

Effects of Sulfamonomethoxine on Parasitemia, Serum Antigen and Antibody Production in Chickens Infected with *Leucocytozoon caulleryi*

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Introduction

Leucocytozoon caulleryi which is the causative agent of chicken leucocytozoonosis was discovered by Mathis and Legar (1909a), and in Japan by Akiba *et al.* (1958).

After the discovery of epidemic leucocytozoonosis in Japan, it was noticed that leucocytozoonosis has caused great damage to the productivity of chickens ; namely reduction of egg production, weight loss or sometimes cause of death. Akiba *et al.* (1963) (1964) (1968) tested pyrimethamine, sulfadimethoxine, sulfamonomethoxine and some other sulfonamides in cases of leucocytozoonosis, and the usefulness of these drugs was recognized by marked reduction of its outbreak. Prophylactic effects of sulfamonomethoxine had been determined by parasitemia, clinical findings and other methods.

Morii and Kitaoka (1970) reported the relationship between age of chickens and the acquisition of immunity to *L. caulleryi*. Morri (1972) suggested a refined method to determine effectiveness of drugs on *L. caulleryi* infection by monitoring parasitemia with combination of a serological assessment. He has shown that serum from chickens at the early stage of *L. caulleryi* infection formed precipitation in agar gel when added

with serum from chicken in the late stage of infection.

However, no exact report has ever documented on relationship between effectiveness of drugs and serological immune response in *L. caulleryi* infection. The present study was conducted in order to investigate the relationship. During administration of feeds containing varying concentrations of sulfamonomethoxine, chickens were inoculated with sporozoites of *L. caulleryi*. The production of serum antigens and antibodies was measured by a serological test developed by Morii (1972) besides the conventional monitoring of parasitemia. The concentration at which sulfamonomethoxine showed effective anti-*Leucocytozoon caulleryi* action is discussed from the interrelationship among parasitemia, serum antigens and antibodies. The concentration of sulfamonomethoxine at which chickens could gain immunity to *L. caulleryi* is also discussed based on the resulted observation on parasitemia, antigens and antibodies after reinfection.

Materials and Methods

Test groups were divided into 10 groups ; each group was respectively given feed containing 10, 20, 30, 40, 50, 75 and 100 ppm of sulfamonomethoxine, and 250 ppm of clopidol for consecutive 29 days from the 2nd day prior to the sporozoite inoculation.

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Afterwards, they were reared by the feed without any drug for an experiment of re-infection of *L. caulleryi*. The infected and non-infected groups were raised by the feed without any drug throughout the experimental period.

Five 21-day-old male White Leghorn chickens each takes from the 10, 20, 30, 40, 50, 75 and 100 ppm of sulfamonomethoxine groups, clopidol group, infected group, and non-infected group were subjected to this test.

The used strain of *L. caulleryi* was isolated from infected chickens in Shizuoka Prefecture in July, 1976 and successively cultivated in the laboratory. Biting midge which sucked the blood of chicken 21 days after exposed to *L. caulleryi* was kept in an incubator at 25 C for 3 days or longer to form sporozoites for next inoculation. The biting midge with sporozoites were ground down by a homogenizer containing Eagle MEM and chicken serum was added to suspend sporozoites, and the adjusted number of the sporozoites was subjected to this test. The number of sporozoites for the first inoculation to the 10, 20, 30, 40, 50, 75 and 100 ppm of sulfamonomethoxine groups, clopidol group, and infected group was 2×10^2 per chicken and for the second inoculation 2×10^3 sporozoites were intravenously injected.

Peripheral blood smears were prepared by ordinary methanol fixation followed by Giemsa staining. *L. caulleryi* parasites in the smears were counted under a microscopy to determine effects of the test drugs.

Presence of serum antigens originating from the second schizont in the chicken and the antigen titer were serologically examined in the serum of 14 days after sporozoites inoculation. Presence of antibodies and development of antibody titer were measured in the sera from chickens 24 days later since the inoculation of sporozoites.

The presence of antigens and the antigen titer were measured in the serum of 13 days after reinoculation of sporozoites and the presence of antibodies and the antibody titer in the serum of 23 days later to a com-

parative study of effects of the test drugs on the acquisition of immunity. These tests were performed by quantitative precipitation method carried out by Morii (1972).

