON THE MONGOLIAN GERBIL AS A SUSCEPTIBLE HOST TO FILARIAL INFECTION COMPARATIVE STUDIES ON GROWTH AND REPRODUCTION AMONG COAT COLOR MUTANTS AND GENETIC ANALYSIS OF COAT COLORS

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Abstract: In order to know the basic nature of the various color mutants of the gerbil as the susceptible host to filarial infection, the growth curve as well as food and water consumption volume and reproduction rates were compared among coat color mutants of the Mongolian gerbil, such as agouti, white spotted-agouti, albino, black and white spotted-black type mutants.

There was almost no significant difference in growth curve and reproduction rates among the color mutants. Namely, mean weights of these newborns and of matures among these gerbils were 3.0 g, and 60 g in male or 52 g in female respectively, and the mean number of newborns among mutants was 4.2 to 4.8. However, the periods of delivery among these mutants varied to wide ranges. As the reasons of the change of the delivery periods, it was considered that the post-partum oestrus occurred in some of the gerbils, and in these gerbils, the delayed implantation of the placenta were seen relatively often and also in some gerbils, the irregular oestrus cycle was existed.

In addition, some genetic studies on coat colors were carried out. It appeared that some genes controlling coat colors exist in the gerbils as in mice or rats, and it suggested that albino and black colors are controlled by autosomal ressesive genes.

The coat color mutants of the gerbils also will be useful animal as the susceptible host to filarial infection.

INTRODUCTION

The Mongolian gerbil usually called jird (*Meriones unguiculatus*) is used as laboratory animal in the field of virology, cerebral neurology, endocrinology and parasitology.

Especially, in the parasitology, the Mongolian gerbil is extensively used as the susceptible host to filarial infection. Biological characters in this animal were examined in 1960's when the gerbil was started to use laboratory animal, but after that, there has been almost no report about them, and the immunological nature of the gerbil has not been made clear at

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all. We succeeded to obtain four kinds of coat color mutants of the gerbil, white spotted-agouti, albino, black and white spotted-black type in our laboratory, so we attempted to study the biological characteristics of the mutants, in order to analyze the infection kinetics of the filarial infection. In this paper, the growth curve and the reproduction rates among the color mutants of the gerbil are described and some genetic studies on the coat colors are carried out.

Materials and Methods

Origin:

The our breeding colony of wild color gerbil called agouti type (Photo. 1) and used generally as laboratory animal was supplied originally from Institute of Medical Science, Tokyo University in 1973. The male coat color mutant named white spotted-agouti type was obtained first from a pet shop in Kawasaki, Japan in January, 1985. The other mutant types of gerbil called albino, black and white spotted-black type were obtained from the matings between the white spotted-agouti type of gerbils.

Description of the mutants:

The white spotted-agouti mutant was characterized by white spots on the crown of the head, the nape of the neck and the tip of the nose (Photo. 2). It also had white feet and nails, the tip of the tail was white, and the ventrum is completely white including the under hairs. The other parts of the coat was agouti color. This male mutant was mated with agouti female at first, and sister-brother mating were made. In the third generation of this mating, the new mutants, such as albino type arose in 1986 and black and white spotted-black type arose in 1987. The albino mutant had entire white coat and red eyes (Photo. 3). The black mutant had entire black coat color except for partially white hairs on the surface of the forefeet (Photo. 4). The white spotted-black mutant had white spots on the same parts of the white spotted-agouti type, and the other parts of the coat were black color (Photo. 5). The colonies of these coat color mutants were also extended with sister-brother matings.

Housing:

Breeding pairs or groups of 5 or 6 mature gerbils were housed in the polycarbonate cage bedded with the white pine shaving, White Flake (Charles River Japan Inc.) in the conventional condition. The diet was a commercial rodent feed, MF (Oriental Yeast Co., LTD.) and fresh water. The room temperature and the humidity were maintained at 24±2°C and 60±5% respectively, and the lighting system was controlled automatically at 12 hours daily between 0800 and 2000 hours.

Growth curve:

For growth curve study, 50 animals at 1, 2, 4, 6, 8 weeks of age, 30 animals at 3 months of age and 20 animals at 4, 5, 6 months of age were used respectively in each color mutant of the gerbil of both sexes.

Food and water consumptions:

Averaged food and water consumptions of each individual gerbil were measured during

Photo. 1 Agouti type.

Photo. 2 White spotted-agouti type.

Photo. 3 Albino type.

Photo. 4 Black type.

Photo. 5 White spotted-black type.

10 days for 20 mature animals in each color mutant of both sexes.

Reproductive rates and periods of the delivery:

Reproductive rates as well as the delivery periods were determined for the 17 breeding pairs each in agouti or white spotted-agouti type, 12 pairs in albino, 8 pairs in black and 7 pairs in white spotted-black type of the gerbil. Breeding pairs were established prior to sexual maturity, and the newborns were usually weaned at 25-30 days of age and monogamous breeding pairs were remained unchanged together.

Genetic studies on the coat colors:

Mating experiments were carried out to examine the inheritance of the coat colors of the mutants using mature gerbils. At first, matings between agouti and white spotted-agouti gerbils were made. F_1 animals with white spotted-agouti color and F_2 hybrids with the same coat color were mated each other. Inheritance of the other coat colors were examined in the same way.

RESULTS

Growth curve:

The curve of body weight changes from birth to 6 months of age is presented in Figure 1. A clear difference between the sexes began to emerge at the time of 6 weeks of age (t-test $p<0.01$). Weight of males exceeded that of females by approximately 14%. There is no significant difference of body weight among coat color mutants at the same age. Mean birth weight was 3.0 g irrespective of sex and of coat color. The mean weight of the mature gerbil of 3 months of age was 63.7 g in agouti male, 53.8 g in agouti female, 60.4 g in white spotted-agouti male, 52.0 g in white spotted-agouti female, 59.5 g in albino male, 51.2 g in albino female, 62.6 g in black male, 55.0 g in black female, 61.5 g in white spotted-black male and 52.0 g in white spotted-black female, as shown in Figure 1.

Food and water consumptions:

The daily food and water consumptions of each mutant of the mature gerbil are shown in Table 1. Mean food consumption was 3.4 to 4.7 g and mean water consumption was 5.0 to 6.7 m*l* for each coat color mutant of both sexes. There was a tendency for male to eat and to drink more volume than female, but there was no significant difference among coat color mutants.

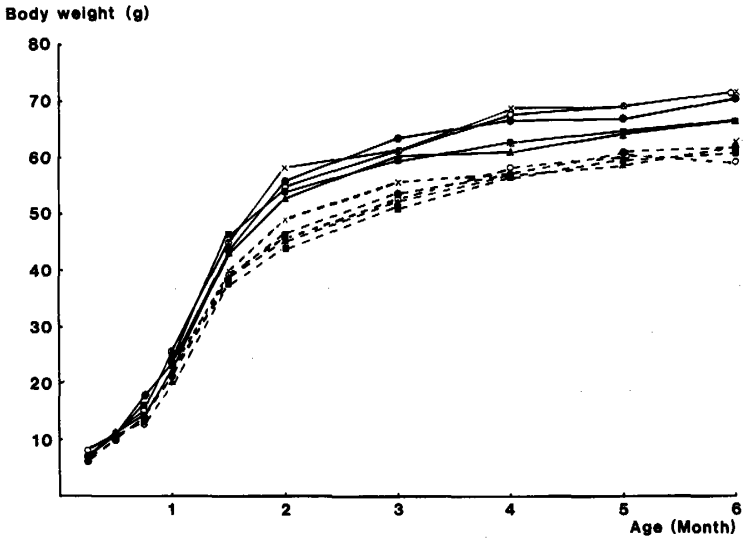

Figure 1 The growth of each mutant of the Mongolian gerbil from birth to 6 months of age.

——— male ····· female

● agouti ■ white spotted-agouti

▲ albino × black

○ white spotted-black

Number of newborns:

Table 2 showed the reproductive physiology of each color mutant. The number of newborns was minimum 1 to maximum 9, and averaged 4.8 in agouti, 4.2 in white spotted-agouti, 4.8 in albino, 4.9 in black and 3.9 in white spotted-black mutant. The number of newborns in each color mutant was almost same.

Sex ratio of newborns:

Sex ratio of newborns of each coat color mutant is shown in Table 3. The ratio of male to female were 1:1.21 in agouti, 1:1.03 in white spotted-agouti, 1:1.29 in albino, 1:1.02 in black and 1:1.21 in white spotted-black, respectively. The number of the female newborns was observed a little more than that of the male in all coat color mutants.

Table 1 Averaged food and water consumptions of each mutant of the Mongolian gerbil

		Body weight (g)	Food (g)	Water (m*l*)
Agouti	male	62.3	4.4	5.4
	female	56.0	4.2	5.0
White spotted-agouti	male	64.2	4.5	5.6
	female	53.7	3.4	5.2
Albino	male	62.0	4.6	6.7
	female	55.0	3.8	5.9
Black	male	65.2	4.7	6.1
	female	56.1	4.3	5.5
White spotted-black	male	63.3	4.5	5.7
	female	55.4	3.9	5.5

N=20

Table 2 Reproductive physiology of each mutant of the Mongolian gerbil

	Pairs	Total number of parturitions	Range of parturitions	Number of newborns	
				Range	Mean
Agouti	17	69	1-10	2-9	4.8
White spotted-agouti	17	75	2-9	1-7	4.2
Albino	12	71	1-11	1-8	4.8
Black	8	23	1-6	2-8	4.9
White spotted-black	7	31	2-10	1-7	3.9

Table 3 Sex ratio of newborns of each mutant of the Mongolian gerbil

	Total	Male	Female	Ratio
				male : female
Agouti	267	121	146	1 : 1.21
White spotted-agouti	294	145	149	1 : 1.03
Albino	300	131	169	1 : 1.29
Black	111	55	56	1 : 1.02
White spotted-black	84	38	46	1 : 1.21

Period of the delivery:

Period of the delivery of each mutant of the gerbil is summarized in Table 4. The periods changed to wide range from 25 days to 186 days. The periods within 30 days were occupied 17.8% to 28.0%. The most propotion of delivery in each coat color mutant was within the range 31-40 days after previous one.

Segregation ratios of crosses:

Table 5 summarizes segregation ratios of experimental matings. In the crosses of spotted-agouti with agouti gerbil, the offsprings showed spotted-agouti or agouti coat color in approximately equal numbers. Matings among F_1 hybrids with spot coat color produced agouti and spot animals. In F_3 generation of spot×spot mating, albino gerbils were appeared. Mating between albino and agouti animals produced gerbils with three different coat colors. All offsprings of albino×albino matings were albino. On the other hand, one pair of F_2 spotted-agouti gerbils produced black and white spotted-black animals. To study the inheritance of these coat colors, spotted-black mated with spotted-agouti gerbils. Their offsprings showed five different coat colors. In the crosses among F_1 animals with spotted-black coat color, four different coat colors were appeared. Mating among black gerbils produced only black animals.

