Studies on Feline Haemobartonellosis II. The Mechanism of Anemia Produced by Infection with *Haemobartonella felis*

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Abstract. The mechanism of anemia in feline haemobartonellosis was examined with 15 cats as experimental animals. *Haemobartonella felis* appeared on erythrocytes 3 to 18 days, or 6.7 days on an average, after inoculation. The erythrocyte count, hematocrit and hemoglobin decreased in value in the 15 cats over a period of appearance of *H. felis* on erythrocytes. They tended, however, to return gradually to their previous levels immediately after the disappearance of *H. felis* from erythrocytes.

On the contrary, the osmotic fragility of the erythrocyte showed a marked increase immediately following the appearance of *H. felis*, but it showed no recovery even when the parasite disappeared from erythrocytes. The direct Coombs test became positive in all the animals for 15 days on an average after the first appearance of *H. felis* on erythrocytes. Erythrophagocytosis by mononuclear cells or rarely by neutrophils was observed in blood smears of 11 animals for 19.5 days on an average from the first appearance of *H. felis*.

From these results, it is conjectured that parasitized erythrocytes may probably be sequestered by reticuloendothelium of the spleen or some other organ, but that some of them may return soon to the circulating blood by getting free from the parasite in spite of some damage caused by the parasite. What damage *H. felis* does to erythrocytes will be studied in future.

In 1971, the occurrence of a clinical case of feline haemobartonellosis was recognized for the first time in Sapporo by the authors. Additional five cases of the disease were found in the next year. The clinical signs and hematological findings of these six cases have been already reported [2]. Especially, of the hematological findings, an increase in osmotic fragility of erythrocytes, positivity of the direct Coombs test, and erythrophagocytosis by mononuclear cells in the peripheral blood smear are of great interest to the authors in view of helping to unravel the mechanism of anemia produced by infection with *Haemobartonella*. Such mechanism has never adequately been elucidated,

though some papers have been published to deal with it [1, 3, 7].

This study was carried out in order to find a solution for this problem on the basis of experimental findings.

Materials and Methods

1. Animals

Seventeen healthy cats kept in Sapporo city were used for this study. Of them, 7 cats were male and 10 female, and 12 were 3 months old and the others 2 to 5 years old. Two of them, 3 months old, a male and a female, served as controls. All the cats were kept under sanitary conditions for over 2 weeks before experimental work began, during which time clinical and hematological examinations were made at six-day intervals.

2. Inoculation

猫へモバルネトラ症に関する研究 II. 猫へモバルネトラ症における貧血の発生機序について: 前出吉光・秦良治 (北海道大学獣医学部家畜内科学教室)

Table 1. Data on 15 cats observed for 30 days after inoculation

Cat No.	Age	Sex	<i>H. felis</i> (Days until positive)	Anemia (Days until anemic*)	Erythrocyte osmotic fragility (Days until increased**)	Coombs test (Days until positive)	Erythrophage in peripheral blood (Days until positive)
1	3 months	male	10	16	12	28	/
2	3 months	male	18	20	20	28	/
3	3 months	male	7	18	12	20	20
4	3 months	female	17	19	20	24	/
5	3 years	female	7	12	8	24	25
6	2 years	male	5	13	12	24	29
7	5 years	female	7	9	16	24	30
8	3 months	male	3	4	4	20	29
9	3 months	male	3	5	4	16	21
10	3 months	male	3	5	4	28	20
11	3 months	female	3	5	4	16	19
12	2 years	female	3	4	8	16	27
13	3 years	female	4	5	4	16	17
14	4 years	female	6	13	8	20	/
15	5 years	female	4	7	8	20	27

Remarks.

* : Days until Hb value become at least 2 gm/100 ml smaller than the preinoculation value.

** : Days until hemolysis was seen in 0.75% saline concentration.

Blood from cats with clinical feline haemobartonellosis was heparinized to prevent coagulation. All the 15 cats were injected intraperitoneally with 1 ml of the blood as soon as possible.