Sera to be tested had been diluted in phosphate-buffered physiological saline (pH 7.2) to prepare two fold serial dilutions starting at 1:2. The titer was expressed as the reciprocal of the highest antigen on antibody dilution that had given a definite ring precipitation.

Results

Prophylactic effects of sulfamonomethoxine on *L. caulleryi* were studied by monitoring parasitemia and also by immunoserological test. The practical concentration of sulfamonomethoxine at which survived chickens produced immunity against reinoculation was determined.

Table 1 shows the results of parasitemia in peripheral blood from 14 days after the first inoculation of sporozoites. Merozoite and gametocyte were detected and clinical findings such as anemia and greenish feces were observed in the 10 and 20 ppm of sulfamonomethoxine groups. Neither merozoite nor gametocyte was demonstrated in the chicken groups given higher concentration of sulfamonomethoxine.

The presence of antigens in the serum of 14 days after the first inoculation of sporozoites is shown in Table 1 and the antigen titer in Fig. 1. Four chickens from the 50 ppm of sulfamonomethoxine group, 1 from the 40 ppm group and 2 from the 30 ppm group were negative, while all chickens from the 10 and 20 ppm of sulfamonomethoxine groups and the control group were positive. The 10 ppm of sulfamonomethoxine group showed antigen titer at 1:32 on the average. However, the titer tended to reduce with increase of the amount of sulfamonomethoxine added, and no antigen was detected in the sera from the 75 and 100 ppm of sulfamonomethoxine groups and the clopidol group.

The presence of antibodies in the serum

Table 1 Effects of sulfamonomethoxine on parasitemia, serum antigen and antibody production in chickens inoculated with *Leucocytozoon caulleryi*

	First inoculation of <i>Leucocytozoon caulleryi</i>				Reinoculation of <i>Leucocytozoon caulleryi</i>			
	Clinical findings Anemia and greenish stools	Parasitemia	Serum antigens 14 days after inoculation	Serum antibodies 24 days after inoculation	Clinical findings Anemia and greenish stools	Parasitemia	Serum antigens 13 days after reinocula- tion	Serum antibodies 26 days after reinocula- tion
Infected Control I	+	5/5 M.G	5/5	5/5	-	0/5 -	0/5	5/5
Infected Control II					+	3/3 M.G	3/3	3/3
Sulfamonomethoxine								
10 ppm	+	5/5 M.G	5/5	5/5	-	0/5 -	0/5	4/4*
20 ppm	+	3/5 M.G	5/5	5/5	-	0/5 -	0/5	5/5
30 ppm	-	0/5 -	3/5	4/5	-	0/5 -	0/5	4/5
40 ppm	-	0/5 -	4/5	5/5	-	0/5 -	0/5	5/5
50 ppm	-	0/5 -	1/5	4/5	+	2/5 M.G	2/5	5/5
75 ppm	-	0/5 -	0/5	0/5	+	5/5 M.G	4/5	4/4†
100 ppm	-	0/5 -	0/5	0/5	+	5/5 M.G	3/4†	4/4†
Clopidol 250 ppm	-	0/5 -	0/5	1/5	+	4/5 M.G	3/5	5/5
Non-infected Control	-	0/5 -	0/5	0/5	-	4/5 -	0/5	0/5