Table 4 Period of the delivery of each mutant of the Mongolian gerbil

	Total number of parturitions	Period of the delivery (days)			
		25-30	31-40	41-50	>51
Agouti	69	18.9 (%)	52.8 (%)	9.4 (%)	18.9 (%)
White spotted-agouti	75	17.8	57.4	7.0	17.8
Albino	71	23.7	57.6	3.4	15.3
Black	23	18.7	43.8	12.5	25.0
White spotted-black	31	28.0	36.0	8.0	28.0

Table 5 Segregation ratios of crosses

Cross	Generation	Total	Phenotype of progeny				
			agouti	spotted	albino	black	spotted-black
agouti×spotted	F_1	76	33	43	0	0	0
spotted(F_1)×spotted (F_1)	F_2	127	39	88	0	0	0
agouti×albino	F_1	109	58	31	20	0	0
albino(F_1)×albino(F_1)	F_2	83	0	0	83	0	0
spotted×spotted-black	F_1	53	8	28	1	4	12
spotted-black(F_1)× spotted-black(F_1)	F_2	68	0	2	6	27	33
black(F_1)×black(F_1)	F_2	59	0	0	0	59	0

spotted: white spotted-agouti
spotted-black: white spotted-black

Discussion

Some reports concerning the coat color mutants of the Mongolian gerbil have been published, for example the study for the white spotted-agouti type of mutants was reported by Waring *et al.* (1978) and that for black type was by Cramlet *et al.* (1974), Waring *et al.* (1980) and Turner *et al.* (1984). However, the present paper is the first report dealing with albino of the mutant and with biological characteristics of the coat color mutants.

We carried out the study of the growth curve, the reproduction rates and inheritance of these coat color mutants of the gerbil and compared with each other in order to analyze the infection kinetics of the filarial infection later.

On the growth curve of the agouti type of the gerbil, Japanese authors of the earlier investigations reported that the body weight was similar in males and females each other (Nakai *et al.*, 1960; Izaki *et al.*, 1985; Tanaka *et al.*, 1988), but some of the foreign investigators pointed out that the weight of males of agouti type always exceeded that of females (Norris *et al.*, 1972; Arrington *et al.*, 1973). In the present works, we could observe the clear difference in body weight between sexes throughout the experiments about the five kinds of the types of the gerbil, such as agouti type, white spotted-agouti type, albino type, black type and white spotted-black type.

In the study of the reproductive physiology of the coat color mutants of the gerbil, the period of the delivery was found to be different to wide range, as the reports of Marston *et al.* (1965) and Arrington *et al.* (1973). Nakai *et al.* (1960) described that the gestation period of the Mongolian gerbil lay between 24 and 26 days. In the present study, the period within 30 days occupied 17.8 to 28.0% in the five kinds of mutants. The period of between 31 to 39 days showed 36.0 to 57.6%, their cause could be explained the delayed implantation of the placenta following post-partum mating. However, the reason why the period delayed over 40 days was not clear. The cause of this phenomenon may related to an irregular and unpredictable time interval of oestrus cycle (Marston *et al.*, 1965) or the high incidence of edema in the ovarian capsule (Tanaka *et al.*, 1988). Further detail experiment is necessary for the reason of the wide range of the delivery periods of the gerbil.

Some genetic studies on coat colors are also described in this paper. As the crosses of spotted with agouti produced spotted and agouti in approximately equal number, spotted mutant thought to be controlled by an autosomal dominant gene. However, the segregation ratio of spotted×spotted fits about 2:1 ratio, but does not fit 3:1 ratio expected for a dominant gene. This fact is similar to the past report by Waring *et al.* (1978). They assigned the gene symbol Sp and that is prenatally lethal gene in homozygous condition. But, present study could not find stillborn young and could not confirm the homozygous lethal gene. In F_3 generation of spotted×spotted mating, albino, black and spotted-black gerbils were appeared. That indicated the existance of the a and the c loci controlling coat colors as in mice or rats. Albino and black coat colors are seemed to be controlled by autosomal ressesive genes, because the mating of albino×albino or black×black produced only albino or black colored young, respectively. But, the crosses of agouti×albino or spotted-black×spotted-black produced various coat colors and the fact made the mode of the inheritance compricative. At any rate, albino and black gerbils were found in F_3 generation of spotted×spotted mating. This indicated that the gene of albino and black coat colors were maintained in our spotted-agouti colony.

As mentioned above, this paper made comparative studies on biological characteristics among coat color mutants and some genetic analysis of coat colors. As a result, coat color mutants have no difference on growth curve and reproductive phisiology. This paper showed that the coat color mutants of the gerbil also would be useful animal as suscestible host to filarial infection.

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フィラリア感染好適宿主としてのMongolian gerbil —被毛色突然変異体の成長と繁殖に関する比較および被毛色の遺伝様式について

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Mongolian gerbil（スナネズミ）の被毛色突然変異体（white spotted-agouti, albino, black およびwhite spotted-black type）について，成長と繁殖に関してagouti typeと比較した。しかし，被毛色間に有意差は見られなかった。出生時体重は3.0g，性成熟後の3カ月齢では各被毛色共，雄は約60g雌は約52gであった。体重の明らかな性差は，生後6週齢から出現した。平均産仔数は4.2から4.8匹，産仔の性比は，雌が雄よりやや多い傾向にあった。分娩間隔の変動は非常に大きく，25～30日のものが全分娩の20%前後，半数以上が31～40日であったが，51日以上のものも20%近くを占めた。このばらつきの原因としては，後分娩発情があること，その際着床遅延が起こること，性周期が不規則であること，および卵巣水腫の発生頻度が高いことなどが考えられた。また，被毛色の遺伝様式についても検討を行った。ラットやマウスと同様の被毛色を制御する遺伝子が，gerbilにも存在することが明らかにされ，albinoおよびblack typeの被毛色は，常染色体性劣性遺伝子によって支配されていることが示唆された。

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REACTIVITY OF MONOCLONAL ANTIBODIES RECOGNIZING THE TEGUMENTAL ANTIGENS OF *PARAGONIMUS OHIRAI* TO OTHER *PARAGONIMUS* SPECIES

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Abstract: The two monoclonal antibodies which were raised by *Paragonimus ohirai* antigens and recognized the different tegumental antigens, were tested for reactivity with other *Paragonimus* species, *P. westermani* and *P. miyazakii*, and with other various helminth parasites, especially trematodes (*Fasciola* sp., *Schistosoma japonicum* and *Clonorchis sinensis*). The monoclonal antibody (MS-Mab) recognizing the tegumental antigen present from the metacercaria to adult stages, reacted with the crude antigens of other *Paragonimus* species. The reactive antigens were localized on the tegumental syncytium and tegumental cells, as previously reported in *P. ohirai*. On the other hand, the other monoclonal antibody (AS-Mab) recognizing the adult main tegumental antigen reacted only with *P. ohirai* antigen. These two monoclonal antibodies did not react with any other helminth adult antigens examined. It is concluded that MS-Mab is genus-specific whereas AS-Mab is species-specific.

INTRODUCTION

There have been a number of papers about comparative analysis of antigens among parasite species or genera. In lung flukes, genus *Paragonimus*, most of the comparative studies have been performed for the purpose of immunodiagnosis of paragonimiasis using Ouchterlony immunodiffusion (Yokogawa *et al.*, 1974), immuno-electrophoresis (Capron *et al.*, 1969; Tsuji, 1975), enzyme-linked immunosorbent assay (ELISA) (Knobloch and Lederer, 1983; Kojima *et al.*, 1983) and SDS-PAGE (Itoh and Sato, 1988). However, there have been few attempts to purify the species-specific or common antigens, and moreover, little attention has been paid to the localization of these antigens on the fluke body.

The surface antigens of a parasite play a crucial role in the immunobiological interaction between host and parasite. We prepared two monoclonal antibodies against the surface antigens of *Paragonimus ohirai*, one (MS-Mab) recognized the surface antigen present in the all development stages from metacercaria to adult and the other (AS-Mab) recognized the major adult-stage surface antigen which is absent in the metacercaria stage (Oikawa and Ikeda, 1989). The present study was undertaken to determine the reactivity of MS-Mab and AS-Mab to the antigens of other *Paragonimus* species.

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Materials and Methods

Parasite

Metacercariae of *P. ohirai* (P.o.), *P. miyazakii* (P.m.) and *P. westermani* (P.w.) were collected from 3 species of crabs; *Sesarma dehaani, Geothelphusa dehaani* and *Eriocheir japonicus,* respectively. Thirty P.w. or P.m. metacercariae were inoculated orally into a dog and twenty P.o. metacercariae into a Wistar rat, and then adult flukes were recovered 3-5 months after infection. The flukes were washed with saline and incubated in several changes of saline for 5 h at room temperature.

Antigen and Ouchterlony immunodiffusion

Each crude adult antigen was prepared by homogenizing adult flukes and extracting with 10 mM phosphate buffered saline (PBS, pH 7.2). The washed flukes were homogenized in PBS by using a Potter-Elvehjem homogenizer. After the homogenate was stirred overnight at 4°C, it was centrifuged at 20,000×*g* for 30 min. The supernatant was dialyzed against PBS and used as a crude adult antigen (5 mg protein/m*l*). Other helminth antigens were prepared as described above. Ouchterlony immunodiffusion was carried out in 1% agarose containing PBS and 0.02% NaN_3. After overnight diffusion between monoclonal antibodies and crude adult antigens, the gels were washed in PBS and distilled water, dried, and stained with 0.5% amidoblack 10 B dye solution.

Immunofluorescent staining

Indirect immunofluorescent staining was performed essentially according to the immunoperoxidase staining described previously (Oikawa and Ikeda, 1989). Briefly, the washed adult flukes were fixed with cold 95% ethanol for 1 day, and then were embedded with paraffin, cut, and deparaffinized according to the standard technique. After being washed with PBS, the fluke sections were incubated with diluted monoclonal antibodies for 1 h, washed in PBS, and then incubated for 1 h with FITC-conjugated anti-mouse IgG antibody (Cappel Laboratories, Westchester, PA) diluted to 1/20. After washing, the sections were mounted with buffered glycerol. The preparations were examined with a fluorescence microscope.

Results

Ouchterlony immunodiffusion of MS-Mab and AS-Mab against crude adult antigens of three *Paragonimus* species is shown in Fig. 1a, b. MS-Mab reacted with the crude antigens of all species examined and produced a fusing precipitin line, whereas AS-Mab did not react with any antigens except the P.o. antigen. To examine the specificity of MS-Mab and AS-Mab to *Paragonimus* antigens, Ouchterlony immunodiffusion was performed against crude adult antigens of various helminth parasites, especially trematode antigens (*Fasciola* sp., *Schistosoma japonicum* and *Clonorchis sinensis*). As shown in Fig. 1c, d, both MS-Mab and AS-Mab produced no precipitin against other helminth antigens.

