Key words: cytochemistry, *Hepatozoon canis*, leucocyte.

*Hepatozoon canis* infection of dogs has been recognized in Asia, Africa, Europe, the Middle East, and the United States, where the tick vector, *Rhipicephalus sanguineus*, is found [4, 9]. In Japan, three cases of dogs infected with *H. canis*, which has not yet been reported until recently, were found from 1989 to 1990 in Miyazaki prefecture [7].

The best definitive diagnosis of *H. canis* infection is to find gametocytes in circulating leucocytes on Giemsa-stained blood smears [1]. Most reports have described that the gametocyte-containing leucocytes are neutrophils [1, 5], although some reports monocytes and neutrophils [3, 4]. However, the kind of leucocytes parasitized by the gametocytes has not yet been determined. The gametocyte-containing leucocytes stained with Wright-Giemsa stain in our cases were similar to neutrophils, because of their segmented nuclei. However, they were somewhat different from normal neutrophils in their morphology. That is, these cells are larger in size, slightly bluish in cytoplasm staining, slightly thick in nuclear probe, and lighter in nuclear chromatin staining, compared with normal neutrophils (Fig. 1). Therefore, a question arose whether the gametocyte-containing leucocytes are neutrophils or monocytes. To clear this question is important for understanding the pathophysiology of canine hepatozoonosis. In the present study, in order to determine whether the gametocyte-containing leucocytes are neutrophils or monocytes, we stained these cells using enzyme cytochemistry specific to the neutrophil or the monocyte, for naphthol AS-D chloroacetate esterase (CAE) or α-naphthyl acetate esterase (ANAE), and then cultured the parasitized leucocytes for a long time.

*Case:* A male Shetland sheepdog, 3 years old, was taken June 11, 1990 to the Veterinary Teaching Hospital of Miyazaki University, developing anorexia, depression, and anemia. This dog was diagnosed as *Babesia gibsoni* infection. Although the dog was first treated with diminazene diacetate (Ganazeqz), babesiosis recurred 2 weeks later, and then organisms like coccidial oocysts were found in the circulating leucocytes on the Wright-Giemsa-stained blood smears [7]. The clinical signs of the dog associated with babesiosis recurred 5 times for 4 months and the gametocytes were observed in leucocytes during the periods. After the 5 cycles of treatment with diminazene diacetate combined with one cycle with tetracycline for 4 months, the dog was recovered from a severe illness, although a very small number of *Babesia* organisms were still detected in the peripheral blood smears.

The gametocyte-containing cells in all polymorphonuclear leucocytes except eosinophils reached only 0.8%, so that parasitized peripheral leucocytes were concentrated by the dextran method [2]. Five ml of heparinized whole blood was mixed with equal volume of 2% dextran (dextran T-500, Pharmacia Fine Chemicals, Sweden) in isotonic sodium chloride solution and allowed to stand for 30 min at room temperature for leucocyte to be separated. The leucocyte-rich supernatant was collected and centrifuged for 5 min at 150 × g. The sediment were smeared on slide glasses, which were kept in a plastic bag at −70°C before use.

Peripheral blood smears of the dog infected with *H. canis* were first stained with Wright-Giemsa stain. Then, ANAE stain was applied to the gametocyte-containing leucocyte blood smears by the method of Yam *et al.* [10]. CAE stain was made by the method of Moloney *et al.* [8]. Then, the sterile circulating leucocytes from the *H. canis*-infected dog were suspended in cold RPMI 1640 medium (Nissui, Co., Tokyo), supplemented with 20% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin to a concentration of 2 × 10⁸ cells per ml of medium. One ml of this suspension was placed in a 24 well plastic dish (Falcon 3047) containing cover slides and incubated in a humidified 5% CO₂ incubator at 37°C for 6 hours. After that, cells that did not adhere to the cover slips were removed by rinsing twice with PRMI 1640.
Nuclei of ANAE-positive cells were difficult to be distinguished, while those of ANAE-negative cells could be seen by the counterstaining. Ninety-five of the 100 gametocyte-containing leucocytes showed the same faint reddish-brown diffuse staining as monocytes. Four of the other ANAE-negative cells could not be decided as neutrophils, because they were similar to monocytes in the nuclear morphology by the counterstaining. Only one showed the same negative staining as neutrophils.

