Decrease in Erythrocyte Survival in *Theileria sergenti*-Infected Calves Determined by Non-Radioactive Chromium Labelling Method

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ABSTRACT. Pathogenesis of anemia in the calves infected with *Theileria sergenti* was investigated from the viewpoint of erythrocyte survival decrease in the circulating blood. For investigation of erythrocyte survival a method of erythrocyte labelling with non-radioactive chromium (⁵⁰Cr) was utilized. It was found that (1) the erythrocyte survival decreased markedly in the *T. sergenti*-infected calves compared with that in the uninfected calves; the survival rate of 25.7% for infected calves and 86.0% for uninfected ones on the fourth day after re-introduction of the labelled erythrocytes into the original donors, and that (2) the survival of non-parasitized erythrocytes in the infected calves was also decreased, which indicates no obvious relationship between parasitism and decrease in survival of erythrocytes.—**KEY WORDS:** anemia, ⁵⁰Cr, erythrocyte, survival, *Theileria sergenti*.

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Bovine theileriosis due to *Theileria sergenti* is prevalent among cattle on pastures in Japan. The affected cattle manifests symptoms represented by anemia, resulting in the decrease in daily gain, loss of body weight, and death in the severest case. From this reason the disease is considered to cause economic loss in the animal husbandry which raises cattle on pastures in this country [3]. However, there have been no fundamental countermeasures developed so far. Elucidation of anemia pathogenesis will be the prerequisite for development of the fundamental countermeasures against *T. sergenti* infection.

As a part of study for elucidating pathogenesis of the anemia, the survival of erythrocytes *in vivo* was examined in the present study by using the method of erythrocyte labelling with non-radioactive chromium [2, 5], which had been developed for the examination of erythrocyte survival in the human patients who were unsuitable for the utilization of radioisotope.

MATERIALS AND METHODS

Experimental animals: Five Japanese Black calves, which were aged 5–6 months and proved not to be infected with *T. sergenti*, were used in the present experiment. Three calves (Nos. 28, 29 and 30) were infected with Ikeda stock of *T. sergenti* through infestation by 150 infected nymphal *Haemaphysalis longicornis* ticks. Another 2 calves (Nos. 26 and 27) were infested by 150 uninfected

nymphal *H. longicornis*. All the calves were raised, until the cessation of experiment, individually in each pen which was free from *H. longicornis*.

Routine blood examination : Number of erythrocytes, packed cell volume (PCV), hemoglobin concentration and percentage of *T. sergenti*-parasitized erythrocytes in Giemsa-stained blood smears were examined at 1–3 day intervals starting from the infestation of calves by ticks until the cessation of the experiment.

Preparation of ⁵⁰Cr-labelled erythrocytes: The enriched stable isotope ⁵⁰Cr, obtained from Oak Ridge National Laboratory, was converted to Na₂⁵⁰CrO₄ according to the report of Kunugiyama *et al.* [4]. The Na₂⁵⁰CrO₄ was dissolved in physiological saline solution and adjusted to give the concentration of 20 µg ⁵⁰Cr/ml.

Re-introduction of ⁵⁰Cr-labelled erythrocytes into the original donors: Approximately 100 ml of the blood was collected at the critical period of parasitemia (28 days post tick infestation; 28 days *pti*) from infected calves and also from uninfected calves, simultaneously. The blood samples were anticoagulated by addition of acid citrate dextrose. They were then mixed with the Na₂⁵⁰CrO₄ saline solution in the ratio of 1 to 10, and incubated for 90 min at room temperature, with frequent gentle stirring, for ⁵⁰Cr-labelling. The bloods were then centrifuged for 15 min at 4°C and plasmas were discarded. Physiological saline solution was added to the sedimented blood cells to restore the original volume. The blood suspensions were re-introduced into the original

donor calves. Each calf was bled 1 day after re-introduction and at 3 days intervals, thereafter. The blood samples were centrifuged for 15 min at 4°C. The deposited blood cells were freeze-dried, sealed in polyethylene sheet bags and stored frozen until neutron irradiation.

