Leukocyte Migration Inhibition in Chickens Immunized with *Eimeria tenella*

Hiroshi ONAGA and Toshio ISHII*

*Nippon Institute for Biological Science,
2221-1 Shin-machi, Ōme-shi, Tokyo 198*

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Abstract. Leukocyte migration inhibition tests (LIT) with chicken peritoneal cells were carried out by the direct and the indirect techniques in chickens immunized with *Eimeria tenella*. In LIT by the direct technique, migration of peritoneal cells was inhibited by adding antigen prepared from the 2nd-generation merozoites. The activities of leukocyte migration inhibitory factor (LIF) were detected from 1 week after a single inoculation with 50,000 oocysts of 3 weeks after the last of 3 repeated inoculations with 50,000 oocysts. Spleen cells obtained from immunized chickens elaborated LIF into the medium when they were cultivated with antigen. In LIT by the indirect technique, supernatant of the cultures of spleen cells from immunized chickens inhibited migration of peritoneal cells collected from normal chickens. The LIF activities detectable by the indirect technique were demonstrated for 5 weeks after the last of 3 repeated inoculations with 50,000 oocysts.

The results obtained by the macrophage migration inhibition test (MIT) or the leukocyte migration inhibition test (LIT) using spleen cells [17], peripheral blood leukocytes [2, 15, 16] or peritoneal cells [13] have been demonstrated to run parallel with the responses of delayed hypersensitivity to purified protein derivative of tuberculin in chickens sensitized with *Mycobacteria*. The MIT or LIT, therefore, has been adapted to a method of studying the role of cell-mediated immunity in chickens.

There have been some reports concerning cell-mediated immunity in chicken coccidial immunity [10, 12, 14], and in mammalian coccidial immunity [4-7]. In these studies, however, the correlation between cell-mediated immunity and resistance of chickens to the parasites, and the duration of cell-mediated immunity after infection

remained to be determined. Morita et al. [10] demonstrated the migration inhibition of splenic cells obtained from chickens infected with *Eimeria tenella*, but the duration of activities of migration inhibitory factor after infection was not shown. Rose [12] used the peritoneal macrophages of chickens infected with *E. tenella* for MIT, and showed rather inconclusive results.

In the present work, migration inhibitions of peritoneal cells from chickens immunized with *E. tenella* were tested by the direct and the indirect techniques of LIT. The results of the observations are presented in this communication.

Materials and Methods

Immunization: Twenty-five 4-week-old White Leghorn chickens were immunized by a single oral inoculation with 50,000 oocysts of *E. tenella*, whereas

* Present address: Department of Parasitology, Nippon Veterinary and Zootechnical College, Musashino-shi, Tokyo 180

forty-two 2-week-old chickens were immunized by inoculating three times at a week interval with 50,000 oocysts each. Eighteen 5-week-old uninoculated chickens were used as controls.

Antigen: Five-day-old chickens were inoculated with 50,000 oocysts of *E. tenella*. The chickens were killed 5 days after the inoculation, and their cecal contents were collected. Merozoites were obtained from the cecal contents by digestion with a solution consisting of 5% chicken bile and 0.3% trypsin in 0.85% saline. After washing with phosphate-buffered saline (PBS), the merozoites were suspended in PBS at a concentration of 5×10^7 /ml, and then subjected to 5 cycles of freezing and thawing. The suspension was centrifuged at 10,000 g for 30 min, and the supernatants were collected, sterilized by means of membrane filtration (0.45 μ m), and stored at -80°C until used as stock antigen.

Direct technique of LIT: Agarose droplet method described by Harrington et al. [3] and Morita et al. [11] was used for all experiments on inhibition of migration.

Immunized chickens were injected intraperitoneally with 0.05 ml/g body weight of 3% corn starch in 0.85% saline to induce peritoneal exudate at specified intervals from 4 days to 6 weeks after a single inoculation or after the last of 3 repeated inoculations. Control chickens were also injected similarly. One day after the injection, chickens were killed by bleeding. Hanks' balanced salt solution (BSS) containing 3 I.U./ml of heparin was injected into peritoneal cavities. After massaging the abdomen, peritoneal cell suspension was collected. After washing with Hanks' BSS, the suspension was centrifuged, and sedimented cells were suspended in almost the same volume of 0.5% agarose (A-45, Nakarai Chemicals, Ltd.) in medium 199 which remained fluid at 37°C . Three 2- μ l droplets of the mixture were placed in each cell culture chamber (4 chamber/slide, Lab-Tek products, Illinois, U.S.A.), and the chambers were cooled at 4°C for 15 min. Each chamber was then filled with 0.4 ml of medium and 0.1 ml of either undiluted (1:5), or 1/2 (1:10) or 1/4 (1:20) diluted stock antigen. The medium was composed of 2.5% heat-inactivated fetal calf serum (FCS), 2.5% heat-inactivated chicken serum, 100 U/ml of penicillin G potassium, 100 μ g/ml of streptomycin sulfate, and 95% medium 199. The chambers were incubated at 40°C for 24 hr in an atmosphere containing 5% CO_2 in the air. Then, migrated cells were fixed in methanol and stained with Giemsa's solution.