The indirect immunofluorescent staining was performed to examine whether P.w. and P. m. antigens recognized by MS-Mab were localized on the same body site as P.o. fluke. The immunostaining of adult fluke sections is shown in Fig. 2-1a, b, c. As observed previously in P.o. fluke, the tegumental syncytium and the tegumental cells were immunostained on P.w. and P.m. fluke sections. Specific immunostaining was not observed on the other tissues. In

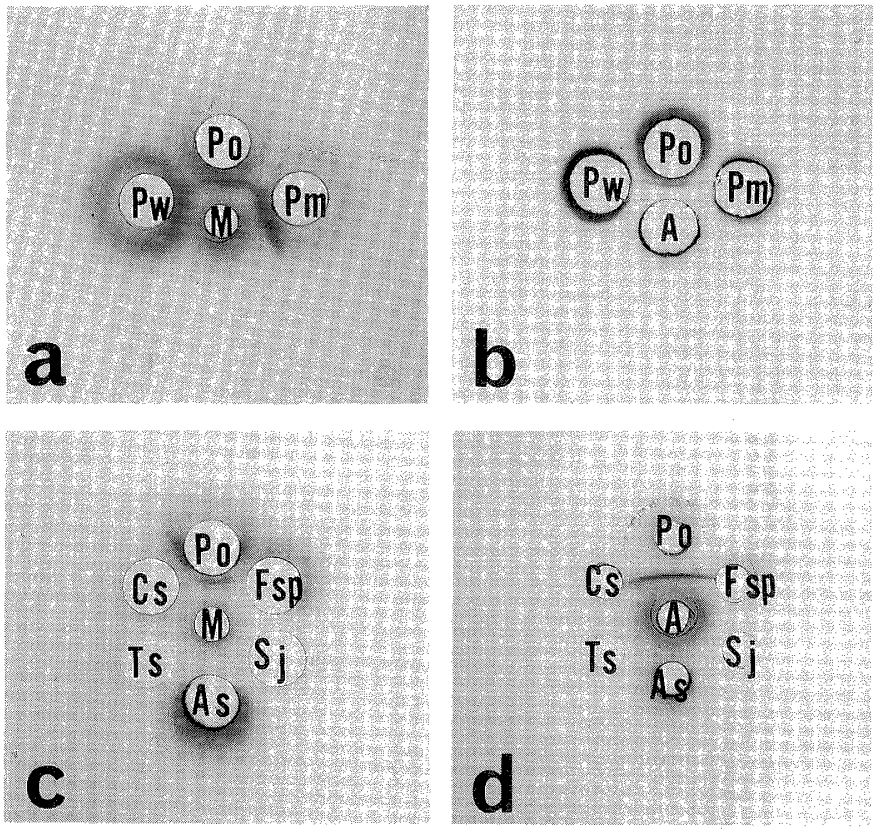

Figure 1 Ouchterlony immunodiffusion of the monoclonal antibodies, MS-Mab and AS-Mab, against crude adult antigens of three *Paragonimus* species and of various helminth parasites. M, MS-Mab; A, AS-Mab; Pm, *P. miyazakii* antigen; Po, *P. ohirai* antigen; Pw, *P. westermani* antigen; Sj, *S. japonicum* antigen; Fsp, *F.* sp. antigen; Cs, *C. sinensis* antigen; As, *Ascaris lumbricoides suum* antigen; Ts, *Taenia saginata* antigen.

the immunostaining with AS-Mab, no immunostaining was observed on P.w. and P.m. sections (Fig. 2-2b, c).

Discussion

The present study clarified that the antigen recognized by MS-Mab was present in all of three *Paragonimus* species examined whereas the antigen recognized by AS-Mab was present only in P.o. species whose antigen raised the monoclonal antibody. As previously reported in P.o. species (Oikawa and Ikeda, 1989), MS-Mab reactive antigen is a tegumental antigen present in the all development stages from metacercaria to adult, whereas AS-Mab reactive antigen is absent in the metacercaria, appears less than one week after infection, and is a main tegumental antigen in the adult fluke. We confirmed that MS-Mab reacted with the antigens on the tegumental syncytium and tegumental cells in P.w. and P.m. flukes as well as in the P.o. fluke. Fujino *et al.* (1989) showed ultrastructually that MS-Mab binds to a

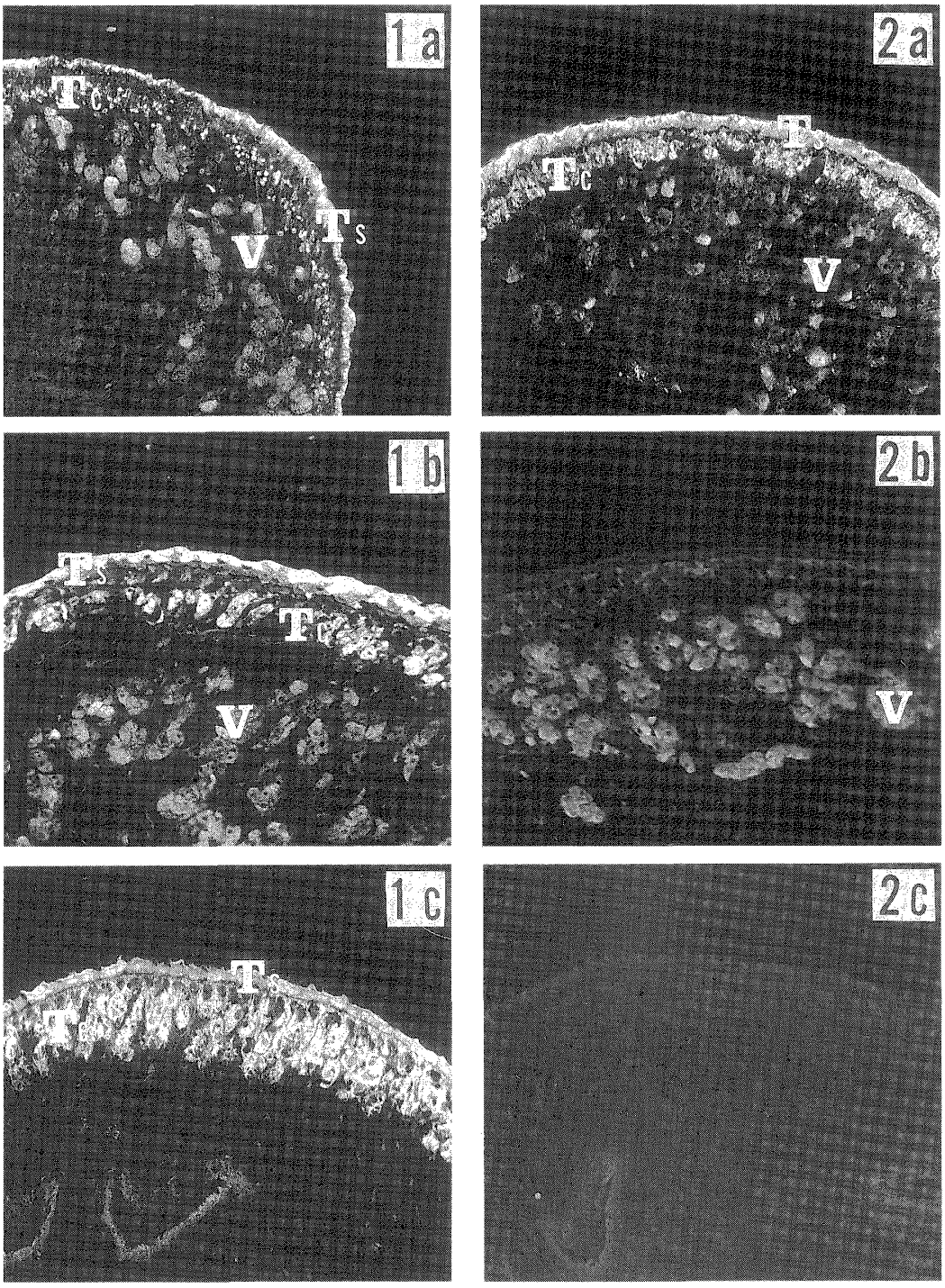

Figure 2 Indirect immunofluorescent staining of MS-Mab and AS-Mab on *Paragonimus* adult fluke sections. a, *P. ohirai* fluke section; b, *P. westermani* fluke section; c, *P. miyazakii* fluke section. MS-Mab bound to the tegumental syncytium (Ts) and tegumental cells (Tc) in all *Paragonimus* species (1a, 1b and 1c), whereas AS-Mab did not bind to any sites except the P.o. tegumental structures (2a, 2b and 2c). Vitelline glands (V) were autofluorescent.

tegumental glycocalyx and some types of tegumental secretory bodies. The AS-Mab reacting antigen was previously suggested, on the basis of the immunostaining features and the presence of the antigen in excretory-secretory products, to be a tegumental glycocalyx different from the metacercaria one. Therefore, the metacercaria type glycocalyx, although it is expressed until the adult stage, is a cross-reactive antigen among the three *Paragonimus* species, and the adult type glycocalyx is a species-specific antigen, at least between P.o. and the others. This stage-dependent difference on antigenic cross-reactivity of glycocalyces is very interesting. In *F. hepatica*, the change in the antigenicity of the tegumental glycocalyx occurs during development in the final host, and the change was estimated to be as a result of adaptation to the host's environment (Hanna, 1980). The present finding that the adult type glycocalyx formed after invasion of the final host is not a cross-reactive antigen, suggests that the glycocalyx antigenicity might alter for adaptation to different suitable final hosts.

The presence of cross-reactive antigens between P.w. and *S. mansoni* and *F. hepatica* adult flukes has been reported (Capron *et al.*, 1969; Oelerich and Nwokolo, 1974; Tsuji, 1975; Hillyer and Serrano, 1983). However, these two monoclonal antibodies were unreactive to crude adult antigens from other adult trematodes (*F.* sp., *S. japonicum, C. sinensis*) and several helminths (*Ascaris lumbricoides suum, Taenia saginata*). Thus, the two monoclonal antibodies are specific to lung flukes, and it is concluded that MS-Mab is genus-specific whereas AS-Mab is species-specific. It is expected that MS-Mab could be applicable for immunodiagnosis of paragonimiasis. AS-Mab might be useful only in paragonimiasis ohirai which is a rare case but was reported as a suspected case by Yamaguchi and Ichinohe (1974). Preparation of monoclonal antibodies against P.w. and P.m. flukes is needed for species-specific immunodiagnosis of paragonimiasis.

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大平肺吸虫外皮抗原を認識するモノクローナル抗体の他種肺吸虫への反応性

池田　照明・及川陽三郎

我々は，大平肺吸虫外皮抗原を認識するモノクローナル抗体2種類（MS-Mab，AS-Mab）を作製したことを前報にて報告した。MS-Mabは，メタセルカリア期から成虫期まで存在する幼虫期主要外皮抗原を認識し，AS-Mabは，終宿主感染後に形成される成虫期主要外皮抗原を認識した。今回，これら2種モノクローナル抗体と他種肺吸虫抗原との交差反応性を検討し，併せて他の寄生蠕虫抗原，特に吸虫類抗原との反応性も検討した。

大平肺吸虫，ウェステルマン肺吸虫および宮崎肺吸虫の各成虫抗原と2種モノクローナル抗体との間でのオクタロニー反応では，MS-Mabは全ての肺吸虫抗原に対して互いに融合する1本の沈降線を形成するのに対し，AS-Mabは大平肺吸虫抗原以外とは反応しなかった。他の寄生蠕虫（吸虫3種，線虫と条虫各1種）の成虫抗原とのオクタロニー反応では，これら2種のモノクローナル抗体は，いずれの抗原に対しても反応性が認められなかった。虫体切片による蛍光抗体法では，MS-Mabはウェステルマン肺吸虫と宮崎肺吸虫に対して，大平肺吸虫の場合と同じく外皮シンチチウムと外皮細胞に結合し，一方，AS-Mabは大平肺吸虫のみにしか結合しなかった。

これらの結果は，MS-Mabが肺吸虫属特異性エピトープを持つ外皮抗原を認識し，AS-Mabが大平肺吸虫種特異性エピトープを持つ外皮抗原を認識することを明らかにした。

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CLINICAL AND EPIDEMIOLOGICAL STUDIES ON A 47KD *PLASMODIUM FALCIPARUM* ANTIGEN

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Abstract: Sera were taken at random intervals from a Japanese patient during the acute to convalescent phases of falciparum malaria. Western blotting study revealed that the 47kD antigenic polypeptide of the parasite was strongly presented by the sera taken during the acute phase of the infection, while the 47kD band became faint as the phase progressed, and serum taken 2 months after the onset of the disease was only weakly reactive to the molecule. During the observation period, the indirect fluorescent antibody titer invariably persisted at 1:256. The reactivity of the serum samples taken at hypo, meso and hyperendemic localities in Sudan was tested by the same method. Regardless of the endemic backgrounds, sera from examinees with currently existing parasitemia or high antibody titers measured by an avidin biotin peroxidase complex enzyme-linked immunosorbent assay (ABC-ELISA), reacted to the 47kD antigenic polypeptide, whereas low-titered sera did not present the 47kD band. Some high-titered sera from the meso or hyperendemic locality showed no reactivity to the 47kD antigen. It was presumed that the high antibody titer of this group was a reflection of accumulated past malaria infections but not of currently active malaria or recent past infection. The 47kD molecule is a useful parasite antigenic polypeptide in terms of defining present and/or recent past infection in the serological survey of malaria.