Number of Positive chickens/Number of test chickens

+ : Positive

- : Negative

* : Accidental death

† : Dead by reinoculation

M : Merozoites detected

G : Gametocytes detected

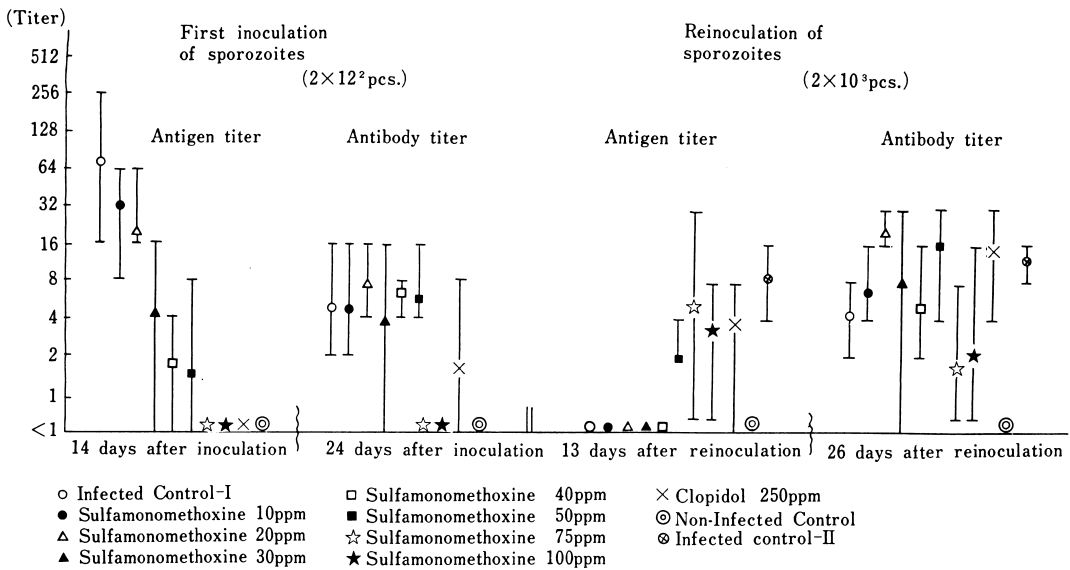


Fig. 1 Effects of sulfamonomethoxine on the development of antigen and antibody production in chickens inoculation with *Leucocytozoon caulleryi*.

Average titers of antigens and antibodies were indicated as geometric means.

24 days after the first inoculation of sporozoites is shown in Table 1 and the antibody titer in Fig. 1. All chickens given 75 and 100 ppm of sulfamonomethoxine, 1 from the 30 and 50 ppm groups and 4 from the clopidol group were negative, while all from the 10, 20 and 40 ppm of sulfamonomethoxine groups and the control group were positive. The antibody titer obtained in the 10, 20, 30, 40 and 50 ppm of sulfamonomethoxine groups were at the similar level to that in the control group, and the clopidol group showed a lightly lower titer than that in the control group. No antibody was detected in the 75 and 100 ppm of sulfamonomethoxine groups.

After *L. caulleryi* disappeared from the peripheral blood following the first inoculation of sporozoites, each group received a larger number of sporozoites than that in the first time. The results of parasitemia in the peripheral blood from the 13th day after the second inoculation are shown in Table 1. Neither merozoite nor gametocyte were observed in the 10, 20, 30 and 40 ppm of sulfamonomethoxine groups and the con-

trol group whose antibodies were positive in the first inoculation of sporozoites. However, merozoite and gametocyte were seen in the 75 and 100 ppm of sulfamonomethoxine groups in which *L. caulleryi* was completely prevented and no antibody was produced.

Table 1 shows the presence of serum antigens in the serum on the 13th day after reinoculation of sporozoites. Fig. 1 shows development of antigen titer. In the 10, 20, 30 and 40 ppm of sulfamonomethoxine groups and the control group in which antibodies were positive in the first inoculation of sporozoites, no antigen was observed in the serum on the 13th day after the reinoculation; therefore, it is presumed that *L. caulleryi* infection with reinoculation of sporozoites was not established. The 75 and 100 ppm of sulfamonomethoxine groups in which no antibody was produced by the first inoculation displayed antigens and infection due to reinoculation of sporozoites, just as parasitemia showed positive with reinoculation of sporozoites.

The presence of antibodies in the serum 26 days after reinoculation of sporozoites

is shown in Table 1 and the antibody titer in Fig. 1. The antibodies in the 10, 20, 30 and 40 ppm of sulfamonomethoxine groups and the control group have changed into positive by the first inoculation of sporozoites. These groups were given the feeds without sulfamonomethoxine from the 28th day after the first inoculation. And furthermore, their prolonged antibodies were detected after the reinoculation of sporozoites. The second inoculation of sporozoites have inhibited the infection for chickens with positive antibodies by the first inoculation of sporozoites. Therefore, dead chickens were not detected in these groups. While, the antibodies were negative in the 75 and 100 ppm of sulfamonomethoxine groups and the clopidol group even after the first inoculation of sporozoites. Those groups were given the feeds without drugs used from the 28th day after the first inoculation. As a result, the antibodies in those groups have changed into positive by the second inoculation of sporozoites. Dead chickens with infection were detected even by the second inoculation of sporozoites.

Discussion

The relationship between effects of sulfamonomethoxine on *L. caulleryi* and the acquisition of immunity to *L. caulleryi* was studied.