Fractionation of ^{50}Cr -labelled erythrocytes: A portion (2 ml) of ^{50}Cr -labelled blood suspension for re-introduction was removed and subjected to Percoll-Conray density gradient centrifugation [9]. The fractions, rich or poor in parasitized erythrocytes, were collected for radioactivity comparison.

Neutron irradiation and radioactivity measurement: The method of thermal neutron irradiation and gamma ray measurement is described in the previous report of Kunugiyama *et al.* [4]. Briefly, the ^{50}Cr -labelled blood samples, which were kept sealed in polyethylene bags, were irradiated by thermal neutron in a reactor at Japan Atomic Energy Research Institute. Radioactivities due to ^{51}Cr and ^{59}Fe , which were produced by neutron irradiation, were measured by a germanium detector with a gamma-ray spectrometer and a pulse-height analyzer. The ratio of $^{51}\text{Cr}/^{59}\text{Fe}$ counts was calculated by photoppeak area of gamma-ray.

RESULTS

Hematological changes detected by the routine blood examination on *T. sergenti*-infected calves: Some hematological changes were detected by the routine blood examination on *T. sergenti*-infected calves. As shown in Fig. 1, *T. sergenti*-parasitized erythrocytes began to appear in the peripheral blood of the infected calves 14–16 days *pti* and became most abundant 30–32 days *pti* to give parasitemia of 2.9–10.8% (an average of 7.8%). PCVs, erythrocyte counts, and hemoglobin concentrations began to decrease 10 days *pti* and gave the minimum value 28–30 days *pti*, followed by a gradual recovery of PCV and hemoglobin concentration. Number of erythrocytes, on the other hand, did not recover within the experimental period of 50 days *pti*. In uninfected calves there were no obvious changes detected until the cessation of the experiment.

Decrease in survival in vivo of ^{50}Cr -labelled erythrocytes: Figure 2 shows the survival of ^{50}Cr -labelled erythrocytes in circulation of the *T. sergenti*-infected and -uninfected calves. The survival rate was expressed by the value of $^{51}\text{Cr}/^{59}\text{Fe}$ according to the procedure described by Drysdale *et al.* [2]. The

Fig. 1. General hematological changes in *Theileria sergenti*-infected and -uninfected calves. Shown are the mean values of 3 infected and of 2 uninfected calves, respectively. ○ = PCV, ● = number of erythrocytes, △ = hemoglobin concentration, ▲ = degree of parasitemia. Arrows show the day when ^{50}Cr -labelled erythrocytes were re-introduced into respective donor calves.

erythrocyte survival in the infected calves decreased significantly 4 days after re-introduction of the labelled erythrocytes up to the value of 25.7%. The decrease in this period was approximately 3.3 times greater in the infected calves than in the uninfected ones. From the day 4 on, the survival rate in the infected calves decreased gradually until almost all the ^{50}Cr -labelled erythrocytes disappeared from circulating blood at the day 16.

Responsibility of both the parasitized and non-parasitized erythrocytes for decrease in erythrocyte survival in circulation of infected calves: In order to determine what kind of erythrocytes, *i.e.* parasitized erythrocyte, non-parasitized one or both of them, is responsible for the decreased erythrocyte survival in circulation of infected calves, radioactivity of parasitized cell-rich fractions and that of non-parasitized cell-rich fractions were compared. The ^{50}Cr -labelled erythrocytes of calf No. 28 were fractionated, prior to re-introduction, to produce the erythrocyte fractions which were different in percentage of parasitized cells, and then radioactivity of these fractions was compared.