Indirect technique of LIT: Spleens were removed from immunized chickens from 1 to 5 weeks after the last of 3 repeated inoculations and from control

chickens. After rinsing in PBS, the tissues were minced with a pair of scissors, and gently teased through a 60-mesh steel sieve. Lymphocytes were isolated by centrifugation at 400 g for 30 min on a Ficoll-Paque (Pharmacia Fine Chemicals). After washing with PBS, lymphocytes were suspended in RPMI 1640 at a concentration of 4.5×10^7 /ml. One point eight ml of the lymphocyte suspension and 0.2 ml of stock antigen or PBS instead of antigen were dispensed to a Falcon dish (35 $\times$ 10 mm). The cultures were incubated at 40°C for 48 hr in an atmosphere containing 5% CO_2 in the air. Supernatants of the cultures were collected by centrifugation and stored at -80°C until used as leukocyte migration inhibitory factor (LIF) in LIT. LIT was performed using peritoneal cells collected from 5-week-old normal chickens by the agarose droplet method in a manner similar to the direct technique. In these tests, 0.25 ml of RPMI 1640 containing 2% FCS and 0.25 ml of supernatant of the lymphocyte cultures were added to a cell culture chamber.

Calculation: The migration distance (MD) of peritoneal cells from the edge of an agarose droplet to the periphery of migration area was measured with an ocular micrometer. The mean MD of one droplet was calculated from measurements of MDs in 4 directions of a droplet (Fig. 1-a). The mean MD in a chamber was calculated from the mean MDs of 3 droplets (Fig. 1-b). The percent migration inhibition was expressed by the following formula:

$$100 - \frac{\text{Mean MD with antigen}}{\text{Mean MD without antigen}} \times 100$$

Percent migration inhibition in groups of immunized and normal chickens was analyzed statistically by the student's t test.

Results

1. Leukocyte migration inhibition as assayed by the direct technique

Peritoneal cells were obtained from chickens immunized by a single inoculation with oocysts 4 days, 1, 2, 3 and 5 weeks after inoculation and from chickens immunized by 3 repeated inoculations with oocysts 1, 2, 3, 4 and 6 weeks after the last inoculation. Peritoneal cells were collected from 4 to 5 chickens at each interval, and assayed for LIF activities. Mean percent migration inhibition and deviation ($p < 0.01$) calcu-

Fig. 1-a

Fig. 1-b

Fig. 1-a. The migration distances of cells were measured at 4 directions from the edge of the agarose droplet to the periphery of migration area.

Fig. 1-b. Three droplets were placed in each chamber.

Fig. 2. Duration of LIF activities after a single inoculation with oocysts.

Fig. 3. Duration of LIF activities after the last of 3 repeated inoculations with oocysts.

lated in each group are shown in Figs. 2 and 3. When inhibitory activities were estimated by comparing the upper level of deviation of migration inhibition in the normal chicken-group with the lower levels of that in the immunized chicken-groups, the LIF activities in the immunized chicken-group could not be detected 4 days after infection, but appeared 1 week after infection. Migration inhibition of peritoneal cells in chickens immunized by a single inoculation with oocysts remained at high levels for 1 to 2 weeks (Fig. 2). Three weeks after inoculation, the lower levels of deviation of migration inhibition in the immunized chicken-group fell on the upper levels of that in the normal chicken-group in the presence of antigen 1:5 and 1:20. On the other hand, migration inhibitory activities in chickens inoculated 3 times with oocysts remained at high levels even at 3 weeks after the last inoculation (Fig. 3). Four weeks after the last inoculation, the lower level deviation of migration inhibition in the immunized chicken-group reached the upper level of that in the normal chicken-group at each dilution of anti-

gen. In all the tests with different dilutions of antigen, the similar patterns of migration were observed.

2. Leukocyte migration inhibition as assayed by the indirect technique

In order to confirm the LIF activities in immunized chickens, the production of LIF was measured by the indirect technique. As shown in Table 1, the spleen cells, the source of lymphocytes, were obtained from 7 immunized chickens 1 week after the last of 3 repeated inoculations with oocysts and from 8 normal chickens. Peritoneal cells collected from normal chickens were not pooled but used as indicator cells.

Percentages of migration inhibitions in the group treated with supernatants of immune lymphocyte cultures (immune group) were higher than those in the group treated with supernatants of normal lymphocyte cultures (normal group). The means of percent migration inhibitions were 37.3 ± 14.5 in the immune group and 2.4 ± 3.6 in the normal group ($p < 0.01$).