Monocytes were CAE-negative, while neutrophils were CAE-positive, showing strong reddish-orange granular coloration in cytoplasm. The gametocyte-containing leucocytes were the same CAE-negative as monocytes (Fig. 3). No CAE-positive cell was observed in the 50 gametocyte-containing leucocytes examined.

Comparative cytochemical properties of leucocytes of several animal species have been described, and cytochemical markers to differentiate neutrophils from monocytes in dogs are chloroacetate esterase (CAE) and nonspecific esterase (ANAE) [6]. Among canine leucocytes CAE activities are strong positive in neutrophils and negative in monocytes, while ANAE activities are negative in neutrophils and moderate positive in most of monocytes and lymphocytes [6]. In the present study, the gametocyte-containing leucocytes were all CAE-negative (50/50) and mostly ANAE-positive (99/100), while no cell was CAE-positive and only five cells was ANAE negative in the 100 gametocyte-containing leucocytes examined. But, four of these five cells could not be decided as neutrophils, judging from their counterstaining. Although only one of the 100 gametocyte-containing cells observed showed neutrophil-like cytochemical properties, this cell might be the neutrophil phagocytizing a gametocyte released from the parasitized monocyte. The cytochemical results in the present study suggest that the gametocyte-containing leucocytes are not neutrophils but monocytes.

One gametocyte each was found in some of the adherent cells after the 10 day cultivation of peripheral medium. Thereafter, the cell cultures were rinsed twice at 24-hour intervals. Ten days later, the cover slips were stained with Wright-Giemsa stain to find the gametocyte-containing leucocytes by light microscopy.

Neutrophils were ANAE-negative, having only faintly grayish cytoplasm and nuclei counterstained with methylgreen. On the other hand, monocytes and lymphocytes were ANAE-positive. Lymphocytes were stained relatively strong reddish-brown, and monocytes showed reddish-brown diffuse coloration with granular reaction in cytoplasm [6]. Cytochemical properties of the gametocyte-containing leucocytes for ANAE were considered to be

Fig. 2. A concentrated peripheral blood smear stained for $\alpha$-naphthyl acetate esterase (ANAE). Four neutrophils (right) are ANAE-negative, have only faintly grayish cytoplasm and nuclei counterstained by methylgreen. On the other hand, a gametocyte-containing monocyte (center) is ANAE-positive, stained relatively reddish-brown with granular reaction in cytoplasm. ($\times 1,000$)

Fig. 3. A concentrated peripheral blood smear stained for naphthol AS-D chloroacetate esterase (CAE). Three neutrophils (upper and right center) are CAE-positive, showing strong reddish-orange granular coloration, while a gametocyte-containing monocyte (lower right center) is CAE-negative. ($\times 1,000$)

Fig. 4. A gametocyte-containing monocyte (center) ten days after cultivation of peripheral blood leucocytes. (Wright-Giemsa, $\times 1,000$)
blood leucocytes from the H. canis-infected dog (Fig. 4). The adherent cells might be monocytes, because non-adherent cells were removed during the cultivation, and the life span of neutrophils in vitro is very short.

Most reports have described that the gametocyte-containing leucocytes are neutrophils, since these cells have the same segmented nuclei as of neutrophils on Giemsa stained blood smears. As mentioned above, however, these cells look somewhat different from normal neutrophils in their morphology with Wright-Giemsa stain. If the gametocyte-containing leucocytes were identified according to their cytochemical properties of ANAE and CAE, these cells could be differentiated as monocytes, especially segmented monocytes. In addition, a considerable number of the circulating polymorphonuclear leucocytes containing no gametocyte also appeared to be segmented monocytes, because they were similar to gametocyte-containing leucocytes in morphological properties by Wright-Giemsa staining. This type of leucocyte was reported in our previous report [7] as neutrophils on the Wright-Giemsa stained blood smears. When the blood smears of the present case of H. canis infection were reexamined based on the new classification in the present study, monocyte counts increased from 11% to 18%, which confirmed more remarkable monocytosis in canine hepatozoonosis.

REFERENCES