Separation of Bovine Erythrocytes Infected with *Theileria sergenti* by “Percoll-Conray” Density Gradient

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**ABSTRACT.** “Percoll-Conray” density gradient centrifugation was applied to separate *Theileria sergenti*-parasitized erythrocytes obtained from three *T. sergenti*-infected splenectomized calves. Administration of dexamethasone was effective in increasing the number of parasitized erythrocytes in *T. sergenti* infected calves, and the mean parasitemia reached 39.9% within 22 days after commencement of dexamethasone treatment. A significant increase in the number of parasitized cells was observed in the low dense fraction following the density gradient centrifugation and reached an average of 2.1 times (maximum percentage of parasitized cells: 64.8%) the pre-centrifuged number of the parasitized cells.---**KEY WORDS:** cattle, erythrocyte, percoll, separation, *Theileria sergenti*.

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Bovine theileriosis, which is caused by an intraerythrocytic protozoan *Theileria sergenti*, is recognized as the most serious disease acquired on pastures in Japan, producing enormous damage to grazing cattle. The main symptom of cattle infected with this disease is serious anemia due to intraerythrocytic parasitism by the protozoan. At present, little is known about the biochemical properties of bovine erythrocytes infected with *T. sergenti*. For biochemical analysis of *T. sergenti*-parasitized erythrocytes, blood showing a high rate of the infection is required. But, the *T. sergenti* infection rate of erythrocytes of bovine theileriosis in field cases is not high. This may be one of the reasons why biochemical studies on *T. sergenti* have not been reported in the past.

Recently, the density centrifugation method has been employed for the separation of parasitized erythrocytes from the blood in some mammalian malarial infections using a variety substances, such as Ficoll [3], Strachten II [4] and Percoll with Hypaque [2] and furthermore, Takahashi *et al.* [8] have observed an enhanced recrudescence of infection in *T. sergenti*-recovered splenectomized calves after treatment with a synthetic corticosteroid.

In this paper, therefore, dexamethasone was used to enhanced erythrocyte infection rate in combination with the density gradient centrifugation method in an attempt to separate *T. sergenti*-parasitized erythrocytes from infected bovine blood.

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**MATERIALS AND METHODS**

**Experimental animals:** Three clinically normal calves (Nos. B-1, 5 and 6), aged 4 to 12 months, were used in this experiment. To prevent any contact with parasite-carrying ticks, the calves were separated from their dams immediately after birth and maintained individually in isolated accommodation. Thereafter, the calves were splenectomized. To prove that the calves were parasite-free, peripheral blood smears were tested regularly by Giemsa staining and indirect fluorescent antibody assay (IFA) before use. The IFA was carried out accord-
ing to the method of Fujinaga and Minami [1], using Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-bovine IgG following absorption of each anti-T. sergenti, Babesia ovata, Anaplasma centrale, or A. marginale bovine serum. These anti-sera were kindly supplied by Dr. T. Minami, the National Institute of Animal Health, Japan.

Parasite strain and inoculation: The Fukushima strain of T. sergenti supplied by Dr. T. Minami was inoculated subcutaneously into the splenectomized calves. The doses of inoculation for B-1, 5 and 6 were 12.8×10^8, 19.2×10^8, and 16.5×10^8 parasitized erythrocytes, respectively.

Dexamethasone treatment: Splenectomized calves infected with T. sergenti were treated intramuscularly with commercial dexamethasone (DEXAMETHASONE INJ. Nippon Zenyaku Co., Koriyama, Fukushima, Japan) from 13–57 days post inoculation. The doses used were 0.15–0.30 mg/kg of body weight, and the treatment was continued for 2–3 weeks. Two calves (Nos. B-5 and 6) were treated with one series of dexamethasone injections, and the remaining calf (No. B-1) was treated three times, once during the initial parasitemia and on two further occasions after the initial parasitemia had declined.

Routine blood examination: Erythrocyte and reticulocyte counts, packed cell volume (PCV), hemoglobin concentration and the number of T. sergenti-parasitized erythrocytes by Giemsa staining were examined routinely in blood samples taken at 1–2 day intervals after inoculation to evaluate the parasitemia of the calves.

