Specific fields: Parasitology

Research Note

Sensitive Enzyme-Linked Immunosorbent Assay (ELISA) to Measure Parasite-Specific Antibodies of Indian Soft-Furred Rats, Millardia meltada

Running head: Sensitive ELISA for *M. meltada*

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ABSTRACT. Immunoglobulin G (IgG) of Indian soft-furred rat, *Millardia meltada*, was purified by an immunoaffinity chromatography and antibodies against it was raised in rabbit. Using this rabbit anti-*M.meltada* IgG antibody, sensitivity of enzyme-linked immunosorbent assay (ELISA) to measure parasite-specific antibodies in the sera of *M.meltada* was markedly enhanced than the previous method using rabbit anti-mouse IgG and rabbit anti-rat IgG antibodies, which could cross-react to *M.meltada* IgG. Since *M.meltada* could effectively produce circulating antibodies against two intestinal helminths, *Strongyloides venezuelensis* and *Nippostrongylus brasiliensis*, the high susceptibility of this animal to an array of parasites seems to be not due to general immunological deficiency.-----KEY WORDS: ~~antibody~~, ELISA, IgG, *Millardia meltada*

The Indian soft-furred rat, *Millardia meltada*, is a wild rodent which inhabits Pakistan, Nepal and Sri Lanka. An inbred strain of *M. meltada* has been established and maintained in the Experimental Animal Center, Miyazaki Medical College. Although biological characteristics of this animal are not well understood, the male animals often develop mammary adenocarcinoma in the inguinal mammary tissues, probably because of their high androgen susceptibility [3]. Recently *M. meltada* has been reported to be susceptible to a filarial parasite, *Acanthocheilonema viteae*, with some sex difference [5]. *M. meltada* was also found to be susceptible to a rat intestinal nematode, *Nippostrongylus brasiliensis*, with sex difference [6]. Such sex difference was found in the expulsive capacity of this animal against *N. brasiliensis* and was regulated by testosterone [6, 7]. In the testosterone-treated female *M. meltada*, goblet cell hyperplasia, a possible candidate of the effector in the expulsion of *N. brasiliensis*, is suppressed almost completely and *N. brasiliensis* worms persists for over 7 weeks [7]. Thus *M. meltada* seems to be a fascinate experimental animal to investigate intestinal mucosal defence mechanisms. Despite of the usefulness of this animal as a experimental model for an array of parasites, *M. meltada* still not be as popular as mice and rats. Therefore, anti-*M. meltada* antibodies have never been available commercially. In this study, we have purified IgG of *M. meltada* and raised rabbit anti-*M. meltada* IgG antibody. This conventional antibody gave satisfying results in ELISA to measure anti-parasite antibody responses of *M. meltada*.

The strain of *M. meltada* has been maintained by sib-mating for over 10 years in the Experimental Animal Center, Miyazaki Medical College. The *M. meltada* used in this study were males and 10-12 weeks old at the start of experiments. Male New Zealand White rabbits of 1-2 kg body weight were purchased from Kuroda Experimental Animal Co. (Kumamoto, Japan). Animals were kept under clean conventional conditions in our laboratory. Normal sera were obtained from mice (C57BL/6), rats (Wistar) and Mongolian gerbils, which were maintained in our laboratory.

Serum samples were obtained from *M. meltada* various time after infection with either *Strongyloides venezuelensis* or *N. brasiliensis*. The animals were infected with either 5000 (for *S. venezuelensis*) or 800 (for *N. brasiliensis*) infective larvae by a subcutaneous injection

as described previously [7] .