3. Observation

a. RBC, Ht, Hb, WBC and blood smear were examined every day at 9:00 AM for 30 days after inoculation. Blood samples were collected by venesection of the auricular vein. The blood was first allowed to flow out freely and then collected directly into pipets, which were used for blood cell counting and hemoglobin determination, and into a microhematocrit capillary tube. Blood smears were also prepared at the same time. Hematological techniques were performed by the standard methods. Blood smears were stained with Giemsa stain and examined microscopically for *Haemobartonella felis* and erythrophagocytosis. Parasitemia was graded as follows.

- + : 30% or less of the erythrocytes were infected with *H. felis*.
- ++ : 30% to 60% of the erythrocytes were infected with *H. felis*.
- +++ : more than 60% of the erythrocytes were infected with *H. felis*.

The osmotic fragility test of erythrocytes and Coombs test were carried out at three-day intervals. Blood collected from the jugular vein was heparin-

ized and used for these tests.

Osmotic fragility was determined by adding 0.05 ml of blood to 5.0 ml of buffered NaCl solution in concentrations ranging from 0.1 to 1.0%. The contents were mixed and allowed to stand at room temperature for 30 minutes. The tubes were then centrifuged at 2,000 rpm for 10 minutes. The hemoglobin content of the supernatant fluid was determined spectrophotometrically at 540 m μ with 1% NaCl solution contained in a tube as blank supernatant fluid. The percentage of hemolysis was calculated to hemolysis in 0.1% NaCl solution assumed as 100%.

The direct Coombs test was carried out as follows. A blood sample was washed four times with 0.9% NaCl solution and made a 50% cell suspension in 0.9% NaCl solution. One drop of the cell suspension was mixed with one drop of rabbit anti-cat globulin serum on a clear slide glass. The mixture was allowed to stand at room temperature for ten minutes and then examined macroscopically for hemagglutination. A control test was conducted in the same manner, except that 0.9% NaCl solution was used instead of rabbit anti-cat globulin serum.

Anti-cat globulin serum was obtained from rabbits which had been injected with 1 ml of a 2% cat globulin solution into the auricular vein five times at three-day intervals. Serum was prepared from

Fig. 1. Changes in peripheral blood of a cat (No. 8) infected with *H. felis* Fig. 2. Changes of erythrocyte osmotic fragility in 15 cats infected with *H. felis*

Remarks.

- ▨ : Osmotic fragility curve for 15 cats before inoculation.
- ▩ : Osmotic fragility curve for 15 cats after the first appearance of *H. felis*.
- ▧ : Osmotic fragility curve for 15 cats after the second appearance of *H. felis*.

the blood collected 10 days after the last injection, inactivated at 56°C for 30 minutes, and then absorbed three times by washed normal feline erythrocytes. The absorbed serum agglutinated a 2% cat globulin solution diluted 1:64 in physiologic saline solution, but not normal feline erythrocytes washed four times with 0.9% NaCl solution.

b. For the purpose of finding the relationship between parasitemia and erythrocyte destruction, 7 cats were examined repeatedly for RBC, Hb, Ht, and *Haemobartonella* in blood smears once during the experimental period. Examinations were started at 10:00 AM and continued at two-hour intervals for 16 or 20 hours.

Results

1. Hematological findings for 30 days after inoculation

The results obtained from the 15 cats are presented in Table 1.

Haemobartonella felis: All the inoculated cats had positive blood smears over a period of 3 to 18 days, or 6.7 days on the average. Thereafter, *H. felis* appeared on erythrocytes at intervals of 1 to 10 days, or 2.6 days on the average. Parasitemia continued over a period of 1 to 13 days, or 3.1 days on the average, during the experimental period.