Akiba (1968) reported that sulfamonomethoxine had an prophylactic effect on *L. caulleryi* at the level of 50 ppm, however, the assessment was performed by clinical findings and parasitemia. The authors studied prophylactic effects of sulfamonomethoxine on *L. caulleryi* using an immunological method developed by Morii (1972) together with the above-mentioned methods, and a slight difference between the present results and the report by Akiba was observed.

The present examination was shown that chickens administered with sulfamonomethoxine were divided into the following three groups.

1) The 10 and 20 ppm of sulfamonomethoxine groups in which anemia and greenish

feces, parasitemia, serum antigens and antibodies were positive, and *L. caulleryi* infection was resisted when challenged by reinoculation of sporozoites.

2) The 30 and 40 ppm of sulfamonomethoxine groups in which clinical findings and parasitemia showed negative, serum antigens and antibodies being positive, and *L. caulleryi* infection being prevented reinoculation of sporozoite was attempted.

3) The 75 and 100 ppm of sulfamonomethoxine groups in which clinical findings, parasitemia, serum antigens and antibodies showed negative, and after reinoculation of sporozoites clinical findings such as greenish feces, anemia and parasitemia were positive in occasional death of chickens.

Infection was established by reinoculation of sporozoites and serum antigens and antibodies turned to positive.

In the 30 and 40 ppm of sulfamonomethoxine groups, parasitemia was not confirmed, but antigens and antibodies were observed in serum. It was assumed that *L. caulleryi* still localizing in some tissue in the chicken. It was considered, therefore, that prophylactic effects of the drug on *L. caulleryi* was reasonably measured when the immunological method was used together with parasite counting.

The prevention of *L. caulleryi* infection will be attained by adding 75 or 100 ppm of sulfamonomethoxine in the feed but the chickens do not gain any immunity to this disease. After withdrawal of sulfamonomethoxine, these chickens should be reinfected with *L. caulleryi* because they have not acquired immunity. Considering the possibility of repeating *L. caulleryi* infections under natural conditions, administration of 30 or 40 ppm of sulfamonomethoxine is recommended, from the fact that these amounts produce immunity to resist the first inoculation of *L. caulleryi* and reinoculation of sporozoites. It will be reasoned to apply this finding to practical use to produce immunity of *L. caulleryi* by drugging.

The produced antibodies persist and infection was resisted even when sporozoites

were inoculated. It is proposed, therefore, that paralleled assay of protective immunity which may be reflected by the development of antibody shown in the present study makes better estimation of prophylactic effects of drugs on *L. caulleryi*.

Onodera has been reported about the blood concentration of chickens, at the time of chickens given 0.2% of sulfamonomethoxine mixed with feed. However, the report on the relationship between the blood concentration and therapeutic effect of each concentration of sulfamonomethoxine mixed with feed, using chickens infected with *L. caulleryi* is not still published. Therefore, we are scheduled to study about them.

Summary

No exact report has ever documented on relationship between effectiveness of drugs and serological immune response in *L. caulleryi* infection. Therefore, to investigate this relationship, we have conducted the study as follows.

The 10 and 20 ppm of sulfamonomethoxine groups in which anemia and greenish feces, parasitemia, serum antigens and antibodies were positive, and *L. caulleryi* infection was resisted when challenged by the reinoculation of sporozoites.

The 30 and 40 ppm of sulfamonomethoxine groups in which clinical findings and parasitemia showed negative and serum antigens and antibodies being positive, and *L. caulleryi* infection was prevented by the reinoculation of sporozoites.

The 75 and 100 ppm of sulfamonomethoxine groups in which clinical findings, parasitemia, serum antigens and antibodies showed negative, and after the reinoculation of sporozoites, clinical findings such as greenish feces, anemia and parasitemia were positive in occasional dead of chickens. Therefore, the infection was established by the reinoculation of sporozoites. As a result, serum antigens and antibodies turned to positive.

Thus, the 30 or 40 ppm of sulfamonomethoxine administration is recommended, from the fact that these amounts produce immunity to resist the first inoculation of *L.*

caulleryi and reinoculation of sporozoites.

It will be reasoned to apply this finding to practical use to produce immunity of *L. caulleryi* by drugging.

The produced antibodies persist and the infection was resisted even when sporozoites were inoculated. It is proposed, therefore, that paralleled assay of protective immunity which may be reflected by the development of antibody shown in the present study makes better estimation of prophylactic effects of drugs on *L. caulleryi*.