INTRODUCTION

Malarial seroepidemiology can be used for the assessment of *period prevalence* or to reinforce data on *point prevalence* (Draper *et al.*, 1972; Voller and Draper, 1982). In particular, it is valuable in the detection of malarious foci following control activities (WHO, 1974). However, high antibody titers shown by the indirect fluorescent antibody test (IFAT) or enzyme-linked immunosorbent assay (ELISA) cannot differentiate recent episodes from the cumulative infections of endemic inhabitants. Western blotting may provide more precise information than the IFAT or ELISA, because it gives details of the different antigenic fractions responding to each of the antibodies present (Thelu and Ambroise-Thomas, 1988). The 47kD *Plasmodium falciparum* (*P.f.*) antigen revealed specifically by the sera at the acute stage of falciparum malaria in a follow-up study on a Japanese imported case is highlighted. In the convalescent stage of the infection, the reactivities of the patient's sera against the 47kD molecule dropped according to the time course, while the indirect fluorescent antibody

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remained at high titer. The finding was applied to a small pilot field study in the Sudan Blue Nile Area. Three spots representing hypo, meso and hyperendemic areas were serologically surveyed and the results by western blotting were compared with declared individual fever histories, antibody measurement and parasite detection. The potential interest of the 47kD antigen, which reflects recent past infection in the epidemiology of malaria, is discussed.

Subjects, Materials and Methods

A severe imported malaria case studied

The case was a 28-year-old Japanese male. He made a trip in Africa and began having fever attacks in Zambia on 12 February 1988. No antimalarial was administered until he returned back to Japan and was hospitalized on 23 February. Unconsciousness, anemia, jaundice and hepatosplenomegaly were recognized on admission. He developed fever to 41°C, and asexual forms of *P.f.* were observed at a density of 17% erythrocytes on his Giemsa-stained thin blood smear. The severe malaria was treated with artemether (Kano *et al.*, 1988), and supplemented with chloroquine. The parasites were eliminated on 26 February and the body temperature dropped to below 37°C on 1 March. Recrudescence did not occur.

Study area in Sudan

The epidemiological study was conducted at the headquarters of the Blue Nile Health Project at Wad Medani, about 150 km southeast of Khartoum (16°N, 32°E). Blood specimens were collected from 3 places: Mobi, which was close to Wad Medani; and Sennar and Sennar Junction, which were localities about 100 km upstream on the Blue Nile from Wad Medani. Malaria transmission was low in Mobi at the time of the sampling (January 1989). Sennar was not within the control area of the Blue Nile Health Project and had a high malaria incidence through September-November (El Gaddal *et al.*, 1985). In Sennar Junction, refugee camps were set out and malaria transmission was very high. Sera were collected by venipuncture from a total of 37 donors, of ages from 9 to 70, and were subjected to antibody measurement by means of the ABC-ELISA. Reactivity of the obtained sera to the 47kD antigenic polypeptide was tested by western blotting.

Parasite antigens

The antigens of the IFAT, ABC-ELISA and SDS-PAGE were obtained from an *in vitro* cultivation (Trager and Jensen, 1976; Miyagami and Waki, 1985) of a *P.f.* strain (GGG strain; donated by Dr. Ambroise-Thomas in 1979) using RPMI medium, type O human red blood cells and 10% human serum.

IFAT

The IFAT was carried out according to a modified method described by Voller and O' Neil (1971). Sera at 4-fold dilutions, 1:4-1:4,096, were overlaid on the antigen spot on a glass slide. Thirty-times-diluted fluorescein conjugated rabbit anti-human IgG (Behring, West Germany) was applied as the secondary antibody. The results were read with an incident light illuminating type fluorescent microscope (Olympus, model BH-RFC, Japan).

ABC-ELISA

The ABC-ELISA was devised by Sato *et al.* (1990) for the detection of malarial antibody. Briefly, the antigen of protein concentration of 250 $\mu g/ml$ was placed in each well of a 96-well U-bottom microtiter plate (Greiner, West Germany). Sera at 2-fold dilutions, 1:32-1:1,024, were applied on the each well bottom. Enzyme reaction was performed by the Vectastain™ ABC kit, which consisted of biotinylated anti-human IgG goat serum and avidin biotin complex horse radish peroxidase (Vector Laboratories Inc., CA). A blue color was generated in the well with a positive serum by the addition of the substrate, 4-chloro-1-naphthol, and the results were read with the naked eye. The ABC-ELISA always showed an excellent correlation with the IFAT results, and this method was designed particularly for field use. In the present study, serological study in Sudan was carried out at the laboratory of the Blue Nile Health Project.

SDS-PAGE and western blotting

Erythrocytes infected with asynchronous *P.f.* parasites at >20% parasitemia were harvested by *in vitro* culture for the antigen preparation. Hemolysis of the infected erythrocytes by treatment with a hypotonic solution (20 mM $MgCl_2$ in PBS pH 7.2) was followed by centrifugation of the lysate at 3,600 rpm for 15 min. After three washings with PBS, the sediment was dissolved in the antigen solution (2.3% SDS, 5% 2-ME, 1 mM PMSF, 0.0625 M Tris-Cl pH 6.8) and preserved at −35°C.

One dimensional SDS-PAGE analysis was performed in 10% acrylamide gels according to the method of Laemmli (1970). The molecular weight markers used in all the experiments were obtained by SDS-PAGE Standards (Bio-Rad, CA).

Western blotting analysis was made by electrotransfer to a nitrocellulose sheet (Clear Blot Membrane-p, ATTO, Tokyo) employing Horizonblot (ATTO, Tokyo) with absorbent papers soaked with blotting buffer (0.1 M Tris, 0.192 M Glycine, 20% methanol). Each serum sample to be studied was diluted 5 times and reacted with a nitrocellulose strip. Secondary antibody to be reacted was 50-times-diluted peroxidase conjugated anti-human IgG goat serum {$F(ab')_2$ fragment specific}, and the substrate for the enzyme reaction was 4-chloro-1-naphthol.

RESULTS

A longitudinal study of the imported case

With the understanding of and in collaboration with the patient, serum samples were taken consecutively from 23 February to 19 April. The collected sera were subjected to both malarial IFAT and western blotting. The IFAT titers against *P.f.* antigen remained at 1:256 from the acute stage through the convalescent stage (Feb. 23-Apr. 19). The time course of the antibody reactivity against malarial antigenic polypeptide is demonstrated in Figure 1. A solid square stands for a lane of electrophoresed parasite antigen fraction, while an open square represents a lane of electrophoresed substance derived from RBCs, which was prepared by the same method used on the infected erythrocytes. The 47kD antigen was specifically and distinctively demonstrated at the acute stage of the infection, however it was noted that the 47kD band became extremely faint at the late stage of convalescence.

The control serum from a healthy donor who had never been abroad showed no

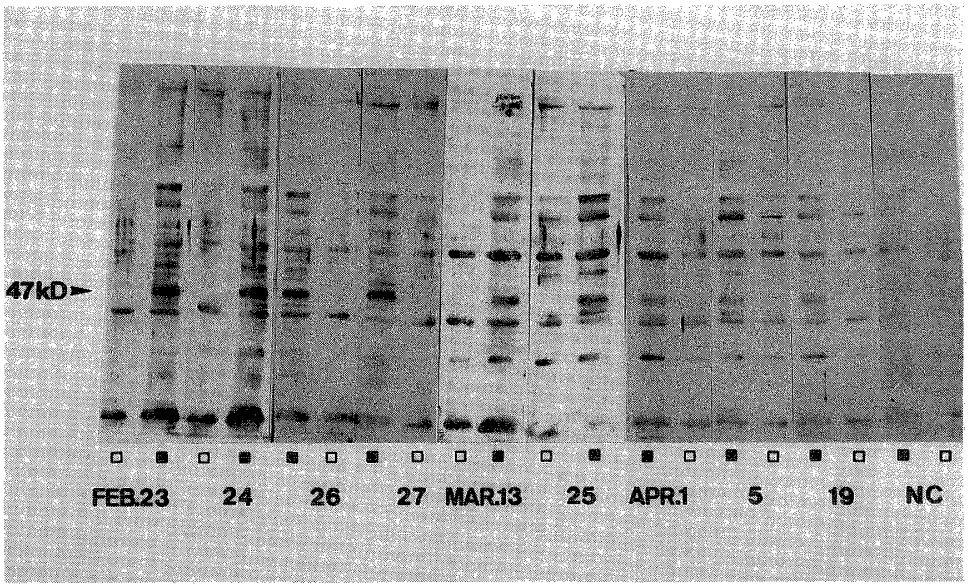

Figure 1 Two months observation on the *P.f.* 47kD antigenic polypeptide presented by the sera taken from a Japanese falciparum case.
(■: *P.f.* antigenic polypeptide, □: RBC antigenic polypeptide, NC: serum from a healthy donor)

reactivity against the 47kD band (NC lane in Figure 1). Neither sera from other healthy donors without a malaria history nor sera from vivax malaria patients reacted the *P.f.* 47kD band by western blotting.

Epidemiological studies in Sudan

A small pilot field study was conducted in the Sudan Blue Nile Area. In Mobi, blood samples were taken from 90 individuals by venipuncture with a syringe or finger-prick method (Kano *et al.*, 1989), and 26 of them showed titers equal to or higher than 1:32 by the ABC-ELISA, while the parasite rate was 7.7%. Ten serum samples taken by venipuncture from adults of ages 35-70 were subjected to western blotting, and the results are shown in Table 1. Only one individual (No. 165) showed the *P.f.* parasite. By the ABC-ELISA test, 4 individuals showed titers equal to or lower than 1:32. Titers at 1:128-1:512 were demonstrated in 6 other examinees. The 47kD band was demonstrated in two individuals, namely No. 165, who showed parasitemia, and No. 179, who manifested a relatively high ABC-ELISA titer at 1:512.

In Sennar, a total of 72 samples were collected at a primary school (54 of 72 showed ≧ 1:32 titer; the parasite rate was 47%). Sera from venous blood were collected from 13 children and 1 teacher and subjected to western blotting (Table 2). Parasites were detected in 5 children, and 11 of 14 showed higher titers than 1:32 by the ABC-ELISA. The 47kD band was demonstrated by those who showed parasitemia or a high ABC-ELISA titer.

In Sennar Junction, refugees were densely concentrated in a camp. All examinees under 20 years of age showed parasitemia, and none over 20 years old showed parasitemia. Table 3 demonstrates that titers equal to or higher than 1:128 were shown by 13 inhabitants, but the

adults showed relatively higher titers than the adolescents. The 47kD reactive antibody was shown in 9 individuals. A 17-year-old male (No. 15) showed parasitemia, but the 47kD band was not demonstrated by his serum.

