Influence of immunosuppressants on the establishment of *Paragonimus miyazakii* in albino rats

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ABSTRACT

The present investigation has shown that combined treatment with dexamethasone and prednisolone or with hydrocortisone and dexamethasone enhances the susceptibility of albino rats to *Paragonimus miyazakii*, presumably by suppressing the host's immune responses. In the rats given these combined treatments, the size and egg production of worms were markedly increased. Use of dexamethasone or prednisolone alone had relatively little influence on the growth and maturity of *P. miyazakii*. In serological analysis and agar double diffusion, considerable differences were recognized between the gamma-globulin fractions and antigen-antibody systems (precipitin bands) of the treated groups and the untreated group. The mechanism of action of these immunosuppressants in this system remains obscure.

Dogs, cats, weasels, wild boars, other carnivores and man have been reported as natural hosts of *P. miyazakii*. In the laboratory, the parasite can reach maturity in the albino rat, but the development of *P. miyazakii* tends to be inferior to that in natural hosts such as dogs and cats, especially in the size of worms and the production of eggs in their uteri (Tada, 1969; Hashiguchi, 1973). These differences between natural and unsuitable hosts (rats) might be due to immunological responses of the host animals.

In recent years there have been many attempts to eliminate the immunological resistance of hosts against various parasites; in most of these cases workers have treated the host animals with immunosuppressants and/or X-irradiation. In helminth parasites, the majority of studies have been carried out with nematodes such as *Ancylostoma*, *Nippostrongylus*, *Trichuris*, *Trichinella*, *Dictyocaulus*, *Gnathostoma* and *Aspiculuris*; there has been little experimental work with trematodes other than schistosomes. Tada (1967a) demonstrated that subcutaneous administration of hydrocortisone showed a little enhancement of the susceptibility of albino rats to *P. westermani*, presumably by the suppression of the host's immune responses.

The work reported here deals with the treatment of albino rats with the immunosuppressants hydrocortisone, dexamethasone and prednisolone, to determine whether suppression of their immune responses would permit a greater number of worms to reach maturity.

MATERIALS AND METHODS

The metacercariae of *P. miyazakii* were obtained from a potamonid crab, *Potamon (Geothelphusa) dehaani*, collected in the endemic area of Kochi, Japan (Hashiguchi *et al.*, 1974). They were administered *per os* to the experimental animals in normal saline, using an injection syringe with a slender vinyl tube.

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Adult albino rats of the Wistar King strain weighing around 200 g were used. They were fed with a commercially prepared diet and water was provided *ad libitum*.

Hydrocortisone acetate (Merck *Cortone*), dexamethasone (Merck *Decadron*) and prednisolone (Merck *Codelcortone*) were administered into alternate thigh muscles of rats beginning 1 day before inoculation of *P. miyazakii* metacercariae and continued every 5 days until necropsy. The dosage levels of these drugs are shown in Table 1. No drugs were given to the rats of the untreated control group (V).

Experimental animals were divided into 5 groups, group I (dexamethasone), group II (prednisolone and dexamethasone), group III (hydrocortisone and dexamethasone), group IV (prednisolone), group V (untreated control). Each group consisted of six or seven male and female rats. During the course of the study, blood samples were obtained by cardiac puncture and the serum samples were subjected to electrophoretic analysis. Agar gel double diffusion (Ouchterlony) was also carried out. The group sera were stored in test tubes at -20°C until required.

Some of the rats in each group were killed on day 30 post-infection, and the remainder were autopsied on day 63 in order to determine the maturity of worms and the number of eggs per gram (E.P.G.) in faeces. In each examination the peritoneal cavity was opened, and the surface of the liver and peritoneal wall was inspected for haemorrhages. The peritoneal surface was flushed with normal saline and the washings were examined for free worms under a dissecting microscope. All of the internal organs were removed separately, then washed with normal saline several times to recover free worms. The liver and lungs were examined for haemorrhages and worm cysts, and then minced with scissors in petri dishes. In order to examine the minced liver and lungs for penetrating worms, thin strips of tissue were pressed between two glass slides.

The majority of worms obtained were flattened and fixed in 70% alcohol. The fixed specimens were stained with carmine and mounted in balsam for morphological examination. Measurements of the worms were made on these stained and mounted worms. Unless otherwise specified, all measurements are in millimeters.

In order to compare the viability of *P. miyazakii* eggs obtained from each group, the eggs were incubated in water at 27°C for two to three weeks, and then examined for the formation of miracidia. Measurements of the eggs were made on living specimens from each group. All of the eggs examined were collected by incubating adult worms in normal saline at 37°C for 12 hours.