RBC, Ht and Hb: The 15 cats showed a decrease in value of RBC, Ht and Hb over the period of appearance of *H. felis* on

Table 2. Relationship between parasitemia and Hb level in 7 cats
(Results of observation at two-hour intervals)

Cat No.	O'clock	10:00	12:00	14:00	16:00	18:00	20:00	22:00	24:00	2:00	4:00	6:00	10:00
2		*6.3	6.3	6.3	6.0	6.1	5.4	4.5	4.3	4.8			6.3
		**++	++	++	++	++	+	+	—	—			—
5		7.1	6.8	7.0	7.0	7.0	6.5	6.1	5.0	5.6	5.8		6.6
		+++	+++	+++	+++	+++	+++	+++	++	—	—		—
6		9.9	8.1	7.8	6.7	6.0	5.5	6.1	6.3	6.8	—		7.6
		++	++	++	++	++	+	—	—	—			—
9		9.7	9.5	8.9	8.0	7.5	7.3	6.7	6.7	6.9			8.1
		++	++	++	++	++	++	+	—	—			—
12		10.1	10.0	10.2	10.4	9.9	10.4	9.4	9.5	9.8	10.6		10.6
		+	+	+	+	+	+	++	+	—	—		—
13		7.3	7.4	7.3	7.9	7.3	7.3	6.6	8.1	7.8	7.6		8.6
		++	++	++	+++	+++	+++	+	—	—	—		—
14		8.1	7.7	7.6	7.7	7.5	7.0	5.8	5.5	5.4	5.6	6.0	6.5
		++	++	++	++	++	++	+++	+++	++	—	—	—

Remarks.

*: Hb value (g/100 ml).

**: *H. felis* on erythrocytes.

+: 30% or less of the erythrocytes were infected.

++: 30% to 60% of the erythrocytes were infected.

+++: More than 60% of the erythrocytes were infected.

erythrocytes (Fig. 1). In 10 cats (Nos. 2, 4, 7–13 and 15), the Hb value was at least 2 g/100 ml smaller than that determined in the preinoculation over a period of 1 to 3 days, or 1.8 days on the average, after the first appearance of *H. felis* on erythrocytes. In the other 5 cats, the Hb value was 2 g/100 ml smaller over a period of 5 to 11 days, or 7.4 days on the average.

RBC, Ht and Hb values in each of the 15 cats showed increase and decrease repeatedly according to the disappearance and appearance of *H. felis* on erythrocytes, reaching the lowest level over a period of 15 to 27 days, or 22.3 days on the average, after the first appearance of *H. felis* during the experimental period. The average lowest values of the 15 cats are as follows: RBC, 2,150,000 within a range of 540,000 to 4,120,000; Ht, 12.5% within a range of 4 to 21.5%; and Hb, 3.8 g/100 ml within a range of 1.2 to 6.2 g/100 ml.

WBC: WBC did not change significantly in any cat.

Osmotic fragility: The changes of erythrocyte osmotic fragility in the 15 cats are shown in Fig. 2. Osmotic fragility increased following the appearance of *H. felis* on erythrocytes. The average of hemolysis in 0.75% saline concentration was 8.7%. Such hemolysis was within a range of 0 to 27%, over a period of 2.1 days on the average after the first appearance of *H. felis*. It was not seen in 0.75% saline concentration in the preinoculation period. It was within a range of 6.2 to 69%, or 37% on the average, over a period of 2.9 days on the average after the second appearance. It was within a range of 44 to 88%, or 69.5% on the average, over a period of an average of 2.1 days after the third appearance. During the experimental period, a marked increase in osmotic fragility was seen in the 15 cats over a period of 10 to 26 days, or 16.7 days on the average, after the first appearance of *H. felis*. Hemolysis in 0.75% saline concentration was within 36.8 to 95.9%, or 76.4% on the average, over that period.

Direct Coombs test: The direct Coombs test became positive in all the inoculated cats over a period of 7 to 25 days, or 15 days on the average, after the first appearance of *H. felis*. The positive test persisted in all the cats to the end of the experimental period, except 3 cats which showed positivity only once during the experimental period.