DISCUSSION

The 47kD antigenic polypeptide of *P.f.* was strongly demonstrated by the sera of the imported Japanese patient at the acute stage of falciparum infection. Although the antibody titer persisted at a high level throughout the observed period, the 47kD band became faintly visible by serum taken during the late convalescent period. This finding was also confirmed

Table 1 Individual records of 10 adults examined in a controlled hypoendemic village, Mobi

No.	Age	Sex	Fever	ABC*	Parasite	47kD
15	42	m	+	<32	−	−
17	53	m	−	256	−	−
53	60	f	+	<32	−	−
106	35	m	−	256	−	−
107	70	f	−	128	−	−
127	35	f	−	<32	−	−
131	65	m	−	256	−	−
165	45	m	−	128	F**	+
176	59	m	+	32	−	−
179	70	m	−	512	−	+

* The reciprocal ABC-ELISA titer
** F, asexual form of the *Plasmodium falciparum*

Table 2 Individual records at a primary school in a mesoendemic locality of Sennar

No.	Age	Sex	Fever	ABC	Parasite	47kD
11		f	−	<32	−	−
14	15	f	+	512	F	+
23	14	m	−	64	−	−
29	11	m	+	1,024	F	+
32	10	m	−	512	−	+
33	9	m	−	256	−	+
37	12	m	−	<32	−	−
38	12	m	−	64	F	+
39	13	m	−	128	−	+
45	14		+	1,024	FG*	+
51	10	f	−	1,024	−	+
66	28	m	+	<32	−	−
69	18		+	64	FG	+
71	14	m	+	256	−	+

* FG, sexual form (gametocyte) of the *Plasmodium falciparum*

Table 3 Thirteen individual records at a hyperendemic refugee camp in Sennar Junction

No.	Age	Sex	Fever	ABC	Parasite	47kD
2	13	m	+	256	F	+
4	18	f	−	512	F	+
6	26		+	128	−	−
9	23	f	−	1,024	−	−
12	12	f	+	512	F	+
13	35	m	+	1,024	−	+
15	17	m	−	256	F	−
16	19	m	+	512	F	+
18	40	f	−	1,024	−	+
19	10	m	+	256	F	+
20	37	m	+	256	−	−
21	60	m	+	1,024	−	+
23	21	m	+	1,024	−	+

in the studies on 4 other imported Japanese falciparum cases both during acute and convalescent period of each (data are not shown). This means that the 47kD reactive antibody specifically reflects present or recent past infection in non-immune patients with one-point falciparum infection.

In the pilot study conducted in the Blue Nile Area in Sudan, 47kD antigenic polypeptide was also demonstrated by the endemic inhabitants showing parasitemia, regardless of their background of endemicity. In hypoendemic Mobi, a considerable background of past infection was estimated by the high titers of the people examined. And yet, the 47kD band was demonstrated by a man with parasitemia and another elderly male with a high antibody titer but no parasitemia. The reflection of present or recent past infection by 47kD reactive antibody was also reasoned in the observation on teenagers living in the mesoendemic area (Table 2): the 47kD band was displayed by sera taken from 5 children with currently existing parasitemia. In principle, the finding was also true in the hyperendemic refugee camp at Sennar Junction: 5 of 6 examinees with parasitemia showed 47kD reactive antibody in their sera. While on the other hand, some donors from the 3 endemic regions who did not manifest detectable parasitemia showed high ABC-ELISA titers and 47kD reactive antibodies. Those people might have cleared parasitemia of recent infection at the time of the present study, because most people in this group was found in mesoendemic and hyperendemic regions. Some of the examinees showed high antibody titers by the ABC-ELISA, but neither 47kD reactive antibody nor parasitemia was demonstrated in them. This group might reflect accumulated infections but not present or recent past infections.

Thus, the 47kD reactive antibody, which reflects the acute and early convalescent phase of falciparum infection in the non-immune Japanese patient, may be a useful immunological indicator in the differentiation of present and/or recent past infection from past malaria accumulation, both of which share high antibody titers by the classical serological methods.

The information about the total experience of malaria of the individuals in a community can be obtained by the serological surveys, which can be called the information on *period prevalence*. This index is particularly useful in the advanced phase of malaria control

program because it becomes difficult to detect parasites by one cross-sectional survey. In the present study it was pointed that the 47kD reactive antibody reflected present and/or recent past infection in the endemic population. Application of the 47kD antigen to a serodiagnostic method as a reference molecular epitope will open a new step in the serological survey of malaria in terms of defining the incidence of the disease within a short period range.

The 47kD molecule in *P.f.* was also reported by another research group. Delplace *et al.* (1985) detected an exoantigen of *P.f.* of the same molecular weight in a culture medium immediately after the merozoites were released. This finding meets our observation using a laser scan microscope (Bio-Rad), which showed that a monoclonal antibody to the 47kD molecule was bound to the surface of the late schizont of cultured *P.f.* (to be reported elsewhere). A wide ubiquity of the 47kD *P.f.* antigen among isolates of various geographic origin was also discussed by Bhatia *et al.* (1987). In our study, the 47kD antigen was also affirmed in another established strain of *P.f.* Thus the antigenic polypeptide is commonly shared in the *P.f.* species.

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熱帯熱マラリア原虫47kD抗原の疫学的意義

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日本人輸入熱帯熱マラリア患者の血清を入院時より治療後にかけて，約2カ月間継時的に採取し，それぞれの血清中の免疫グロブリンと反応する熱帯熱マラリア抗原分子につき，ウェスタン・ブロット法により調べた。その結果，感染初期血清中の免疫グロブリンが特異的に47kD抗原と結合すること，しかし，入院後57日目の血清は，47kD抗原との反応性が著しく弱まってしまうことが見出された。この間，間接蛍光抗体法によって測定された特異的免疫グロブリンの力価は，1：256と変りなく高値を示した。同様の事実が，4名の日本人熱帯熱マラリア感染においても確認された。この知見は，47kD抗原特異的免疫グロブリンは，現在の熱帯熱マラリア感染，もしくは，極めて近い過去において起こった熱帯熱マラリア感染を反映する特徴的な抗体であることを示している。日本人患者において見出された以上の知見をもとに，スーダンにおいて野外調査を行い，低流行地，中等度流行地，高度流行地の住民につき，ABC-ELISA法により抗体価を測定した。さらに，それぞれの流行地において血清が得られた例について，各血清と47kD抗原との反応性をウェスタン・ブロット法により調べた。その結果，現在末梢血液中に熱帯熱マラリア原虫が証明された例では，1例以外はすべて47kD抗原結合性免疫グロブリンが確認された。また，現在原虫は証明されないが，極めて近い過去に感染を受けたと想定される住民から採取された高抗体価血清も，47kD抗原と強く反応した。しかし，一部の高抗体価血清では，47kD抗原との反応性が示されなかった。これらの血清供与者は，過去のマラリアの累積結果が高抗体価として表現され，現在および近い過去において熱帯熱マラリア感染はなかったグループと判断された。以上の実験結果から，熱帯熱マラリア原虫の47kD抗原は，疫学上，現在もしくはごく近い過去のマラリア流行を捉える上で，有用な抗原であると考察された。

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APPLICATION OF TWO TYPES OF *TRYPANOSOMA CRUZI* AMASTIGOTES OF DIFFERENT VIRULENCE TO ELISA FOR CHAGAS' DISEASE

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Abstract: *Trypanosoma cruzi* amastigotes derived from fibroblast cultures and from cell-free liquid medium were comparatively evaluated as solid-phase antigens of ELISA for Chagas' disease. Amastigotes were obtained from the high- and low-virulent clones of the Tulahuen strain and from the G-1 strain. They were sonicated and directly applied into wells of a microplate to use insoluble particles as antigens. Fifty-one sera from patients of Chagas' disease including one acute case (with Romana's sign), 10 sera from patients of cutaneous leishmaniasis including one serum of mixed infection with Chagas' disease and 10 sera from patients of toxoplasmosis including three of mixed infection with either Chagas' disease or leishmaniasis were examined. All amastigotes from different sources and from different clones or strains showed considerable correlation with each other. Slightly higher sensitivity was found in amastigotes of high-virulent clones and slightly higher specificity in those from cell-free culture. One acute case was detected as positive by these methods although it was negative by the usual ELISA using epimastigotes.

INTRODUCTION

Chagas' disease caused by *Trypanosoma cruzi* (*T. cruzi*) is still a major public health problem in South America (Moncayo-medina, 1987). Various serological diagnostic methods have been developed to detect patients and to examine the epidemiological condition (Meirvenne and Ray, 1985). The immunofluorescence antibody test with formaldehyde-fixed culture forms (epimastigotes) seems to be most sensitive and specific among those (Camargo,

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1974). However, the enzyme immunoassay has been getting popular because of its easiness and rapidity (Anthony *et al.*, 1974). Some reports indicated that the use of amastigotes as antigens gave better sensitivity than that of epimastigotes (Gam and Nevo, 1977; Araujo and Guptill, 1984; Cossio *et al.*, 1985) but this advantage was limited to the use of whole bodies of fixed amastigotes, suggesting that some important antigens were insoluble in water. Kitagawa *et al.* (1987) reported the successful use of insoluble antigens in water for enzyme immunoassay. In the present work, we attempted to use insoluble antigens from amastigotes of two clones and one strain of *T. cruzi* in enzyme immunoassay to detect patients of Chagas' disease. Furthermore, two sources of amastigotes, namely derived from fibroblast cell cultures and from cell free tube cultures (Brenner and Chiari, 1965) were comparatively examined for their usefulness as solid-phase antigens.

Materials and Methods

Parasite

The high-virulent clone (H-23) and the low-virulent clone (L-38) were derived from the single strain Tulahuen (Kanbara *et al.*, 1987) which had been obtained from NIH, Bethesda, U.S.A. through Keio University. The strain G-1 was obtained from Prof. I, Tada, Kumamoto University Medical School, Japan as an isolate from a patient in Guatemala in 1986.

Preparation of amastigote antigen

1. Amastigotes in cell cultures

Culture forms of each clone or strain were inoculated into fibroblast cultures from ICR mouse skins and cultured in MEM with 10% newborn bovine serum at 37°C in 5% CO_2 atmosphere. Medium change was done every 2-3 days. When most of fibroblasts were infected, clusters of amastigotes appeared in overlaid medium with free trypomastigotes. They were collected and washed in saline by low-speed centrifugation at 1,200 rpm for 5 min to remove trypomastigotes. The clusters were broken by repeated passing through a needle with 23 G, and the number of amastigotes was counted on a hemocytometer and suspended in saline at concentration $10^8/ml$.

The suspensions were sonicated with 60 W for 2 min and diluted with 10 mM Tris buffer (pH 8.6) upto the concentration of $5\times10^6/ml$. The volume of 200 μl dilution was applied to each well of a microplate and incubated at 37°C for 4 h to prepare a solid-phase antigens. The solid-phase antigens were fixed in 3% formalin and unused spaces were blocked by 1% casein.

2. Amastigotes in cell-free tube cultures

Trypomastigotes of each clone or strain developed in infected fibroblast cultures were isolated by passing through a CM-cellulose column (Kanbara and Nakabayashi, 1983), inoculated into test-tubes containing LIT media with a slight modification and incubated at 27°C. They were transformed to amastigotes within 120 h and proliferated mainly as amastigotes during several passages. The amastigotes were processed in the same manner as described above.