The methods for purification of *M. meltada* immunoglobulin G (IgG) and preparation of rabbit anti-*M. meltada* IgG were basically as same as the previous report [4]. Briefly, *M. meltada* were immunized subcutaneously with 100 μg of bovine serum albumin emulsified in complete Freund's adjuvant (CFA) followed by bi-weekly immunizations with the same amount of BSA emulsified in incomplete Freund's adjuvant (IFA). Sera were obtained from the animals before and after the immunization and tested for the binding to BSA in ELISA as described below. *M. meltada* IgG was purified as anti-BSA antibodies by immunoaffinity chromatography using BSA-coupled Sepharose-4B. Purity of the *M. meltada* IgG was checked in 15% polyacrylamide gel electrophoresis (PAGE) under reducing condition. Rabbits were immunized with 200 μg of purified *M. meltada* IgG in CFA, followed by two immunizations of 100 μg of *M. meltada* IgG in IFA. Sera were collected before and after the immunization and tested for the reactivity and specificity to *M. meltada* IgG by various ways as described in the RESULTS section. In brief, immunoreactivity of the rabbit antisera against *M. meltada* and other rodent immunoglobulins (species specificity) was tested by Ouchterlony's methods and the specificity against *M. meltada* IgG by immunoelectrophoresis.

Binding of *M. meltada* sera to BSA or parasite antigens was measured in ELISA. Wells of microtiter plate was coated with BSA (1 μg/ml) or parasite antigens (10 μg/ml), and incubated overnight at 4 °C. After washing and blocking the plate, various concentrations of *M. meltada* sera were added to the wells and the binding of *M. meltada* IgG to the antigens was detected either by horse radish peroxidase (HPO)-labelled rabbit anti-mouse IgG (1:1000 dilution) and HPO-labelled rabbit anti-rat IgG (1:1000 dilution) or rabbit anti-*M. meltada* IgG (1:1000 dilution) followed by HPO-labelled swine anti-rabbit IgG (1:1000 dilution). H_2O_2 and O-phenylenediamine dihydrochloride were used as substrate and color indicator. Optical densities (OD) were measured 20 min later at 490 nm.

IgG of *M. meltada* was purified as anti-BSA using immunoaffinity chromatography as mentioned previously in detail. Purity of the *M. meltada* IgG was checked by PAGE under reducing condition (Fig. 1). Only two sharp bands of 55 kDa and 25 kDa corresponding to

heavy and light chains of immunoglobulin were observed. After having raised rabbit antibodies to the purified *M. meltada* IgG, purity was also checked by immunoelectrophoresis. The rabbit anti-*M. meltada* IgG antiserum produced sharp single arch against purified *M. meltada* IgG, while it could recognize at least two IgGs present in the normal *M. meltada* serum (Fig. 2).

Specificity of rabbit antisera raised against purified *M. meltada* IgG was tested by an Ouchterlony's double diffusion in agar. Strong precipitin band was produced against normal *M. meltada* serum, whereas far weaker bands were observed against sera of other rodents (Fig. 3).

To demonstrate the advantage of the use of rabbit anti-*M. meltada* IgG in measuring *M. meltada* antibodies, sera from BSA-immunized *M. meltada* were tested for the binding to BSA in two different ELISA methods; in one system either HPO-labelled rabbit anti-mouse IgG or HPO-labelled rabbit anti-rat IgG were used for detection of *M. meltada* IgG while in the other system a combination of rabbit anti-*M. meltada* IgG and HPO-labelled swine anti-rabbit IgG was used. As shown in Fig. 4, HPO-labelled rabbit anti-mouse IgG could detect binding of *M. meltada* IgG to BSA only at lower dilutions of sera and the maximum OD values were less than 0.9. Also HPO-labelled rabbit anti-rat IgG could detect binding of *M. meltada* IgG to BSA only at lower dilutions of sera and maximum OD values were less than 1.7. On the other hand, rabbit anti-*M. meltada* IgG followed by HPO-labelled swine anti-rabbit IgG could detect binding of *M. meltada* IgG to BSA at much higher dilutions all the way up to 10^{-5} and the maximum OD values were about 2.7.

To confirm further the advantage of the use of rabbit *M. meltada* IgG in measuring *M. meltada* antibodies, sera from *M. meltada* infected with *S. venezuelensis* or *N. brasiliensis* were tested for the binding to the corresponding parasite antigens in two different ELISA methods as described above. As shown in Figs. 5 and 6, regardless of the parasite used for infection, parasite-specific antibodies were detected most sensitively when a combination of rabbit anti-*M. meltada* IgG followed by HPO-labelled swine anti-rabbit IgG was employed.

