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Electrophoretic Studies on Enzymes in *Paragonimus* spp.
IV. Comparison of Allelic Frequencies of Glucosephosphate Isomerase Isozymes in Natural Populations between *P. ohirai* and *P. sadoensis*

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Introduction

To discriminate among species of parasites, gel electrophoresis has been used in a variety of parasites, such as species belonging to the genus, *Trypanosoma* or *Leishmania* (Taylor and Muller, 1979). In many studies reported hitherto, glucosephosphate isomerase (GPI) variations have been recognized among species in parasitic protozoa (Carter, 1973; Baker *et al.*, 1978; Shirley, 1979; Kreutzer and Christensen, 1980). Carter (1973) found that enzyme forms of GPI differentiate *Plasmodium* species and its strains. Miles *et al.* (1977) reported that the electrophoretic patterns of GPI differed between domestic and sylvatic stocks of *Trypanosoma cruzi*. They emphasized that their results supported the epidemiological evidence which the domestic and sylvatic transmission cycles in a rural area do not overlap. In parasitic helminths, on the other hand, several attempts have also been made to examine GPI variation (Vrijenhoek, 1978; Agatsuma and Suzuki, 1981). Recently, Agatsuma (1981a, b) investigated GPI isozymes in natural populations of Japanese lung flukes, *Paragonimus miyazakii* and *P. iloktsuenensis*, clearly differentiating two species with electrophoretic patterns of GPI isozymes. Furthermore, he found that considerable variations of this enzyme occur in natural populations of each species.

The present study deals with GPI isozymes of other two lung flukes, *P. ohirai* and *P. sadoensis*, using starch gel electrophoresis. Their morphological features are closely allied with each other, but, they have been considered to be distinct species, especially owing to the differences in their morphology of metacercariae and in their host specificity (Kawashima *et al.*, 1967; Miyazaki *et al.*, 1968). It is, therefore, of interest to know the degree of genetic differences among them. The current paper reports on the extent of GPI polymorphism in these two species.

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Materials and Methods

Parasite

Metacercariae of *P. ohirai* were obtained from the crab host, *Sesarma dehaani*, collected at the Sendai river, Sendai, Kagoshima Prefecture, and at the Maruyama

river, Kinosaki, Hyogo Prefecture, during July to August 1980. *P. sadoensis* metacercariae were obtained from *Geothelphusa dehaani* at small streams around the mouth of the Ohkura river in Sado Island, Niigata Prefecture, in March 1980. Adult worms of each species were harvested from the lung cysts of the albino rats (Wister) inoculated with the metacercariae 40–50 days after inoculation. The adults obtained were washed thoroughly with 0.8% physiological saline, and then stocked in a deep freezer at −80 C until required for electrophoresis.

Preparation of enzyme extracts

The extracts of parasites were prepared by homogenizing individually in 0.1 ml of 0.1 M phosphate buffer solution (pH 7.5) using a teflon homogenizer in an ice water bath. Then, the homogenates were centrifuged at 3,000 rpm for 3 min at room temperature. The supernatants obtained were used for electrophoresis. As a control run, the samples of normal rat lung extracts were also prepared in the same procedures as those of the parasites.

Electrophoretic procedures

Electrophoresis was carried out in a 12% horizontal starch gel at 4 C for 14 h with a constant-current power supply of 70 mA. Samples were absorbed on to 7×6 mm strips of filter paper, and then inserted into gels. Electrophoretic buffer for the electrode was composed of 0.1 M Tris-Maleic acid, containing 0.1 M Na_2EDTA, 0.01 M $MgCl_2$ and 0.13 M NaOH (pH 7.4), and the buffer solution for the gel was obtained by diluting the electrode buffer ten times. After a run, a slide of gel was stained for GPI. GPI bands were made visible by incubation in the dark at 37 C for 15 to 20 min with a solution containing 0.1 M Tris-HCl (pH 8.0), 0.1 mg/ml MTT tetrazolium, 0.1 mg/ml phenazine methosulfate, 0.1 mg/ml NADP, 3.7 mg/ml $MgCl_2$, 0.1 U/ml glucose-6-phosphate dehydrogenase and 0.15 mg/ml fructose-6-phosphate. These methods for electrophoresis were essentially based on those of Shaw and Prasad (1970) and Nozawa *et al.* (1977).