Migration inhibitions 1, 3 and 5 weeks after the last of 3 repeated inoculations were

Table 1. Leukocyte migration inhibition assayed by the indirect technique

Origin of spleen cells	Mean migration distance (μ m)		Percent migration inhibition
	Antigen	PBS	
Immunized chickens*	453	809	44.0
	188	258	27.2
	870	1062	18.1
	450	708	36.4
	1108	1408	21.3
	80	177	54.8
	513	1263	59.4
Normal chickens	735	710	-3.5
	458	443	-3.4
	991	1028	3.6
	610	635	3.9
	703	761	7.9
	573	480	1.2
	1035	1115	7.2
	960	980	2.0

* At 1 week after the last of 3 repeated inoculations with oocysts.

** Means of percent migration inhibitions in the immunized and normal chicken-groups ($p < 0.01$).

Fig. 4. Duration of LIF activities assayed by the indirect technique after the last of 3 repeated inoculations with oocysts.

assayed in the groups consisting of 5 chickens each. As shown in Fig. 4, the means of percentages of migration inhibitions and deviations in the immune groups were higher than those in the normal group at all the observation points.

Discussion

In the present work, the LIF activities were detected by the direct technique of LIF using chicken peritoneal cells from 1 to 3 weeks after inoculation with oocysts of *E. tenella*. Rose [12], in a similar experiment using the capillary method of MIT, obtained equivocal results. Since a large number of cells are required to operate the capillary method of MIT, we used the agarose droplet method for LIT. The tests were operated easily, and required rather a small number of cells. The difference between the results obtained by Rose and by the present authors might be due to the difference in techniques used.

In a preliminary experiment of this work using the indirect technique, when RPMI 1640 containing 10% FCS was used for cultivating lymphocytes as well as LIT, the difference in the percentages of migration inhibitions between immunized and normal chicken-groups was too small to be analyzed

statistically. On the other hand, satisfactory migration inhibition responses were observed when FCS was omitted from the medium, but the attachment of cells to the glass surface was remarkably lowered. In the present work, therefore, the concentration of serum contained in the medium was reduced to 5% in the direct technique and 2% in the indirect technique of LIT.

There is a possibility that the cells obtained from immunized chickens were coated by specific antibodies via the Ig receptors. Vlaovic et al. [16] described that cytophilic antibodies rendered normal peripheral blood leukocytes sensitive to inhibition by antigen, and rabbit anti-chicken IgG serum abolished leukocyte migration inhibition due to cytophilic antibodies. In another experiment, the peritoneal cells obtained from chickens 3 and 5 weeks after the last of 3 repeated inoculations with oocysts of *E. tenella*, after being treated with rabbit anti-chicken IgG serum by the method described by Vlaovic et al. [16], were used for the LIT (Onaga and Ishii, unpublished data). The results obtained with these cells were almost the same as those obtained by using chicken peritoneal cells which were not treated with rabbit anti-chicken IgG serum (Fig. 3), but the sensitivity of response in LIT was a little low. In addition, it has been said that the indirect method of MIT or LIT using indicator cells collected from normal animals was highly specific to detect MIF or LIF activity [1, 17]. In the present experiment, migration of unimmunized chicken peritoneal cells was inhibited by adding supernatant of immune lymphocyte-cultures containing antigen.

In the present experiments, LIF activities were detected for 3 weeks by the direct technique, whereas 5 weeks by the indirect technique. The difference in these results might

be due to different sensitivities of both techniques. It has been reported that the immune state of chickens to coccidial infection exists for a few month [7, 8]. Findings presented here suggest that cell-mediated immunity plays a role in immune responses of chickens to coccidial infection, especially in the early stage of infection. However, the exact relationship between cell-mediated immunity and resistance of chickens against coccidial infection remains to be elucidated.

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要 約

Eimeria tenella 免疫ヒナにおける白血球遊走阻止反応: 大永博資・石井俊雄 (日本生物科学研究所) —ヒナの腹腔細胞を用いて, 直接法ならびに間接法による白血球遊走阻止試験によって *Eimeria tenella* に対するヒナの免疫を検討した. 直接法では, 腹腔細胞の遊走は第2代メロゾイトから調製された抗原の添加により阻止された. 白血球遊走阻止因子の活性は 50,000 のオーシスト投与後1週で検出され, 50,000 のオーシストを3回反復投与した後, 最終投与より3週間持続した. 免疫ヒナの脾細胞を抗原とともに培養した場合, 間接法において正常ヒナの腹腔細胞の遊走を阻止し得る因子が培養液中に産生された. 間接法では, 白血球遊走阻止因子の活性は 50,000 のオーシストの3回反復感染後5週まで検出された.