Egg counts were made on faecal samples of albino rats in each group between the 46th and 63rd days of infection, by means of the methods reported by Hunter *et al.*, (1948); one gram of faecal sample was collected from each group according to the schedule shown in Fig. 1 and each faecal sample was treated and then the eggs in faecal sediments were counted under the microscope. In addition, numbers of eggs in the uteri of the mounted specimens were examined to secure the maturity of individual worms.

All sera were analysed with a cellulose acetate electrophoresis cell (Model SE-2, Toyo Kagaku Sangyo) supplying constant current. Serum samples were placed on a cellulose acetate membrane (*Seaparax*: Jooko Sangyo Co. Ltd.), and then subjected to 0.8 mA per 1 cm membrane for 50 minutes. The dried strips of cellulose acetate membranes were scanned in a Densitrol DMU-2 (Toyo Kagaku Sangyo), and the relative percentages of albumin, alpha-, beta- and gamma-globulins were determined. The amount of total serum protein was determined by the micro-Kjeldahl technique (g protein per 100 ml serum).

Adult worms of *P. miyazakii*, recovered from cysts in the lungs of infected rats and stored at -20°C , were added to 10 volumes (W/V) of phosphate buffer, pH 7.4. The worms

were homogenized for 5 minutes at 0°C and centrifuged for 15 minutes at 3000 r.p.m. The supernatant was stored at -20°C as the antigen for the agar double diffusion technique.

Ouchterlony plates were prepared by flooding 5 by 11 cm slides with 9.5 ml of 0.9% agar. The central well was filled with *P. miazakii* antigen and the other wells with serum samples of rats from each group. The position of precipitin bands was recorded by drawings and photographs.

RESULTS

Effect of drugs on rats

Direct effect of the immunosuppressants on the rats were reflected in decreases of the body weight. In the untreated control group (V) no marked fluctuation of the mean body weight was recorded throughout the experiment. The animals in groups II, III and IV decreased in weight during the period from the beginning to the end of the experiment. In addition, some of the animals in groups I and IV died, presumably from the administration of the drugs, during the course of the study. In these animals, internal organs such as lungs, liver and kidney, appeared to be damaged, showing abscess-like haemorrhages.

Worm recovery

Pertinent data are summarised in Table 1. No significant differences were found among the average recoveries in the treated groups and the control. Worm cysts were found on the 30th day in the lungs of all rats of the treated groups, whereas no cysts were observed in any animal of the controls. Average numbers of worm cysts in groups I, II, III, IV and V were 2.0, 2.4, 3.2, 2.3 and 1.4, respectively, between the 30th and 63rd days of infection; percentages of the worms recovered from these cysts per total worms recovered were 50.9%, 68.6%, 83.7%, 69.6% and 33.3%, respectively.

TABLE 1

Paragonimus miazakii worm recoveries from rats in each group between the 30th and 63rd days of infection: 20 metacercariae each were administered *per os* to the animals

Group*	No. of rats examined	Recovery rates	No. of worms recovered from				No. of cysts in lungs
			abdominal cavity	liver	pleural cavity	cysts in lungs	
I	7 (2)**	37.9%	14	4	8	27	14
II	7 (1)	36.4%	2		14	35	17
III	6	40.8%			8	41	19
IV	6 (2)	38.3%	2		12	32	14
V	7	42.9%	16	7	17	20	10

* I: dexamethasone (2 mg/rat every 5 days), II: prednisolone and dexamethasone (10- and 2 mg/rat every 5 days, respectively), III: hydrocortisone and dexamethasone (25- and 2 mg/rat every 5 days, respectively), IV: prednisolone (10 mg/rat every 5 days), V: untreated control

**Numbers of dead animals recovered during the period of the experiment, presumably from the administration of the drugs

Worm growth

In the treated groups (I-IV) the worms had a tendency to show a slightly greater mean length and width than those from the untreated control group (V) on day 30 post-infection. On day 63, however, the mean lengths of worms in groups I, II, III, IV and V were 5.0 mm, 7.5 mm, 7.3 mm, 5.1 mm and 5.2 mm, respectively. Thus, the worms in groups II and III showed a markedly greater size compared with those in the other groups. In addition the worms recovered from the rats in groups II and III had significantly more eggs in their

TABLE 2

Cellulose acetate-electrophoretic analysis of serum proteins in experimental rats treated with and without various immunosuppressants on day 30 postinfection