Sera

Fifty one patient antisera of Chagas' disease including one antiserum obtained from an acute phase patient showing Romana's sign, ten antisera from patients of cutaneous leishmaniasis including one antiserum of mixed infection with Chagas' disease and ten antisera

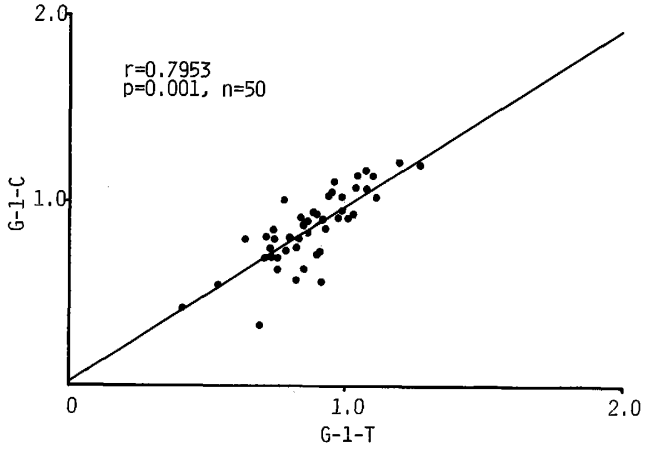

Figure 1 Correlation between serum titers against amastigotes of G-1 strain derived from fibroblast cultures (G-1-C) and those derived from cell-free tube cultures (G-1-T).

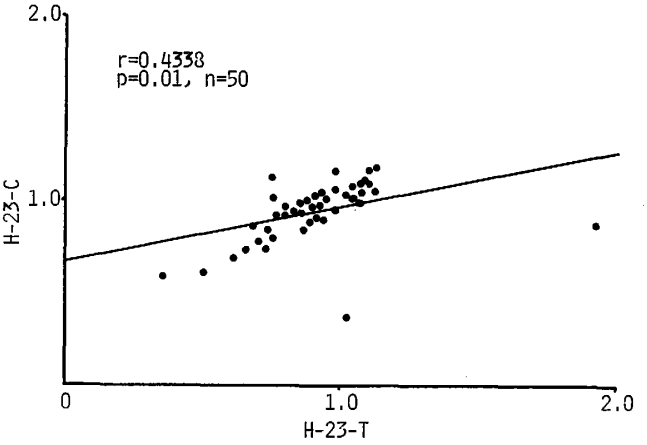

Figure 2 Correlation between serum titers against amastigotes of H-23 clone derived from fibroblast cultures (H-23-C) and those derived from cell-free tube cultures (H-23-T).

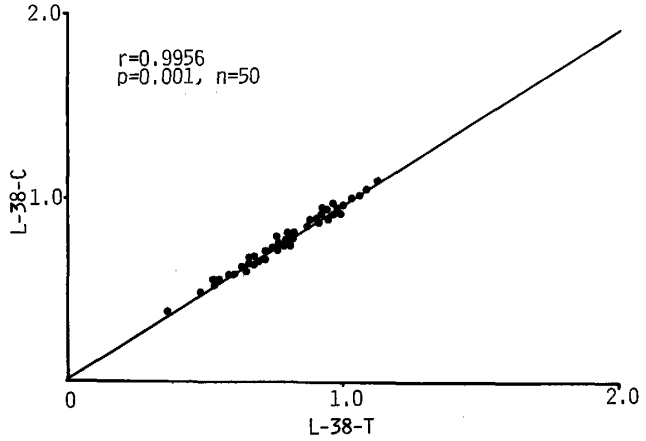

Figure 3 Correlation between serum titers against amastigotes of L-38 clone derived from fibroblast cultures (L-38-C) and those derived from cell-free tube cultures (L-38-C).

from toxoplasmosis patients including three of mixed infection with Chagas' disease and leishmaniasis were used enzyme-linked immunosorbent assay (ELISA) technique. Each 50 μl of sera at 1/100 dilution was put into each well and the microtiter plates were incubated at 37°C, 1 h. After washing with PBS-Tween three times, 100 μl of 1:4,000 dilution in 1% BSA-PBS of goat anti-human IgG conjugated with peroxidase was added and incubated at 37°C for 1 h. After washing with PBS-Tween three times, 100 μl of substrate solution containing 0.5 mg/ml of o-phenylene diamine and 0.02% of H_2O_2 in 0.05 M citrate-phosphate buffer, pH 5.0 was added and incubated at 37°C for 30 min. The stop solution (10 μl of 8 N H_2SO_4) was, then, added and the optical density at 492 nm was read.

Statistical analysis of differences of mean values and correlations in ELISA titer were done by t-test.

RESULTS

In the comparison of ELISA titers between the two methods of antigen preparation in G-1 strain and two clones of Tulahuen strain H-23 and L-38, there were significant correlations between two methods for antigen preparation, namely, $r=0.7953$ ($p<0.001$), 0.4338 ($p<0.01$) and 0.9956 ($p<0.001$), respectivery (Figs. 1, 2 and 3). Though the significances were not found in the differences of mean values between the two methods in two strains (G-1 and H-23), a significance was found in the difference of mean value in L-38 strain ($p<0.005$) (Table 1). It means that the microtiter plate coated with amastigote antigens from tube cultures as an antigen was more reactive than with amastigote antigens from cell cultures. Analyzing the relationship between virulent and low virulent clones (H-23-C and L-38-C), there was a significant correlation between two clones, namely, $r=0.6653$, $p<0.001$ (Fig. 4). The mean value of the ELISA titers in H-23-C was higher than that of L-38-C and it's difference is statistically significant ($p<0.001$) (Table 1). It is suggested that the antigenicity of virulent strain against patient antisera is more reactive than that of low virulent strain. In comparison of ELISA titers between G-1 and each Tulahuen strain, there were also significant correlations, namely, $r=0.6270$ ($p<0.001$), $r=0.8741$ ($p<0.001$) (Figs. 5, 6). The mean value of ELISA titer in G-1 strain was lower than that of virulent clone and higher than of low virulent clone (Table 1). Each difference of the mean value was statistically significant ($p<$

Table 1 The comparison of ELISA titers in different strains of amastigotes

Strains	Chagas' disease	Negative	No. of patients less than mean±2SD
G-1-C	0.850±0.191	0.396±0.098	3
H-23-C	0.942±0.153	0.491±0.127	5*
L-38-C	0.752±0.155	0.302±0.088	1*
G-1-T	0.876±0.159	0.380±0.084	0
H-23-T	0.904±0.218	0.315±0.069	0
L-38-T	0.772±0.162	0.300±0.094	1*

Values are presented as means±SD.

*: No. of positive includes an acute case.

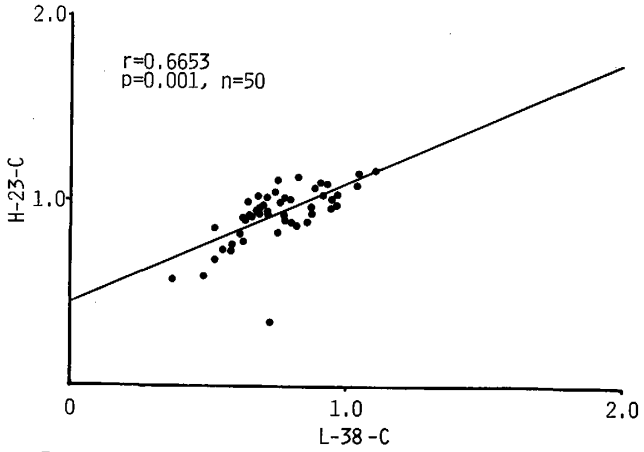

Figure 4 Correlation between serum titers against amastigotes of H-23 and L-38 clones derived from fibroblast cultures (H-23-C and L-38-C).

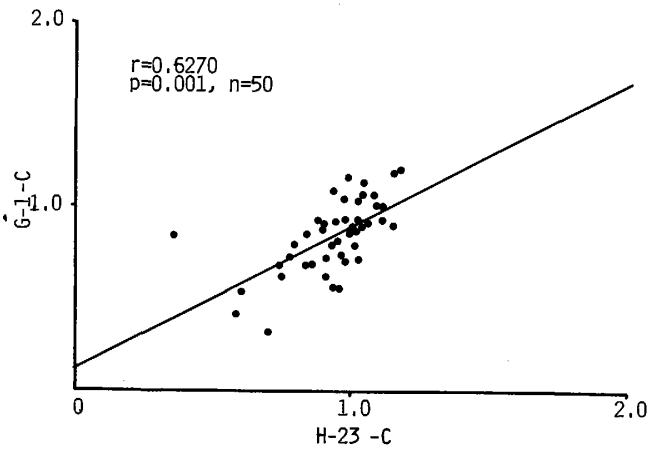

Figure 5 Correlation between serum titers against amastigotes of G-1 strain and H-23 clone derived from fibroblast cultures (G-1-C and H-23-C).

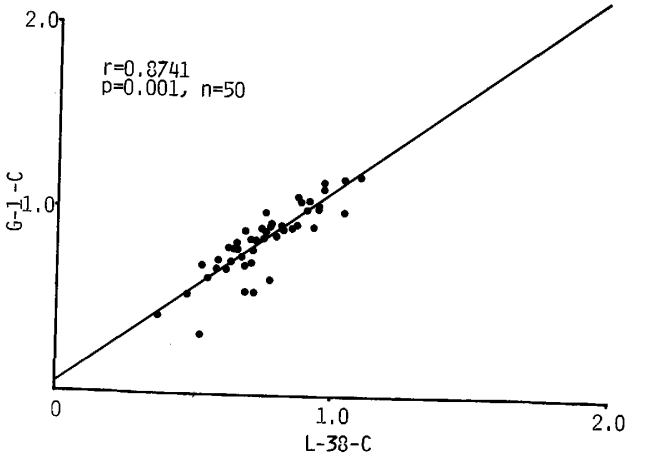

Figure 6 Correlation between serum titers against amastigotes of G-1 strain and L-38 clone derived from fibroblast cultures (G-1-C and L-38-C).

0.001, $p<0.001$, respectively).

It means that the antigenicity of G-1 strain against patient antisera is less reactive than that of virulent clone and more reactive than that of low virulent clone. As an antiserum obtained from a patient in acute phase was not screened as a positive case in the ELISA system which used epimastigote as an antigen for solid-phase, we tried to analyze whether this new system can be applied to screening. Nine antisera from cutaneous leishmaniasis patients and seven antisera from toxoplasmosis patients without mixed infection with Chagas' disease was used as a negative control.

The ELISA system using G-1-T or H-23-T were satisfactory if more than mean value± 2SD was considered to be positive. In these system an antiserum from a patient in acute phase was also evaluated to be positive.

Discussion

Antigenicity of *T. cruzi* has a wide variation among developmental stages and also among isolates or strains (Nussenzweig *et al.*, 1963; Kloetzel *et al.*, 1975; Bongertz and Dvorak, 1983; Morgado *et al.*, 1985; Okanla *et al.*, 1982; Flint *et al.*, 1984; Aranjo and Remington, 1981). Moreover, antibodies produced in infected mammals have a big diversity according to the course of infection and to the individual difference (Grögl and Kuhn, 1985). Therefore, serological diagnostic methods depend on detection of common antigens in prepared specimens for antigens, which are mainly derived from culture forms (epimastigotes) of a certain strain. In this point, the higher sensitivity and specificity in the immunofluorescein assay is quite reasonable because it uses total antigens including insoluble antigens. Kitagawa *et al.* (1987) introduced the method to apply insoluble antigens to ELISA for quantification of some bacterias. Since some works (Gam and Neva, 1977; Aranjo and Guptill, 1984; Cossio *et al.*, 1985) revealed that the use of amastigote antigens was more sensitive than that of epimastigote antigens, we used insoluble antigens of amastigotes of different clones or strain which were cultured in different ways and compared their efficiency as solid-phase antigens.

The results demonstrated good correlation between amastigotes from two sources and also between different strain or clones.