Results

Electrophoretic patterns of the parasites differed from those of the host tissue (rat lung), as previously described by Agatsuma (1981a, b).

In *P. ohirai,* three different phenotypes of GPI were found in Sendai population, while only one was found in Kinosaki population (Fig. 1). Those found in Sendai showed either single band (a of Figs. 1, 2), or two types of triplet bands (b, c of Figs. 1, 2), and the phenotype in Kinosaki population appeared as a single band (a of Figs. 1, 2). Mobilities of the single band found in these two populations were identical with each other and also with those of the slowest band of the triplet-type phenotypes. On the other hand, only one phenotype was found in *P. sadoensis* population. This phenotype appeared as a single band which was identical with the single band of *P. ohirai* in mobility (a of Figs. 1, 2). Chi-square (X^2) value of the difference from the theoretical value calculated on the basis of Hardy-Weinberg's equilibrium are given in Table 1. There is no significant deviation from Hardy-Weinberg proportion. The distribution of phenotypes of banding patterns suggested that the GPI protein may possess dimeric structure. These results obtained here were very similar to those of previous studies with *P. miyazakii* and *P. iloktsuenensis* (Agatsuma, 1981a, b). Therefore, the same assumption was again proposed as those described previously; GPI isozymes of the present two species were a dimer protein, and were controlled by a single locus. This would be supported also by

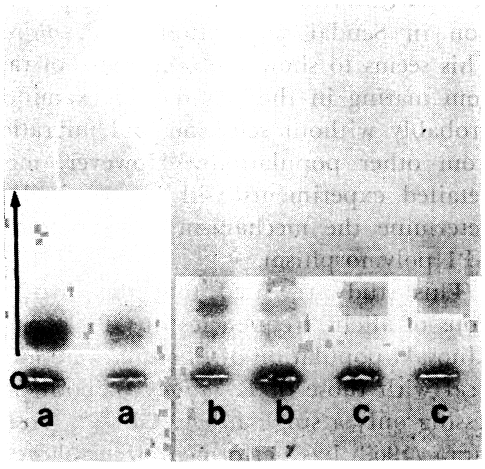

Fig. 1 Photograph of three phenotypes of GPI isozymes found in *P. ohirai* and *P. sadoensis* by means of starch gel electrophoresis.
o; origin a; $GPI^{1.00}/GPI^{1.00}$
b; $GPI^{1.00}/GPI^{2.30}$ c; $GPI^{1.00}/GPI^{2.80}$

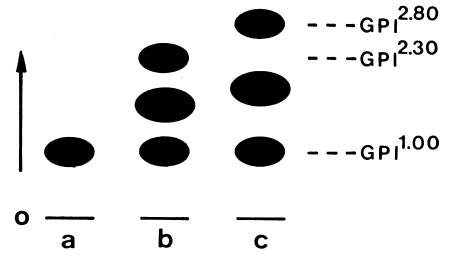

Fig. 2 Diagrammatic representation of GPI isozymes in *P. ohirai* and *P. sadoensis*. The a, b and c show the same genotypes as those in Fig. 1.

Table 1 Genotype frequencies of glucosephosphate isomerase (GPI) in *P. ohirai* and *P. sadoensis*

Species & Localities		Genotype frequencies of GPI isozymes							
		1.00/1.00§	1.00/2.30	1.00/2.80	2.30/2.30	2.30/2.80	2.80/2.80	No.	X^2
P. ohirai									
Sendai	obs.†	83	20	4	0	0	0	107	1.70*
	exp.‡	84.37	17.67	3.62	0.92	0.38	0.04		
Kinosaki		68	0	0	0	0	0	68	
P. sadoensis									
Sado		85	0	0	0	0	0	85	

* $0.70>P>0.50$ (df=3)
† observed numbers
‡ expected numbers calculated, based on the Hardy-Weinberg law
§ Numbers, 1.00, 2.30 and 2.80 show $GPI^{1.00}$, $GPI^{2.30}$ and $GPI^{2.80}$, respectively.