Erythrophagocytosis: In 11 cats, erythrophagocytosis by mononuclear cells or rarely by neutrophils was observed in blood smears stained with Giemsa stain over a period of 13 to 26 days, or 19.5 days on the average, after the first appearance of *H. felis*.

Two cats which served as controls showed no significant hematological changes.

2. Findings at two-hour intervals

Seven cats (Nos. 2, 5, 6, 9, 12–14) were used for this experiment. The results are shown in Table 2. RBC, Ht and Hb generally decreased in value over the period of appearance of *H. felis* on erythrocytes and increased following the disappearance of the parasite. In 5 cats (Nos. 2, 5, 6, 9 and 14), Hb decreased by 2 to 4.4 g/100 ml, or 2.9 g/100 ml on the average, over a period of 10 to 16 hours after the beginning of observation. It increased, however, by 1.1 to 2.1 g/100 ml, or 1.6 g/100 ml on the average, over a period of 4 to 12 hours after the disappearance of the parasite.

Discussion

The mechanism of anemia in haemobartonellosis has never been elucidated. In their electron microscopical studies of *H. felis*, Small and Ristic [6] described that *H. felis* seemed to penetrate or erode the erythrocyte partly at the point of its attachment. Their findings seem to suggest that the anemia of *H. felis* infection may be caused by the direct action of the parasite over the erythrocyte. Simpson et al. [4] reported, however, that *H. felis* invaded the

host cell by rhopheocytosis. Simpson and Love [5] also found in their electron microscopical studies of *H. bovis* that there was no ultrastructural evidence for hemolysis of erythrocytes associated with severe parasitemia. Venable and Ewing [7] described that *H. canis* appeared to grow, without rupturing the plasmalemma of the host erythrocyte, and that erythrophagia seemed to remain as the primary factor in the resulting anemia.

The present experiments revealed the decrease of erythrocytes, increase of erythrocyte osmotic fragility, positivity of the direct Coombs test, and appearance of erythrophages in the peripheral blood. These findings were the same as observed in clinical cases [2]. From the results of the present experiments, it is elucidated that erythrocytes in an infected cat decrease rapidly following the appearance of *H. felis* on them, but that they tend to return gradually to the previous level after the disappearance of the parasite from the host cell. Especially, from the data given in Table 2 and Fig. 1, it appears that the recovery of the erythrocyte count occurs immediately after the disappearance of *H. felis* from erythrocytes.

On the contrary, osmotic fragility increased markedly after the first appearance of *H. felis*. It increased continually, without recovery, even when the parasite disappeared from erythrocytes. From these results, it is conjectured that parasitized erythrocytes may probably be sequestered by the reticuloendothelial system in the spleen or some other organ, that some of them may soon return to the circulating blood, being freed from the parasite, that these erythrocytes may suffer some damage by the parasite, and that as the result of the damage, they may increase in osmotic fragility. Osmotic fragility showed a further increase in accordance with the frequent appearance of *H. felis* on erythrocytes. The fact seems to

be a result of increase of damaged erythrocytes in the blood, which may lead to the production of anti-erythrocyte antibody and also the occurrence of phagocytosis by macrophages. Thus it is probable that anemia may proceed to a severer stage.

Kreier and Hall [1] studied the relationship between erythrocyte survival and *H. muris* infection by carrying out an experiment on transfusion of Cr⁵¹-labeled blood from *H. muris*-infected rats to uninfected ones. From the results of their experiment, they indicated that the actual loss of erythrocytes from the *H. muris*-infected blood was less than the expected loss when all the erythrocytes parasitized with *H. muris* were irreversibly damaged. They concluded that all the parasitized erythrocytes were not irreversibly damaged. Their results seem to lend support to the authors' hypothesis that some of the parasitized erythrocytes may return to the circulating blood after being freed from the parasite, though affected with some damage.

What damage *H. felis* does to erythrocytes will be studied in future.

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