The present results clearly demonstrate that the sensitivity of the detection of parasite

specific antibodies in the sera of *M. meltada* by the ELISA using rabbit anti-*M. meltada* IgG was 2-4 times higher than that using either rabbit anti-mouse IgG or rabbit anti-rat IgG. Although such results were predictable, appropriate antiserum raised against *M. meltada* IgG has never been sold commercially, in spite of the uniqueness of *M. meltada* as an experimental host for various parasites. Since *M. meltada* has shown androgen-dependent significant sex difference in the susceptibility to various parasite [5, 6], this animal seems to be a good model to investigate the regulatory mechanisms of androgenic steroid hormone in the host defence. However general immunological background of *M. meltada* including antibody producibility to parasites has never been studied, probably because adequate assay methods has not been available so far.

As the recommended methods for purification of immunoglobulins, combination of ion exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-200 has widely been used in many laboratories [2]. More recently the use of protein-A or-G affinity chromatography was recommended for the purification of IgG [1]. Compare to these, immunoaffinity method described here is simple and less expensive and can theoretically be applicable to any animal species [4]. In fact, we could successfully prepare rabbit anti-*M. meltada* IgG within 2 months using only five *M. meltada* immunized with BSA. Since we have made a bulk of rabbit anti-*M. meltada* IgG antisera, portions of this could be distributed upon request with no obligation.

In the present study, we have found that *M. meltada* could produce circulating antibodies against two species of intestinal helminths. The antibody titers rose up further with time during the periods of infection. These results suggest, though not completely rule out, that the high susceptibility of *M. meltada* to various parasites is not due to defective antibody production. Application of the sensitive ELISA methods to an array of parasite infections in *M. meltada* would solidify this possibility.

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Legends to Figures

Fig. 1. SDS-PAGE of normal *M. meltada* serum (whole serum) and purified *M. meltada* IgG. Lanes 1-3: normal *M. meltada* serum (0.5 μl, 0.025 μl and 0.0125 μl from lane 1 to lane 3 respectively). Lanes 4-6: purified *M. meltada* IgG (2.0 μl, 1.0 μl and 0.5 μl from lane 4 to lane 6 respectively). M: molecular markers.

Fig. 2. Immunoelectrophoretic pattern of purified *M. meltada* IgG. R: rabbit anti-*M. meltada* IgG; MIgG: purified *M. meltada* IgG; NMS: normal *M. meltada* serum.

Fig. 3. Specificity of rabbit anti-serum against *M. meltada* IgG by an Ouchterlony's method. Center well: rabbit anti-*M. meltada* IgG antiserum (1:2 dilution); M.m: normal *M. meltada* serum; rat: normal rat serum; mouse: normal mouse serum; gerbil: normal Mongolian gerbil serum.

Fig. 4. Titration of *M. meltada* antibody against BSA by ELISA. ●: detected by rabbit anti-*M. meltada* IgG followed by HPO-labelled swine anti-rabbit IgG; ○: detected by HPO-labelled rabbit anti-mouse IgG; ■: detected by HPO-labelled rabbit anti-rat IgG.

Fig. 5. Detection of parasite specific antibodies in the sera of *M. meltada* infected with *S. venezuelensis*. Symbols are the same as Fig. 4. All sera tested were diluted at 1: 100.

Fig. 6. Detection of parasite specific antibodies in the sera of *M. meltada* infected with *N. brasiliensis*. Symbols are the same as Fig. 4. All sera tested were diluted at 1: 100.