Table 2 Frequencies of GPI alleles in *P. ohirai* and *P. sadoensis*

Species & Localities		Allelic frequencies			Total alleles
		$GPI^{1.00}$	$GPI^{2.30}$	$GPI^{2.80}$	
P. ohirai	Sendai	0.888	0.093	0.019	214
	Kinosaki	1.000	0.000	0.000	136
P. sadoensis	Sado	1.000	0.000	0.000	170

the fact that the ratios of phenotypes agreed with Hardy-Weinberg expectation for these isozymes. Thus, the most common allele at GPI locus is designated $GPI^{1.00}$, and other alleles were assigned numbers relatively to the mobility of

$GPI^{1.00}$. Then, the genotypes of three kinds of phenotypes found in Sendai population of *P. ohirai* will be $GPI^{1.00}/GPI^{1.00}$, $GPI^{1.00}/GPI^{2.30}$ and $GPI^{1.00}/GPI^{2.80}$ (Fig. 2). Based on this hypothesis, three alleles, $GPI^{1.00}$, $GPI^{2.30}$ and $GPI^{2.80}$ will occur in Sendai population of *P. ohirai,* while only one allele, $GPI^{1.00}$, in Kinosaki population of *P. ohirai* and in Sado population of *P. sadoensis.* The frequencies of these three alleles were estimated on the basis of the presumptive genotype frequencies. As shown in Table 2, GPI locus was polymorphic in Sendai population of *P. ohirai,* but monomorphic in Kinosaki population of *P. ohirai* and in *P. sadoensis* population. No difference was found between the natural populations of the latter two species in the distribution of allelic frequencies.

Discussion

The present study suggested that GPI isozymes found in *P. ohirai* and *P. sadoensis* were a dimer protein, controlled by a single locus. This suggestion has been also made in other *Paragonimus* species, *P. miyazakii* and *P. iloktsuenensis* (Agatsuma, 1981a, b). Thus, these features of GPI are considered to be common in the species belonging to the genus, *Paragonimus.* It was also found that in *P. ohirai,* GPI locus revealed polymorphism in Sendai population, but monomorphism in Kinosaki population. GPI polymorphism has so far been observed in parasitic helminths, i.e., *Paragonimus miyazakii* and *P. iloktsuenensis* (Agatsuma, 1981a, b), and *Contracaecum* sp. (Vrijenhoek, 1978), and in also other animals, Colias butterflies (Watt, 1977), and plants (Gottlieb and Weeden, 1979). In the species of the genus, *Colias,* it was suggested that GPI polymorphism may be maintained by natural selection (Watt, 1977). In this study, it was demonstrated that there occurred no significant difference of departure from Hardy-Weinberg proportion in Sendai population of *P. ohirai.* This seems to show the occurrence of random mating in the population examined, probably without selection and migration from other populations. However, more detailed experiments will be required to determine the mechanism relating to the GPI polymorphism.

This study revealed that the distributions of allelic frequencies in GPI locus of Kinosaki population of *P. ohirai* were identical with those of *P. sadoensis*; both possessing only a single allele, $GPI^{1.00}$. Yoshimura (1969) has performed electrophoretic studies on the whole body proteins to compare *P. sadoensis* with *P. ohirai* collected in Izu Peninsula, and concluded that the electrophoretic patterns were essentially identical with each other. Thus, the present results partly support those of Yoshimura's studies (1969), though only one enzyme was employed as a marker in comparison of both species.

The present denominations of GPI alleles were done as reported previously (Agatsuma, 1981a, b), based on their relative mobilities. Thus, direct comparison is possible between the present results and the previous ones. According to this, any common allele was not found between *P. ohirai* and *P. miyazakii,* while *P. ohirai* and *P. iloktsuenensis* share two alleles, $GPI^{1.00}$ and $GPI^{2.30}$, and the former allele was most common in both two species. From the above, *P. ohirai* and *P. sadoensis* are considered to be genetically more allied to *P. iloktsuenensis* than *P. miyazakii.*

Up to date, little genetic analysis of the parasitic helminths has been made. The GPI variants detected in natural populations will serve as a good marker of inheritance in *Paragonimus* species.