Density gradient centrifugation: Blood samples used in the density gradient were heparinized. On the basis of the routine blood examinations, these blood samples were collected at three different stages of parasitemia, i.e., Stage 1 (at the beginning of the dexamethasone injection regime), Stage 2 (during the rising dexamethasone enhanced parasitemia) and Stage 3 (at the end of the dexamethasone injection regime), respectively (Fig. 1). Fifty ml of heparinized blood were collected each time and centrifuged at 3,000 rpm for 15 min., at 4°C. After the plasma was removed, the pellets were suspended in 50 ml of ice-cold phosphate buffered saline solution (PBS). The suspension was centrifuged at 3,000 rpm for 15 min. at 4°C, and the supernatant was removed again. The pellet was washed 3 times in cold PBS, and then the cell number was adjusted to 6–7×10^9/mm^3 in PBS. The contaminated leukocytes in the blood samples were removed as follows by the method of Nakao et al. [6]. Twenty-five ml of a standard concentration of cells in PBS were loaded into a glass column, 3 cm diameter, which was packed into a 9 cm length with a 1:3 v/v mixture of Sulphoethyl cellulose (SE-23 Serva, Heidelberg, West Germany) and Sephadex G-25 (medium grade, Pharmacia, Uppsala, Sweden), the mixture was equilibrated with 5 mM MgCl_2 containing PBS (Mg-PBS). After the cells entered the column, an additional 25 ml of Mg-PBS were applied. Eluted erythrocytes were again washed by cold PBS and adjusted to approximately 10^7 cells/mm^3. From the standard suspension of erythrocytes, each 1 ml of suspension was mixed with 20 ml of 4 solutions of different specific gravities with Percoll (Pharmacia, Uppsala, Sweden) and Conray (Daiichi Seiyaku Co., Tokyo, Japan) (a 65% aqueous solution of the iodinated compound iotalamate) being used to give specific gravities of 1.122 (medium A), 1.132 (medium B), 1.159 (medium C), or 1.170 (medium D) g/ml, respectively (Table 1). The Percoll-Conray mixtures at the different specific gravities were centrifuged at 35,000 G for 20 min., 4°C. After density gradient centrifugation, cell fractions were recovered from the top of each gradient using a density gradient fractionator (DGF-U, Hitachi, Ltd., Tokyo, Japan).
Table 1. Composition of each Percoll-Conray density gradient medium

<table>
<thead>
<tr>
<th>Component</th>
<th>(A) 1.222</th>
<th>(B) 1.132</th>
<th>(C) 1.159</th>
<th>(D) 1.170</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percoll</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Conray</td>
<td>15</td>
<td>18</td>
<td>22</td>
<td>25</td>
</tr>
<tr>
<td>H₂O, distilled</td>
<td>43</td>
<td>40</td>
<td>36</td>
<td>33</td>
</tr>
</tbody>
</table>

a) Calculated from measured results in a chemical balance.

These cells were then washed three times with ice-cold PBS and the pellets were suspended in 0.3 ml of cold PBS. The percentage of parasitized erythrocytes in each fraction was counted by Giemsa staining.

RESULTS

Changes in parasitemia following dexamethasone treatment: Dexamethasone treatment was effective in enhancing the number of *T. sergenti*-parasitized erythrocytes in infected cattle. Before the administration of dexamethasone, parasitized erythrocytes were rarely detected in peripheral blood (Fig. 1), but the number of parasitized erythrocytes increased markedly in all calves, after 7–12 days of dexamethasone treatment. In calf B-1, dexamethasone was given on two further occasions at 1–2 months' intervals in the same manner as the first treatment, and the number of parasitized cells increased uniformly. In three calves, the percentage of parasitized cells reached a mean peak of 39.9% parasitemia within 22 days after initiation of dexamethasone treatment. When treatment ceased, the number of parasitized erythrocytes decreased immediately in two calves (Nos. B-1 and 6), but in the remaining calf (No. B-5), the number of parasitized erythrocytes remained above 35%, and the animal died at 4 days later. During the experimental period, reticulocyte counts were rarely detected in the peripheral blood except at death in B-5.

Separation of parasitized erythrocytes by the density gradient fractionation: The distribution of *T. sergenti*-parasitized erythrocytes at Stage 3 of medium B followed by Percoll-Conray density gradient centrifugation is shown in Fig. 2. Among the four different gradient media, parasitized erythrocytes were concentrated most obviously in gradient medium B, which had a specific gravity of 1.132. In the lighter gradient medium A, however, a greater number of erythrocytes rose to the top of the gradient. In the heavier gradient media C and D, the erythrocytes were concentrated at the bottom of the gradient, and neither the distribution of erythrocytes nor the recovery rate of parasitized cells was superior to that of medium B. Medium B was therefore used for the fractionation of *T. sergenti*-parasitized erythrocytes in subsequent experiments. During the course of the parasitemic reaction the distribution of recovered erythrocytes shifted gradually toward the top of the gradient, and increases in number of the parasitized cells were always observed in the denser and lower level of the recovered erythrocytes, where the peak concentrations of erythrocytes occurred. At Stage 3, significant increases in the number of parasitized cells appeared in the denser and lower fractions (fractions 5–7, specific gravities between 1.133 and 1.141), and the
number of parasitized cells reached a mean of 2.1 times (maximum percentage of parasitized cells were shown in No. B-1 as 64.8%) higher than the pre-centrifuged one, a similar tendency being observed in all stages of parasitemia.