Fig. 1

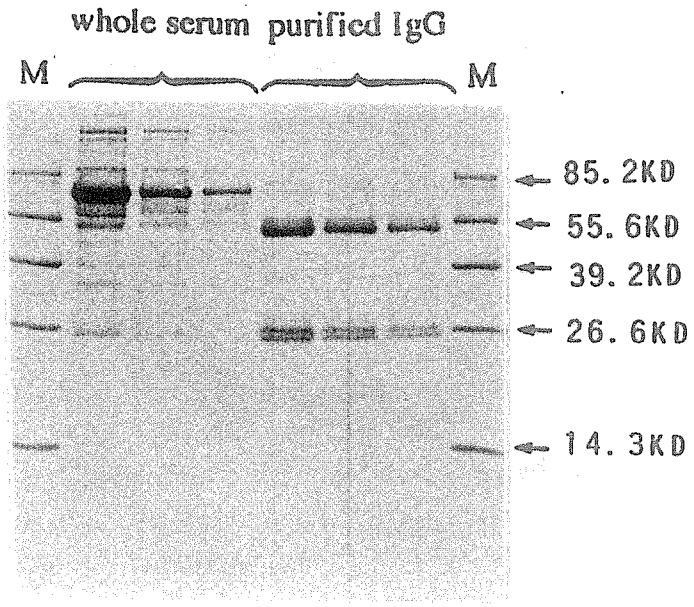


Fig. 2

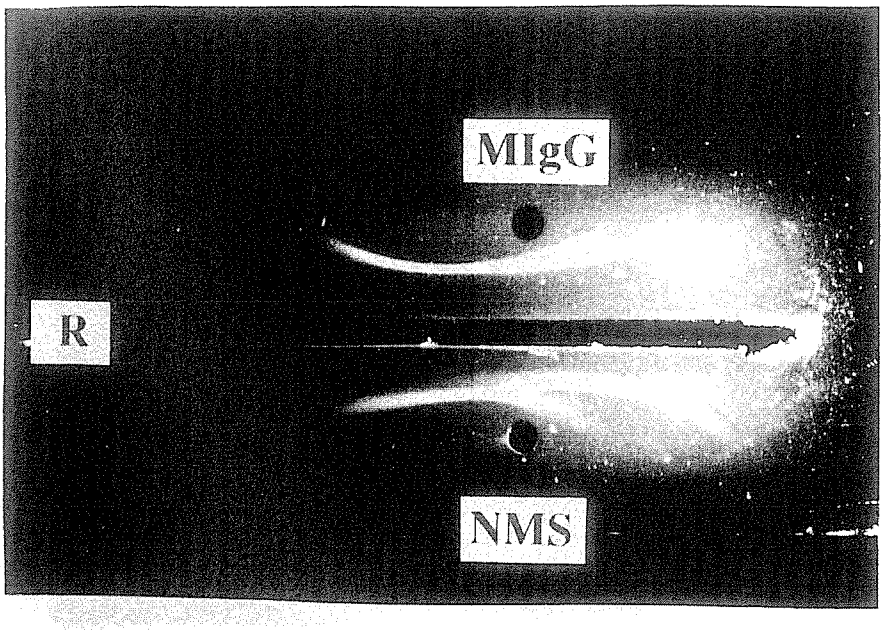


Fig. 3

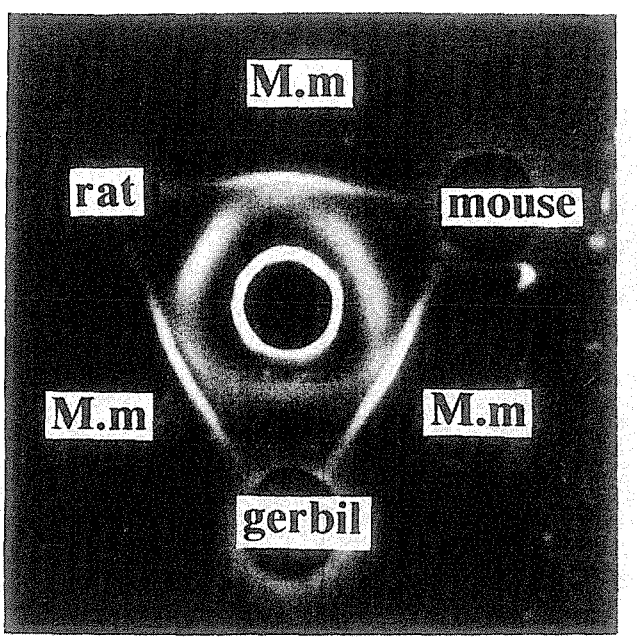


Fig. 4

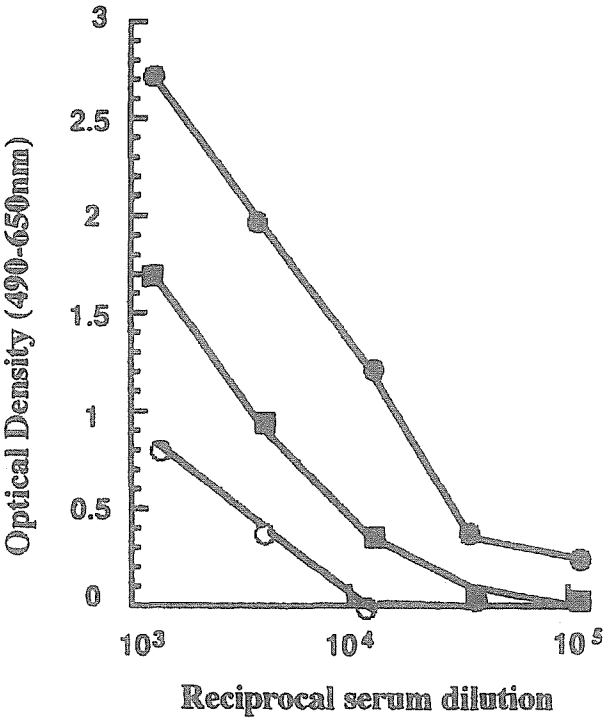

Optical Density (490-650nm)
3
2.5
2
1.5
1
0.5
0
10^3
10^4
10^5
Reciprocal serum dilution

Fig. 5