Summary

Studies on glucosephosphate isomerase

(GPI) were performed in Japanese lung flukes, *Paragonimus ohirai* and *P. sadoensis*, using starch gel electrophoresis. In *P. ohirai*, three different phenotypes (single band or triplet bands) of GPI were found in Sendai population, while only one (single band) was found in Kinosaki population. In *P. sadoensis*, only one phenotype (single band) was found. From the distribution of phenotypes of banding patterns and no deviation from the Hardy-Weinberg equilibrium, it was considered that GPI isozymes may be of dimeric structure and be controlled by a single locus with three alleles (*P. ohirai*) or only one (*P. sadoensis*). Based on this hypothesis, three alleles, $GPI^{1.00}$, $GPI^{2.30}$ and $GPI^{2.80}$, might occur in Sendai population of *P. ohirai*, while only one allele, $GPI^{1.00}$, in Kinosaki population of *P. ohirai* and in *P. sadoensis*. The estimation of allelic frequencies suggested that GPI locus was polymorphic in *P. ohirai* of Sendai, and monomorphic in *P. ohirai* of Kinosaki and in *P. sadoensis*, and also that there is no difference in distribution of allelic frequency between the Kinosaki population of *P. ohirai* and *P. sadoensis* population.

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肺吸虫類アイソザイムの電気泳動法による研究
IV. 大平肺吸虫および佐渡肺吸虫の自然集団における glucosephosphate isomerase アイソザイムの対立遺伝子頻度の比較

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本研究では，大平肺吸虫および佐渡肺吸虫の glucosephosphate isomerase (GPI) について自然集団における遺伝的変異の頻度をデンプンゲル電気泳動を用いて調べた．大平肺吸虫のメタセルカリアは，鹿児島県川内および兵庫県城崎のクロベンケイから，又，佐渡肺吸虫のメタセルカリアは新潟県佐渡島のサワガニからそれぞれ採集し，ラット感染後，40～50日目に成虫を回収した．これらの成虫を一匹ごとにホモジナイズ，遠心して，その上清を電気泳動に用いた．泳動法および酵素発色法は，Shaw and Prasad (1970) に従った．その結果大平肺吸虫の川内集団には変異がみられ，3種類の表現型が検出された．それらは1本のバンドのみをもつもの，および3本のバンドをもつもので，3本のバンドをもつものには移動度の異なる2種類の表現型があった．しかし城崎の集団では，変異はみられず，すべて1本のバンドをもつ個体であった．このバンドの移動度は川内集団の1本のバンドをもつ個体のものと一致した．一方，佐渡肺吸虫においては，すべて1本のバンドの個体のみであり，これらの移動度は，大平肺吸虫のそれと同じであった．この1本のバンドの移動度は，3本のものの最も陰極側のバンドと同じであった．以上のように GPI アイソザイムは，大平肺吸虫の城崎集団と佐渡吸吸虫の集団では変異はみられなかったが，大平肺吸虫の川内集団では多型を示し，その頻度分布はハーディーワインベルグの法則から計算された期待値に一致した．これらのことから，両種肺吸虫の GPI アイソザイムは2量体と考えられ，その遺伝様式は既報の他種肺吸虫と同様に，単一遺伝子座により支配されているものと推定された．この仮説により，大平肺吸虫の川内集団には，対立遺伝子 $GPI^{1.00}$, $GPI^{2.30}$, $GPI^{2.80}$ が，城崎の大平肺吸虫および佐渡島の佐渡肺吸虫には $GPI^{1.00}$ のみが存在するものと考えられた．このことは，城崎の大平肺吸虫と佐渡島の佐渡肺吸虫が唯一の遺伝子 $GPI^{1.00}$ を共有していることを示唆し，両種はきわめて近い類縁関係にあるものと推察された．