The first interesting point was that amastigotes derived from tube cultures gave better specificity than those from cell cultures because of lower level of titers among negative controls. This might be due to purity of antigens and meant that amastigotes in tube cultures kept the common antigens with those in cell-cultures as far as the ELISA test used in the present work was concerned. The second point was the high-virulent clone showed better sensitivity and the G-1 strain, which was not cloned and kept medium virulence, showed middle sensitivity. This indicated that the additional antigen which were commonly detected by patients existed in high-virulent amastigotes. Some of the additional antigens in high-virulent amastigotes were probable to be recognized by an acute patient although only one case was available in the present work. Since we prepared solid-phase antigens in Japan, the fixation step which generally reduced antigenicity was essential because of long transportation at unfavorable temperature. The present work revealed that amastigotes grown in cell-free system were available and more useful than those in cell cultures. Amastigotes in cell-free media are possible to continuously propagate in devised media (Pan, 1978) and to be used

as easily as epimastigotes. Therefore, various trials to examine the suitable quantity and quality of amastigotes antigens, effects of various fixatives and the suitable blocking solutions will develop the sero-diagnosis for Chagas' disease.

ACKNOWLEDGEMENT

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Trypanosoma cruzi から得た2種の Amastigote を抗原として用いた ELISA 法の試み

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マウス線維芽細胞内で増殖した Amastigote（Am-C）および cell-free の液体培地中で増殖した Amastigote（Am-T）を固相抗原とした ELISA 法を用いて，患者血清に対する反応性の検討を行った。Amastigote は G-1 株および Tulahuen 株の強毒，弱毒クローンから得られたものを用いた。抗原として用いた Amastigote は超音波破砕した後，不可溶性部分を含んだままで，マイクロタイタープレートに固相として用いた。血清は1例の急性期患者を含む51例のシャーガス病患者血清，1例のシャーガス病との混合感染患者血清を含む10例の皮膚リーシュマニア症患者血清，および3例のシャーガス病，もしくは皮膚リーシュマニア症との混合感染患者を含む10例のトキソプラズマ症患者血清を用いた。その結果，調整法の異なるそれぞれの Amastigote の間，およびクローン間，株間でもそれぞれにおいてかなり高い相関が見られた。強毒株を固相抗原として用いた ELISA において高い反応性が，また cell-free の液体培地中で増殖した Amastigote を用いた ELISA において高い特異性が見られた。通常の Epimastigote を用いた ELISA 法で陰性であった1例の急性期患者血清は，G-1-T，H-23-T において陽性と判定された。

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症例報告

静岡県藤枝地域に発生したランブル鞭毛虫症

市澤　知子[1]・加藤　裕美[1]・持塚いずみ[1]・
栗田　雅史[1]・瀬野尾一孝[2]・鈴木　了司[3]

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ランブル鞭毛虫は全世界に分布し，特に熱帯，亜熱帯地方では高い寄生率を示すことが知られている。我が国においても，かつては各地に感染者が存在したが(猪木ら，1950; 片峰ら，1963)，最近では海外渡航者の増加と共に，帰国者中に本虫の感染者が増えていることが問題となっている（中林，矢野，1987)。

著者らは，1988年春以来，藤枝市立志太総合病院の患者，特に人間ドック受診者より，ランブル鞭毛虫嚢子保有者を検出したので，これらの保有者について疫学的考察を加えた。

方　法

1988年3月から1989年12月末までの1年10カ月の間，ドック受診者2,839名，入院患者1,524名，計4,363名の糞便の寄生虫検査をホルマリンエーテル法による集卵法で実施し，ヨード染色，ギムザ染色などを併用した。

ランブル鞭毛虫保有者については，問診，またはアンケートにより，その生活環境，その他について調査を行うと共に，保有者の血液検査，生化学検査，免疫血清学的検査，尿および糞便検査を行った。

成　績

1）感染状況と疫学的調査

ドック受診者からは24名 (0.9%) とその家族1名，および入院患者からは3名 (0.2%) の計28名 (0.6%)にランブル鞭毛虫嚢子が検出された。そのうちの3例については胆汁採取を行い，いずれも栄養型を確認した。また別の1名から，小形アメーバを見出した。保有者の月別検出状況を図1に示すと，1988年3月に第1例が認められて以来，同8月，11月，12月に各1名，1989年1月に1名，2月に2名，3月に3名，4月に1名，5月に5名，6月に2名，7月に1名，8月に2名，10月に4名，11月に1名，12月に2名が検出された。1989年5月が最も多く，次は10月であった。

保有者の年齢層は，41歳から78歳(平均53.1歳)で，中高年齢層に多い。

性別では，男性26名（被検者3,035名中0.9%)，女性2名（被検者1,328名中0.2%）で男性に明らかに多い。

職業別では，電力会社勤務4名，建築関係会社勤務4名，水産物取扱業3名，鉄工会社勤務2名，自動車学校教官2名，船舶工業会社勤務1名，教員1名，温水器会社勤務1名，金属工業会社勤務1名，採石センター勤務1名，自営業4名，無職4名である。

保有者の居住地を示したものが図2である。保有者は病院の存在する藤枝市（10名）を中心に，

1　藤枝市立志太総合病院臨床検査科
2　藤枝市立志太総合病院消化器科
3　高知医科大学寄生虫学教室

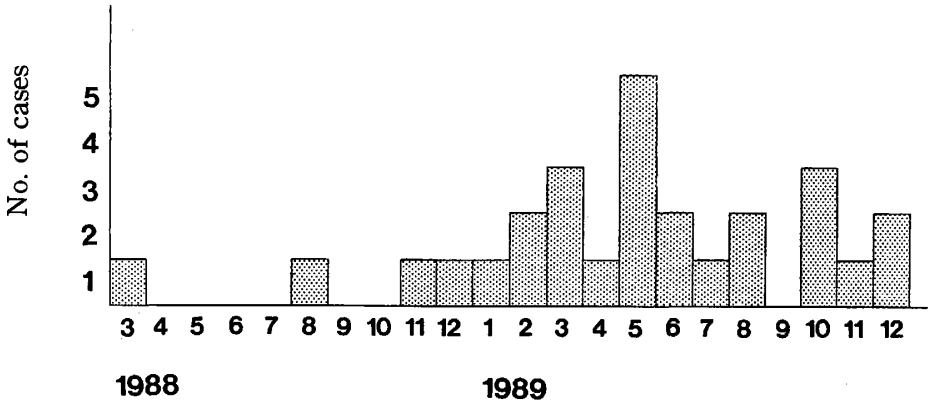

Figure 1 Cases of giardiasis by date of stool identification between March 1988 and December 1989.

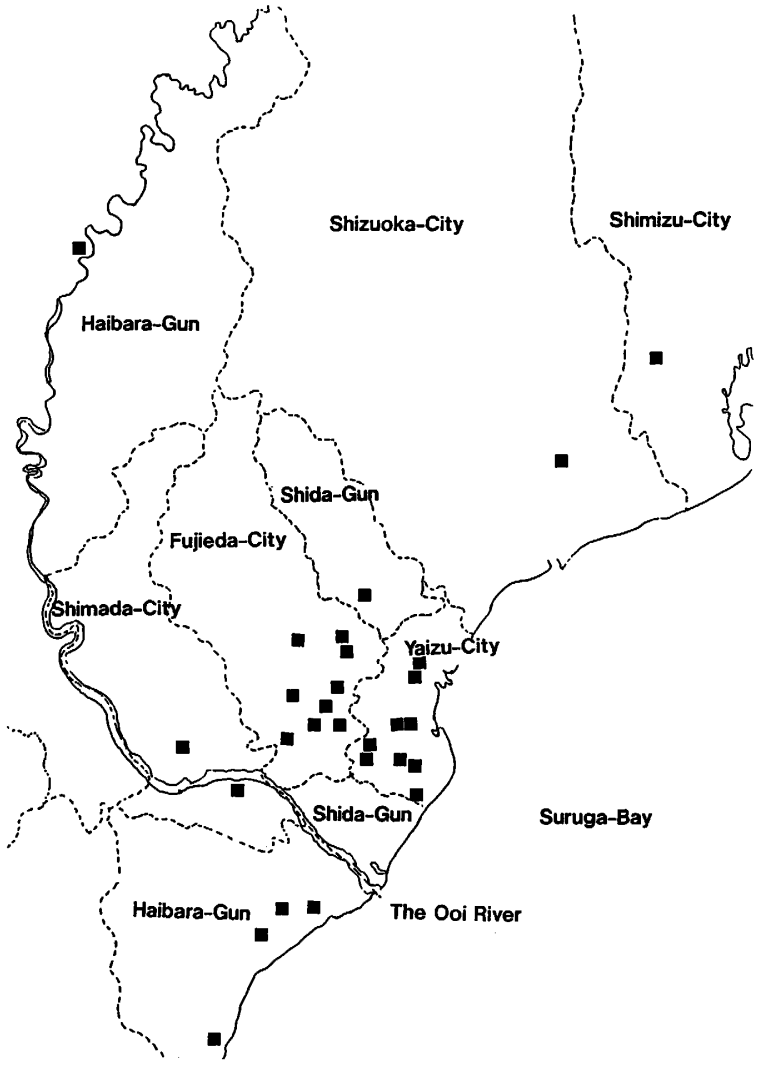

Figure 2 Geographical distribution of giardiasis in Fujieda and its neighboring towns.

焼津市（９名），島田市（２名），榛原郡（５名），静岡市（１名），清水市（１名）であった。受診者に対する保有者の割合を調べたところ，市町村間には差はない。

飲料水の利用については，保有者28名中，市上水道のみを利用している者25名(89.3%)，自家水道と市上水道を併用している者２名，自家水道のみを利用している者１名で，上水道利用者に多い。

ペットの飼育については，調査ができた25名中現在飼育している人11名(44%)，過去に飼育していた人は３名（12%），飼育していない人は11名（44%）であった。現在ペットを飼育している人は，犬10名，ネコ４名，アヒル，ウサギ各１名であり，犬を飼育している者が多い。過去においては，ネコの飼育２名，ブタの飼育１名，サルの飼育１名である。

保有者で，海外旅行の経験者は28名中11名（39.3%）で，渡航先はグアム，サイパン，アメリカ，台湾，メキシコ，ポルトガル，韓国，香港，マレーシア，オーストラリア，フィリピン，西ドイツなどであり，旅行時期はそれぞれの受診時期の１カ月前から10年以前であった。また，保有者の家族周辺が海外旅行をしている者は６名おり，渡航先はカナダ，ハワイなどである。

保有者のうち，小さな子供のいる者は４家族で，その内訳は３名が幼稚園児，１名が未就園児である。

同性愛の経験の有無については，問診などの調査での確認は困難であった。

２）臨床症状および臨床検査

保有者28名のうち，自覚症状を有するものは９名で，そのうち消化器系の自覚症状を訴えた者は７名(25%)（下痢２名，腹痛２名，嘔気１名，残胃感２名，食欲不振２名，体重減少１名）であった。一方，慢性胃炎と診断された者は15名(54%)で，胃X線検査において胃粘膜にびらん，萎縮，および肥厚などが見られた者である。このほか，胃潰瘍１名，胃ポリープ１名，胃潰瘍はん痕２名，その他に胆嚢ポリープ４名，胆石１名，直腸ポリープ２名，S状結腸ポリープ１名，大腸癌１名が認められた。

28名の血液検査においては，５名に白血球数の増加が見られ，１名に白血球数の減少が見られた。赤血球数は，１名に軽度減少，２名に軽度の増加が見られた。Hb値は８名に軽度から中等度の低下が，Ht値は４例に軽度の低下が見られた。

血液像は，５名に好塩基球の軽度増加，その他５名にリンパ球の軽度増加が見られた。血液沈降速度は，３名に昂進が見られたほかは正常域内にあった。

肝機能検査では，γGTPのみ高値を示す者４名，LDHのみ高値を示す者１名，γGTP・GOT・GPTに高値を示す者２名，ALP・γGTP・GOT・GPTともに高値を示す者１名であり，８名の肝機能検査値になんらかの異常が見られた。CRP値は２名に，軽度の増加が見られたのみである。梅毒反応は１名に陽性が認められた。