Group	No. of serum samples	Albumin	Fractions of serum protein*				A/G ratio	Total protein (g/100 ml)
			alpha 1	alpha 2	beta	gamma		
I	4	47.3 ± 4.51	16.9 ± 1.00	6.2 ± 1.52	9.9 ± 2.67	19.7 ± 1.16	0.9 ± 0.15	6.6 ± 0.43
II	4	54.6 ± 5.14	11.2 ± 4.17	7.3 ± 1.17	11.5 ± 4.37	15.5 ± 2.70	1.3 ± 0.26	6.6 ± 0.23
III	3	53.1 ± 9.30	14.1 ± 4.29	8.4 ± 1.59	8.7 ± 3.78	15.6 ± 3.29	1.1 ± 0.45	6.6 ± 0.73
IV	3	55.1 ± 2.65	13.4 ± 6.05	5.5 ± 1.44	10.4 ± 5.84	15.6 ± 2.48	1.2 ± 0.12	7.6 ± 0.21
V	4	52.2 ± 9.86	15.7 ± 5.06	4.8 ± 2.62	7.8 ± 1.09	20.6 ± 4.31	1.2 ± 0.54	7.3 ± 0.81

* Relative protein concentration in *per cent* (with standard deviation)

FIG. 1. The appearance of eggs in faeces of rats treated and not treated with immunosuppressants. I: dexamethasone (2mg/rat every 5 days), II: prednisolone and dexamethasone (10- and 2mg/rat every 5 days, respectively), III: hydrocortisone and dexamethasone (25- and 2mg/rat every 5 days, respectively), IV: prednisolone (10mg/rat every 5 days), V: untreated control.

uteri than worms from the other groups. This difference was apparent also when faecal egg outputs from day 46 onwards were compared (Fig. 1). Furthermore, the differences between the treated groups (I–IV) and the untreated group (V) were also reflected in the degree of miracidial formation in the eggs; the percentages of eggs with miracidia to those without were 81.7% in group II and 82.8% in group III but only 29.2% in the control. Moreover, the eggs of the worms recovered from the rats treated with immunosuppressants were longer and wider than those from the controls.

Serological analysis

The results of cellulose acetate-electrophoresis studies on serum samples from rats infected with *P. miyazakii* in each group are shown in Table 2. In comparison with untreated control group (V), the albumin fraction in sera from treated animals (groups I–IV) showed no marked differences, ranging from the mean percentage value of 47.3% in group I to 55.1% in group IV, while the value in the control was 52.2%. On the other hand, in the globulin fractions the amount of gamma-globulin was relatively low in groups II, III and IV varying between 15.5% and 15.6%, compared to 19.7% in group I and 20.6% in group V (control). However, the other globulin fractions showed a variation in mean percentage values, with no apparent relationship to the treatment of the groups. Averages of albumin per globulin (A/G) ratios were 1.2 in group V and 0.9 to 1.3 in groups I, II, III and IV on the 30th day of infection. Total serum proteins were 7.6 g/100 ml in group IV and 7.3 in group V; but only 6.6 in groups I, II and III.

The formation of precipitin bands in agar double diffusion plates is shown in Table 3. Three serum samples out of four taken on day 30 in group II showed no precipitin bands and the number of bands in the other treated groups was zero to two, while all serum samples from the untreated control group produced three to five bands.

TABLE 3

Number of antigen-antibody bands appeared in agar double diffusion plate in serum samples from rats in each group treated with and without various immunosuppressants on day 30 postinfection

	Group				
	I	II	III	IV	V
No. of serum samples tested	4	4	3	3	4
No. of bands appearing in agar diffusion plate	2, 1, 1, 0	1, 0, 0, 0	1, 1, 0	1, 1, 0	5, 4, 3, 3

DISCUSSION

The results reported in the present paper show that the growth and maturation of *P. miyazakii* in albino rats could be enhanced by treatment with immunosuppressants. The administration of prednisolone and dexamethasone (group II) or hydrocortisone and dexamethasone (group III) resulted in better worm growth and maturation, suggesting that doubly treated rats in groups II and III were made more susceptible to *P. miyazakii* than rats treated with dexamethasone only (group I) or prednisolone only (group IV). In the case of the rats treated with hydrocortisone only, Tada (1967a) also demonstrated that the drug did not have any significant effect on the establishment of *P. miyazakii*. From these results, it is suggested that combined treatment of such drugs is more effective in suppressing the host's immune responses against *P. miyazakii*.