Fig. 2. Survival of ^{50}Cr -labelled erythrocytes in circulation of *Theileria sergenti*-infected and -uninfected calves. Survival rate was expressed by the value of $^{51}\text{Cr}/^{59}\text{Fe}$ ratio. The rate at each day was calculated in proportion to the rate 100% at the day 1. $\circ$, $\square$ and $\triangle$ = infected calves (Nos. 28, 29 and 30, respectively), $\bullet$ and $\blacktriangle$ = uninfected calves (Nos. 26 and 27, respectively).

The result is shown in Fig. 3. The fraction No. 10, which was most abundant of parasitized erythrocytes, did not show the highest radioactivity, while the fraction No. 4, which was rich in non-parasitized cells and poor in parasitized ones, did show the highest radioactivity. It was found, therefore, that the non-parasitized erythrocytes could also be labelled with ^{50}Cr and that both the parasitized and non-parasitized erythrocytes are responsible for the decreased survival of erythrocytes in blood circulation of the infected calves.

Furthermore, hematological examination on erythrocyte population in the blood used for ^{50}Cr -labelling revealed rare presence of abnormal erythrocytes such as reticulocytes and/or macrocytes, which indicated little concern of such abnormal erythrocytes with the decrease in erythrocyte survival in the present examination.

DISCUSSION

It was demonstrated first in the present study that the survival of erythrocytes in the circulating blood of *T. sergenti*-infected calves decreased more rapidly than that of uninfected ones and also that the decrease occurred not only in parasitized erythrocytes, but also in non-parasitized ones. The reason for this survival decrease is not clear yet.

Two possible reasons can be considered for cause of the survival decrease; intrinsic defects of erythrocyte itself and extrinsic influences on erythrocytes. Some hereditary diseases like thalassemia, erythrocyte enzymopathy, hemoglobinopathy or spherocy-

Fig. 3. Radioactivity of the blood fractions, rich or poor in *Theileria sergenti*-parasitized erythrocytes. a) Radioactivity was expressed by 10^{-2} values of $^{51}\text{Cr}/^{59}\text{Fe}$ ratio of each fraction. $\circ$ = $^{51}\text{Cr}/^{59}\text{Fe}$ ratio, $\triangle$ = parasitized cells.

tosis are categorized into the intrinsic defect. On the other hand, influences of parasitism, malfunction in vascular endothelium like disseminated intravascular coagulation (DIC), non-specific acceleration of erythrophagocytosis, denaturing of blood plasma, etc. are categorized into the extrinsic influence.

In case of *T. sergenti* infection it is hard to consider that the erythrocyte survival decrease is due to the intrinsic defects of erythrocytes because erythropoietic function in the *T. sergenti*-infected calves seems not to be damaged [8].

Extrinsic influences on erythrocytes seem to be the most probable reason for erythrocyte survival decrease in this infection. Influence of parasitism was denied by the fact that even non-parasitized erythrocytes shortened their life. Influences of malfunction in vascular endothelium and non-specific acceleration of erythrophagocytosis seem not to be of the primary importance, because they have not been observed in *T. sergenti* infection so far.

The most probable reason for erythrocyte survival decrease seems to be the influence of denatured blood plasma, because the evidence suggesting denaturing of blood plasma has been reported for another protozoan infections [1, 6, 7].

Rosenberg *et al.* [7] reported on production of the antierythrocyte antibody in blood plasma and responsibility of the antibody for decreased erythrocyte survival *in vivo* in *P. falciparum* infection. In *T. sergenti* infection, however, production of such antibody has not been reported so far. Further study will be needed for demonstration of the antibody.

On the other hand, Clark *et al.* [1] reported on production of oxygen-derived free radicals *in vivo* in *Plasmodium* infection. In addition, Quinn and Wyler [6] reported on decrease in erythrocyte survival induced by intravenous administration of the free radicals. The finding in the experiment performed by one of the present authors and his associates [10] also suggests a possibility of free radical involvement in *T. sergenti* infections. Further studies are needed for demonstration of free radical involvement and elucidation of the reason for erythrocyte survival decrease in *T. sergenti* infection.

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