免疫グロブリン値は，23名中６名にIgGの軽度の減少が，６名にIgAの増加が見られた。また，10名にIgMの増加が見られ，６名にIgEの増加が見られた。その他，便潜血反応検査は２名に陽性が見られた。

考　察

１）保有状況

1988年３月から，1989年12月末までの１年10カ月の間に，藤枝市立志太総合病院の主としてドック入院患者から，28名のランブル鞭毛虫の保有者が見出された。本病院がこの地域の中核病院であるため，藤枝市周辺の居住者に保有者が多いが，その保有者数は２年近い期間とはいえ，現今の我が国の衛生状態から考えると高率といわざるをえない。

さらに保有者の検出状況を見ると，1988年２月以前においては，３年間にわたり2,048名に，同一の糞便検査法を実施しているにもかかわらず，１例も検出されていないことから，1988年３月以降になんらかの感染の機会があったものと思われる。特に28名の保有者のうち，13名は１－２年前に本院のドックを受診しているが，本虫が検出されていないことからこの事実を裏づける。年間の月別保有者の検出数は５月に多く，10月がそれに続くが，

保有者の絶対数も少ないうえ，5月は受診者も多いので，正しい季節的な消長を示しているものかどうかは今後の検討に待ちたい。保有者が中高年齢者層に多いのは，検査の対象者がドックの受診者であるので当然であろう。ただし，ドック受診者と入院患者とでは，前者に圧倒的に保有者が多いことについては，入院患者の病名など対象を十分に把握できなかったため，比較することが困難であったが，角ら（1986）も人間ドック受診者が一般の受診者より，寄生率が高いことを指摘している。

2）感染経路

ランブル鞭毛虫の人への感染要因としては，(1) 海外旅行による感染，(2) ペットなどの動物との接触による感染，(3) 人からの感染（保育園内感染，病院内感染），(4) 日和見感染症として，免疫不全に随伴する感染とホモ愛好者間の感染，(5) 飲料水の汚染（塩素その他の処理不完全。下水，ゴミ，動物の糞などによる水道汚染。食品および食器の汚染，その他）などがあげられる。

(1) 海外に出かけての感染は，Waltzer *et al.* (1971) や Andersson *et al.* (1972) らが指摘しているように世界中に多くの報告があり，日本でも青年海外協力隊員（山浦ら，1980）や海外旅行者の症例（中林，矢野，1987）や，インドシナ難民の症例（建野ら，1981）が存在する。今回の症例では海外旅行の経験者が，保有者28名のうちの11名であったが，その旅行時期が受診時の半年から10年以前であり，これら保有者が時を同じくしての集団旅行ではないことから，主要な感染経路としては考えにくい。

(2) ペットなどからの感染については，現在ペットを飼育中の者は調査ができた25名中11名であった。荒島ら（1990）は東京都の犬で，80頭中12.5%に本虫の感染を見出している。今回は，ペットの検便が困難なため，結論を出しえなかったが，人のランブル鞭毛虫が犬や猫に感染するとすれば，保虫宿主としての係わり合いのあることを容易に推定できる。しかし，ペットの種類の多様性などから，少なくとも同一の感染源と考えることは否定的である。

(3) 保育園や幼稚園などでの本虫による下痢症の流行は欧米では多発しているが，今回の保有者の大部分が幼児などの子供がなく，また，この期間に保育園などで，原因不明の下痢などの消化器疾患が流行したという情報はなく，子供からの感染も考えられない。

保有者の中には，同一勤務先を持つ保有者が存在した。たとえば，同一電力会社からの受診者200名中に4名の保有者が見られた例では，2名は別個の営業所で働いており，互いに関係はない。しかし，他の2名は住所は異にしているが，同一営業所に勤務していた。また，自動車学校では1988年に21名，1989年には23名がドックに入院し，それぞれ1名と2名の保有者を認めた。このうちの1名は同一人で，未治療のため2回とも陽性となり，実際には2名であった。さらに鉄工会社で20名中2名の保有者が存在したことなど，集団の検査で複数の保有者を認めたことは，職場での感染の可能性を示唆した。

病院内感染は，ドック受診者にはあらかじめ糞便採取容器を渡しておき，2日分の糞便を家庭で採取して持参するシステムであり，検査器具はディスポーザブル，または高圧滅菌したものを使用していることなどから，検査時に混入することはない。しかも，本病院内の医療関係者に本症の発生を見ていないことからも，院内感染の可能性はない。

(4) 性行為などによる本虫の感染が，近年注目されつつあるが，このような経験の有無については確認が困難であった。免疫不全による感染は，著しい低下を保有者に認め得ないことからも考えにくい。

(5) 水系による本虫の集団発生は，世界各地で報告されているが（Craun, 1984），その大部分は，水道処理場の故障や処理の不完全，上流におけるビーバーなど動物の排泄物による水道の汚染が原因である。今回の保有者の大部分は上水道を利用しているが，居住地の水源は焼津市，藤枝市，榛原郡，島田市などを含む4市10町では，大井川の表流水をダムに貯水し，浄水処理された広域水道を1988年5月から利用し始めている。しかし，現在は一部の地区が利用しているに過ぎず，大部

分は各市町村ごとに，大井川の異なった地点からの地下水をそれぞれ水源としている。たとえば，保有者の多い藤枝市では，一部の地区が広域水道を使用するのみで，地区により取水場所の異なった地下水を利用している。しかも，その地下水は互に混和されていない。焼津市では，市内全域にわたり，地下水と広域水道とが混和されて配水されているが，広域水道の使用量は全量の1/10程度で少ない。静岡市と清水市では，安倍川の表流水と，それぞれ異なった地下水とを水源としている。

一方，藤枝市，焼津市，榛原郡などの住民を対象とする屎尿処理施設は，大井川の取水地より下流にあり，これら施設よりの汚染は考えられない。また，取水地付近には大量に家畜を飼育しているところはなく，糞便による汚染も考えられない。

このように，市町村により同一の水源を利用している訳ではなく，また一部の使用されている広域水道はその利用以前から保有者が見出され，保有者の発生も長期にわたっていることなどから，水系からの感染を否定する方向にある。

また輸入食品，特に汚染野菜類による本虫の感染の可能性もあるが，それらについては調査を行っていないので，可能性のみをあげておく。

感染経路に関しては，今回の調査からは特定し得なかったが，最近の国内の調査では，星加ら（1980）は岡山県内の2カ所で約5,000名の検査で0.08%の寄生率を，真子（1982）は福岡県の食品業者のうち，下痢便の1,237名から1.1%，健康者1,107名から0.4%の陽性者を検出し，角ら（1986）は1980年から5年8カ月の間に，首都圏の病院の一般受診者9,431名中0.5%，人間ドック受診者5,325名中0.7%の陽性者を検出し，関戸ら（1988）は1983年から1988年までの5年間に，埼玉県大宮市内の病院で，入院および外来患者6,168名中10名（0.16%）を検出している。このほか，海外渡航歴のない本症の報告（津嶋ら，1985）が散見されている。

最終的な結論を出すには，感染源，周辺地区の調査を今後行う必要があるが，この地域には不顕性感染者が存在し，人から人への感染，特に職場での伝播が行われていると推定する方が妥当と考えられる。

また，寄生虫症に対する意識の低さ，検査の軽視化と不十分さなどから，本症の存在を見逃している可能性がある。たとえば，この地域周辺の複数の病院では，人間ドックの検査においても薄層塗抹法を用いており，過去2年間に本虫が全く検出されていない。鈴木（1982）は寄生虫の検査に当たり，集卵法（MGL法）の採用を指摘しているが，今回の著者らの成績および角ら（1986）や関戸（1988）の成績が，すべて集卵法によって得られていることを改めて留意する必要があろう。

なお志太郡在住の1名から，小形アメーバの保有者が検出された。真子（1982）は福岡市で，鈴木（1982），角ら（1986）および関戸ら（1988）は首都圏で，本アメーバを検出していることから，ランブル鞭毛虫同様，検査法によっては見出されるものと考える。

3）臨床症状

ランブル鞭毛虫は，便中に排出され，経口摂取された嚢子が小腸上部で脱嚢して栄養型となり，十二指腸，空腸上部に寄生し，下痢，軟便，腹痛，食欲不振，吐気，嘔吐などの消化器症状を示すばかりか，しばしば胆嚢炎様症状を呈することが知られている。今回の保有者について実施した臨床検査成績からは，血液検査，免疫血清学的検査，尿および糞便検査などのデータに一定の傾向がほとんど認められなかった。

慢性胃炎と診断されたものは15名であったが，自覚症状を訴えたものは7名にすぎず，臨床検査値，その他を考慮すると，本虫が既存の症状を悪化させていることは想像に難くないが，ランブル鞭毛虫とこれら胃炎との直接の関連性は比較的少ないと考えられた。

肝機能検査値に異常を示した8名の内，γGTPのみ高値を示した者は糖尿病によるもの（1名），またγGTPのみ（1名），γGTP・GOT・GPT（2名）の上昇のあった3名は脂肪肝によるものと考えられる。しかし，胆嚢壁肥厚，拡張などが見られたため胆石が疑われていた症例は，ALP 41.3，γGTP 455，GPT 111，GOT 50，LDH 281，AMY 90で，明らかな肝機能障害を認め，ランブル鞭毛虫によると推定した。その他のLDH，

γGTPの上昇の3名は軽度の上昇であり，他の検査値を考慮すると，ランブル鞭毛虫との関係は少ないと考える。しかし治療後の検査を行っていないので，推定の域を脱しえない。この3例のうち，1名は肝超音波検査により日本住血吸虫症を疑う像が見られたため，肝バイオプシーを実施したところ，日本住血吸虫卵を認めた。

近年，免疫不全に伴う下痢症から本虫がしばしば見出されるという報告が多く，本虫の感染，発症が免疫と関係があることは明らかであるが，受診時の免疫グロブリン値のみから見ると，IgMが増加している者が23例中10例と多く，それらの関係を示唆するが，特に免疫状態の低下とは考えられず，また感染前，あるいは駆虫後の測定が行われていないため，結論を出しえなかった。

まとめ

1988年3月より1989年末まで，藤枝市立志太総合病院において，主として人間ドックの入院患者より，28名のランブル鞭毛虫症の患者が認められた。

保有者について，特定の感染源は見出されなかったが，人から人への経路でこの地域において伝播しているのではないかと推定した。

保有者の症状では，明らかにランブル鞭毛虫に関連があると考えられる症状を示したものは，肝機能に異常のあった1名であった。そのほかに慢性胃炎と診断され，消化器系になんらかの症状を示したものが15名存在した。

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Case note

GIARDIASIS IN FUJIEDA AND ITS NEIGHBORING TOWNS, SHIZUOKA PREFECTURE

TOMOKO ICHIZAWA[1], HIROMI KATO[1], IZUMI MOCHIZUKA[1], MASASHI KURITA[1], KAZUTAKA SENOH[2] AND NORIJI SUZUKI[3]

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Giardia lamblia cysts were found in the stool of 25 patients for health screening and in 3 inpatients at the laboratory of the Fujieda City Shida General Hospital, Shizuoka Prefecture from March 1988 to December 1989.

Epidemiologic investigation showed that there are no the common source such as drinking water, travel or animal contact. It may be given as a conclusion that human giardiasis in Fujieda and its neighboring towns are indigenous and the transmission appears mainly to occur from person to person.

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Case note

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