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Sulfamonomethoxine の *Leucocytozoon caulleryi* 感染 鶏におけるパラシテミアと血清抗原ならびに抗体産生におよぼす影響

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鶏の *Leucocytozoon caulleryi* (以下 L. c.) 感染の予防には, pyrimethamine, sulfadimethoxine, sulfamonomethoxine 等が使用されてきた。これら予防薬の効果は主として末梢血液中への原虫出現の有無により判定されていた。そこで本実験では, 薬剤投与中の鶏に, sporozoite を人工的に接種し, パラシテミアと第2代の schizont に由来し, 血清中に産生される血清抗原およびその抗体産生, さらに免疫獲得性に対する供試薬剤の影響を比較検討した。

実験に供した L. c. 1976年7月, 静岡県下で分離し, 実験室で継代しているものを, 試験鶏は21日齢の白色レグホン系の雄を各群5羽ずつ使用した。

試験群は, sulfamonomethoxine (SMM) 10, 20, 30, 40, 50, 75, 100 ppm, Clopidol (cp) 250 ppm の群を設定し, 薬剤無添加中雛用飼料にそれぞれ配合し, sporozoite 接種2日前から, 29日間連続して与えた。鶏血清加 Eagle MEM 液に浮遊させた sporozoite を1羽当り 2×10^8 個ずつ静脈内に接種した。

Sporozoite, 初回接種後14日目からのパラシテミアは, SMM 10, 20 ppm 群では merozoite および gametocyte が検出され, 貧血, 緑便等の臨床所見も認められた。SMM, 30, 40, 50, 75, 100 ppm 群では merozoite, gametocyte の出現を認めなかった。

Sporozoite, 初回接種後の抗原は, SMM 50 ppm 群では 4/5 羽, 40 ppm 群は 1/5 羽, 30 ppm 群は 2/5 羽が陰性で, 10, 20 ppm 群, 対照群では全例が陽性であった。抗原価については, SMM 10 ppm 群は平均32倍を示したが, SMM 75, 100 ppm 添加群と Cp 群は抗原が検出されなかった。

Sporozoite 初回接種後, 24日目の抗体については, SMM 75, 100 ppm 群は, 全例が陰性であったが, Cp 群は 4/5 羽が陰性, SMM 30, 50 ppm 群は 1/5 羽が陰性, 10, 20, 40 ppm 群と対照群においては全例が陽性であった。SMM 10, 20, 30, 40, 50 ppm 群では, 対照

群とほぼ同程度の抗体価が得られ, Cp 群では対照群よりやや低い値を示した。しかし, SMM 75, 100 ppm 群では, 抗体が全く検出されなかった。

初回の sporozoite を接種してから, 血液から原虫が消失した後, 各群の鶏に1羽当り sporozoite 2×10^8 個を再接種した。パラシテミアの結果については, 初回の sporozoite 接種で抗体が陽性になった SMM 10, 20, 30, 40 ppm 群と対照群では, 再接種後, merozoite および gametocyte の出現は認められなかった。しかし, 75, 100 ppm 群のように完全に L. c. をおさえてしまい, 抗体が産生されなかった群では, sporozoite 再接種により, merozoite および gametocyte が検出された。

初回の sporozoite 接種で抗体が陽性になった SMM 10, 20, 30, 40 ppm 群と対照群においては, 再接種後13日目の血清抗原は認められず, 再接種による感染は成立しなかったと考えられる。しかし, 初回の接種により, 抗体の産生されなかった SMM 75, 100 ppm 群は再接種によりパラシテミアが陽性を示したのと同様に抗原も出現し, sporozoite 再接種をした為の感染が認められた。

Sporozoite 初回接種により抗体を産生した SMM 10, 20, 30, 40 ppm 群, 対照群等では持続した抗体を持っていたが, SMM 75, 100 ppm 群, Cp 群等では, sporozoite 再接種時の感染により産生された抗体が認められた。また, 初回の sporozoite 接種により抗体が産生されなかった SMM 75, 100 ppm 群では, sporozoite の再接種後, 感染を阻止する抗体がないため死亡鶏が認められた。

以上の成績から, 本症に対する薬剤の効果は, 感染鶏におけるパラシテミア, 血清抗原抗体産生の有無, 免疫獲得性等について, 比較検討することにより適確な判定ができるものと考えられる。