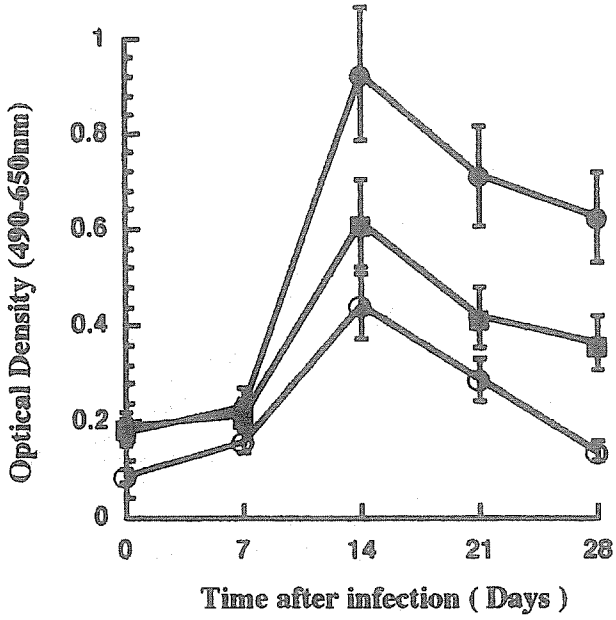
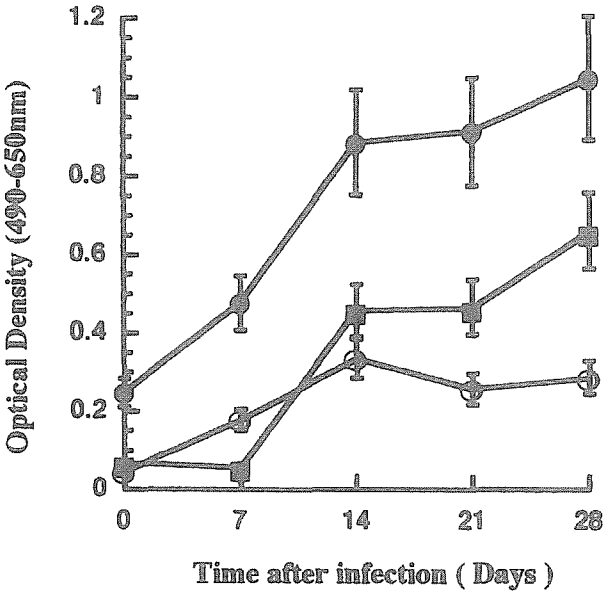

Optical Density (490-650nm)
1
0.8
0.6
0.4
0.2
0
0
7
14
21
28
Time after infection (Days)

Fig. 6


Optical Density (490-650nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
7
14
21
28
Time after infection (Days)