In this study, some of the rats in the treated groups died during the course of the experiment, showing abcess-like haemorrhages in their internal organs. In addition a steady decrease in the body weight of the rats was apparent in groups II, III and IV. These facts suggest that the dosage of the drugs used in the present study were too high.

It is of interest to note that the formation of cysts in the lungs was apparent in all rats treated with immunosuppressants (groups I-IV) by the 30th day of infection, while no cysts were found in the lungs of untreated control rats until day 63 (group V). This is the most remarkable difference between the treated groups and the untreated control group. Tada (1967a), working with hydrocortisone treated rats infected with *P. westermani*, reported that the number of worm cysts in the lungs was greater in treated animals. This tendency was also found in the present experiment; the average numbers of worm cysts per rat in each group were 2.0 to 3.2 in the treated groups and only 1.4 in the control. The number of worms recovered from cysts in the lungs was greatest in group III (hydrocortisone and prednisolone), being 83.7% of the total worms recovered between the 30th and 63rd days of infection. The corresponding values were 50.9% to 69.6% in groups I, II and IV and 33.3% in group V (control). The high value in group III suggests that combined treatment with hydrocortisone and prednisolone results in the production of conditions that are most favourable for *P. miyazakii* in this system.

The worms recovered from groups II and III showed greater sizes than worms from the other groups, being 7.46 mm and 7.30 mm in average length, respectively, while, in the case of naturally infected animals, the largest worm, parasitic in cats measured 12.6 mm in length (Kamo and Hatsushika, 1966). From these differences in the sizes of worms, it is presumed that there might be additional immunological and physiological factors, as yet unknown, which may exist in this unsuitable host relationship.

Eggs were found in the faeces of rats in groups II, III and IV on the 46th day of infection, thereafter the number of eggs in the faeces showed a steady increase especially in groups II and III. In groups I and V, however, eggs were first recognized on the 51st day of infection and then a few eggs appeared in the faeces until the end of experiment. These findings were confirmed by the examination of the eggs in the uteri of worms on the 63rd day of infection; in groups II and III, the majority (72% to 90%) of worms had eggs in their uteri, while 54% of the worms in group V (control) had no eggs. In addition, the size of eggs deposited and the percentage formation of miracidia in these eggs were also greater in groups II and III than in the control group (V). These results also suggest that the rats treated in groups II and III provided better conditions for the establishment and survival of *P. miyazakii* in the rats.

Changes in serum protein fractions during human and animal paragonimiasis have been reported. Kruideneir and Katoh (1959), working with rats infected with *P. kellicotti*, demonstrated by paper electrophoresis that a distinct increase occurred in the beta-globulin fractions, but little or no changes occurred in the actual albumin and alpha-globulin fractions though relative decreases were apparent. According to Tada (1967b), in the longterm infection of rats with *P. miyazakii* gamma-globulin levels attained their peak on the 30th day. In this study, the serum samples from rats were also analyzed on day 30. The most noticeable difference in the serum fractions among the present groups was found in gamma-globulins; the fractions showed lower percentages in groups II, III and IV than those in groups I and V. The differences may be caused by the suppression of the host's immune responses.

Three to five bands (antigen-antibody systems) appeared on agar gel diffusion plates using control serum (group V), whereas no bands or only one band was found in groups II, III and IV; up to two bands were recognised in group I. These facts suggest that antibody production was greatly suppressed in the animals given immunosuppressive treatments. From the results reported hitherto, it is suggested that around five precipitin bands appear on agar gel diffusion plates during the period of peak levels in antibody productions in animals and humans infected with *Paragonimus*, if the hosts do not receive any treatment with immunosuppressants (Yogore *et al.*, 1965; Seed *et al.*, 1966; Tada, 1967a).

In the present study, the susceptibility of albino rats to *P. miyazakii* seemed to be markedly enhanced by combined treatment with immunosuppressants, resulting in better growth and maturation of the parasite. It is still difficult, however, to come to clear conclusions from this experiment regarding the relative importance of immunological and physiological factors in determining development of *P. miyazakii* in such unnatural host. In order to clarify these points, more detailed information on the action of particular drugs against this trematode parasite in the host animals are needed.

ACKNOWLEDGEMENTS

We express our appreciation to Dr. D. Wakelin, The Wellcome Laboratories for Experimental Parasitology, University of Glasgow, Bearsden Road, Glasgow, for his critical reading of the manuscript, and we are also grateful to Messrs H. Hiraoka, S. Kono, K. Watanabe, S. Banda and M. Yamaguchi and Miss E. Okabayashi for their assistance throughout the experiment.

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Accepted 28 October, 1976.