DISCUSSION

In the present study, dexamethasone treatment was shown to effectively enhance the parasitemia in *T. sergenti*-infected splenectomized calves. After the treatment, the number of parasitized cells was increased markedly, confirming the previous observation of Takahashi et al., [8]. The maximum percentage of parasitized cells in the three calves examined reached a mean of 39.9%, and this value was substantially higher than that seen in naturally infected calves. The explanation for recrudescences of theileriosis due to dexamethasone treatment is not clear, although corticosteroids have been used as immunosuppressants and it is likely that the phenomenon caused by dexamethasone is due to suppression of the immune system in the host animal.

Several density gradient methods have

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**Fig. 1.** Changes in percentage of packed cell volume (PCV) and *Theileria sergenti* parasitized erythrocytes with dexamethasone treatment: ○=PCV, △=*T. sergenti* parasitized erythrocytes. ▲1, 2, 3 shows the stages of parasitemia at which density gradient centrifugation was done.

**Fig. 2.** Distribution of *Theileria sergenti* parasitized erythrocytes in density fractions (at stage 3 in parasitemia): ○=calf No. B-1, □=No. B-5, △=No. B-6 and ●=specific gravity of erythrocytes fraction.
been devised to separate infected erythrocytes from uninfected ones in mammalian malarial parasites. Lunde and Powers [3] applied concentrated schizont-containing *P. knowlesi*-infected erythrocytes to a discontinuous Ficoll gradient. Many schizont-containing cells were presented in a layer at the interface of the 20% and 25% Ficoll bands. McAlister and Gorden [4] also separated *P. berghei*-infected cells into different developmental stages on a discontinuous Stracten II gradient. However, there are some disadvantages in using these gradient media to study erythrocyte metabolism, for example, Ficoll causes erythrocyte agglutination and Stracten II is unacceptably crude.

Recently, Gruenberg and Sherman [2] tried to recover *P. falciparum*-infected erythrocytes from a continuous Percoll-Hyapaque gradient as a part of their study on erythrocyte plasma membrane. In the results, they recovered in the uppermost layer of the gradient a 95% homogeneous population from 40 percent of the schizont-infected erythrocytes originally present. Percoll consists of colloidal silica particles, coated with polyvinylpyrrolidone. This material neither penetrates cell membrane nor has cytotoxicity [7], so is considered suitable for use in research on erythrocyte metabolism.

In the present study, we added Conray to Percoll. As the result, an average of 2.1 times the number of pre-centrifuged *T. sergenti*-parasitized erythrocytes was recovered from low dense fractions of the density gradient. The reason for the high recovery of *T. sergenti*-parasitized cells obtained from the low dense fraction is not clear, but the speculation of Miller and Chein [5] concerning *Plasmodia*-parasitized erythrocytes might be applicable to our results, *i.e.*, invaded protozoa cause host erythrocytes to enlarge but to be less dense than erythrocytes, thus causing the host-parasite complex to have less density. The recovery rate of *T. sergenti*-parasitized erythrocytes in this study, was apparently lower than that of *plasmodia*. The reason of the difference may be due to the difference in the size between two protozoa.

The technique described herein is expected to provide a high concentration of *T. sergenti*-parasitized cells for use in studies on the biochemical properties of parasitized cells.

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REFERENCES


要约

バーコール・コンレイを用いた密度勾配法による *Theileria sergenti* 感染赤血球の分離：八木行雄・古内進・清水真也1（農林水産省家畜衛生試験場東北支場，1家畜衛生試験場）—— *T. sergenti* 人工感染摘脾牛3頭から得られた血液について，バーコール・コンレイ密度勾配遠沈を行ない *T. sergenti* 寄生赤血球の分離を試みた。*T. sergenti* 感染牛にアキサメサゾンを投与することにより寄生赤血球数は増加し，投与開始後，22日以内に39.9%に達した。密度勾配遠沈により，寄生赤血球は比重の分画で著しく増加し，遠沈前の平均2.1倍（最大寄生率64.8%）に達した。