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Invasion of *Babesia gibsoni* into Mouse Erythrocytes

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Evidence of invasion of *Babesia gibsoni* organisms into mouse erythrocytes was demonstrated after inoculation of the organisms from artificially infected dogs into mice.

Severe anemia of dogs suffering from *B. gibsoni* occurs not only in Asia but also in North America (Robert et al. 1985). But, very little research on *B. gibsoni* has been done, especially with regard to the development of specific drugs. Currently, drugs are still being used that result in severe side effects and are not 100% effective (Kuttler 1988b). In spite of increased need for effective treatments, it has become difficult to do research using dogs as experimental animals because of its cost as well as the ethical aspects involved. Therefore, alternative experimental methods for *B. gibsoni* without dogs would be desirable.

Experimental methods to study piroplasms without a specific host include; in vitro cultures, which has been described in many reports since the 1980's (Kellermann et al. 1988); and erythrocyte-replaced mice, which are prepared by injection of bovine erythrocytes into severe combined immunodeficiency (SCID) mice in order to infect *Theileria sergenti*, a bovine-specific haemosporina (SCID-Bo system; Tsuji et al. 1992). In addition, despite high host specificity of *Babesiidae* in general (Kuttler 1988a; Dalgliesh 1993), establishment of artificial infection models in non-specific hosts would be also one of the worthwhile experimental strategy because some species of *Babesiidae* have been succeeded in infections in non-definitive hosts (Brandt et al. 1977; Gorenflot et al. 1991; Healy and Ristic 1988). Therefore, though *B. gibsoni* has not been reported in any hosts

except *Canidae*, we have attempted to establish an alternative artificial infection model for *B. gibsoni* by inoculating the organisms into different mouse strains in the present study.

Organisms of *B. gibsoni*, supplied by Azabu University (Kanagawa, Japan) and maintained by serial inoculations of parasitized erythrocytes (PE) into intact dogs in our laboratory, were inoculated into the splenectomized dog which was the source of PE for the inoculation into mice. When the percentage of PE (PPE) reached approximately 15% in the splenectomized dog, approximately 14 days after inoculation (DAI), PE were collected and $1x10^{8}$ PE (approximately 0.2 ml of blood) were inoculated into each of the 25 mice.

C57BL, BALB/c and SCID mice, purchased from CLEA Japan (Japan), were used for the present study. In total, 5 groups of 5 mice each were set as follows: untreated C57BL, BALB/c and SCID, and dexamethason-treated C57BL and SCID mice. Dexamethason (one mg/kg body weight/day, intraperitoneal injection) was administrated to each mouse in the respective dexamethason-treated groups, from the day before inoculation to 4 DAI, to suppress the function of the immune systems (Fauci 1985; Sell 1987).

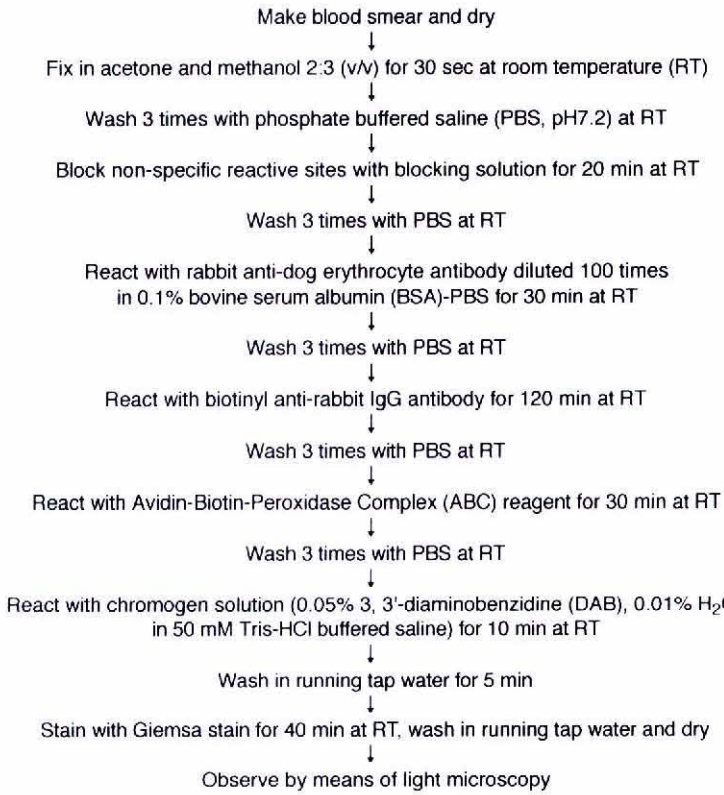

Figure 1. Procedure of immunocytochemical staining with Vectastain® ABC kit.

Blood smears were prepared by bleeding from the tail vein every other day. The immunocytochemical staining method shown in Fig. 1 [which was in according with Fishleder et al. (1984) with anti-dog erythrocyte antibody (Inter-Cell Technologies, Inc., U.S.A.) and Vectastain® ABC kit (Vector Lab., U.S.A.)] was employed to classify the erythrocytes into four types: non-parasitized mouse erythrocyte (NME), non-parasitized dog erythrocyte (NDE), parasitized mouse erythrocyte (PME) and parasitized dog erythrocyte (PDE). Observation was terminated after observation of a series of at least two smears without PDE, NDE and PME. Both kinds of blood from a dog and a mouse, of which volumes and erythrocyte counts were measured exactly, were mixed in vitro, and then five smears were prepared from it and stained. Approximately two thousands erythrocytes were observed on each them and were classified into dog's and mouse's erythrocytes. The results were suitable to the expected values statistically by the test for the proportion.

As shown in Fig. 2, NME, NDE, PME and PDE could be distinguished by observations of both the brown-colored dog erythrocytes stained with deposited DAB (3,3'- diaminobenzidine) and the visible parasites in the erythrocytes showed with Giemsa staining. Changes of percentage of each classified erythrocyte, which were calculated from a thousand observed erythrocytes, in the mice are shown in Fig. 3.

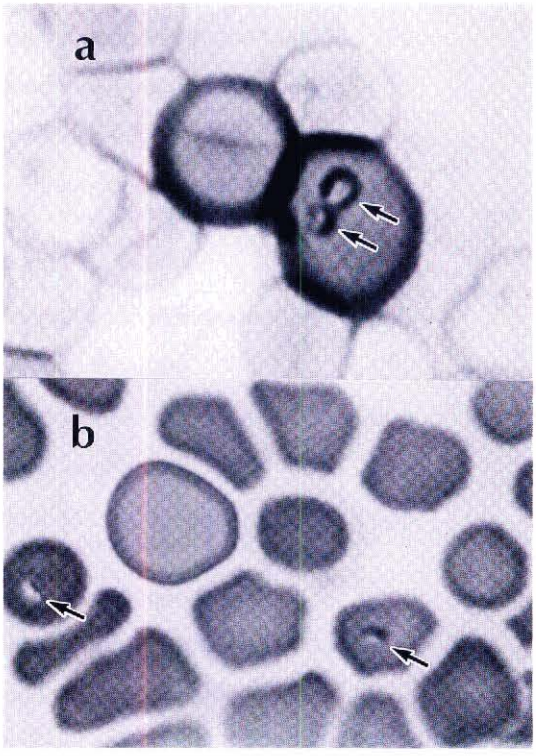

Figure 2. Differential staining of dog and mouse erythrocytes. a: Two dog erythrocytes are distinguished by their frown color, and one of them has two organisms (arrows). b: Two parasitized mouse erythrocytes (arrows) and a brown-stained dog erythrocyte are seen.

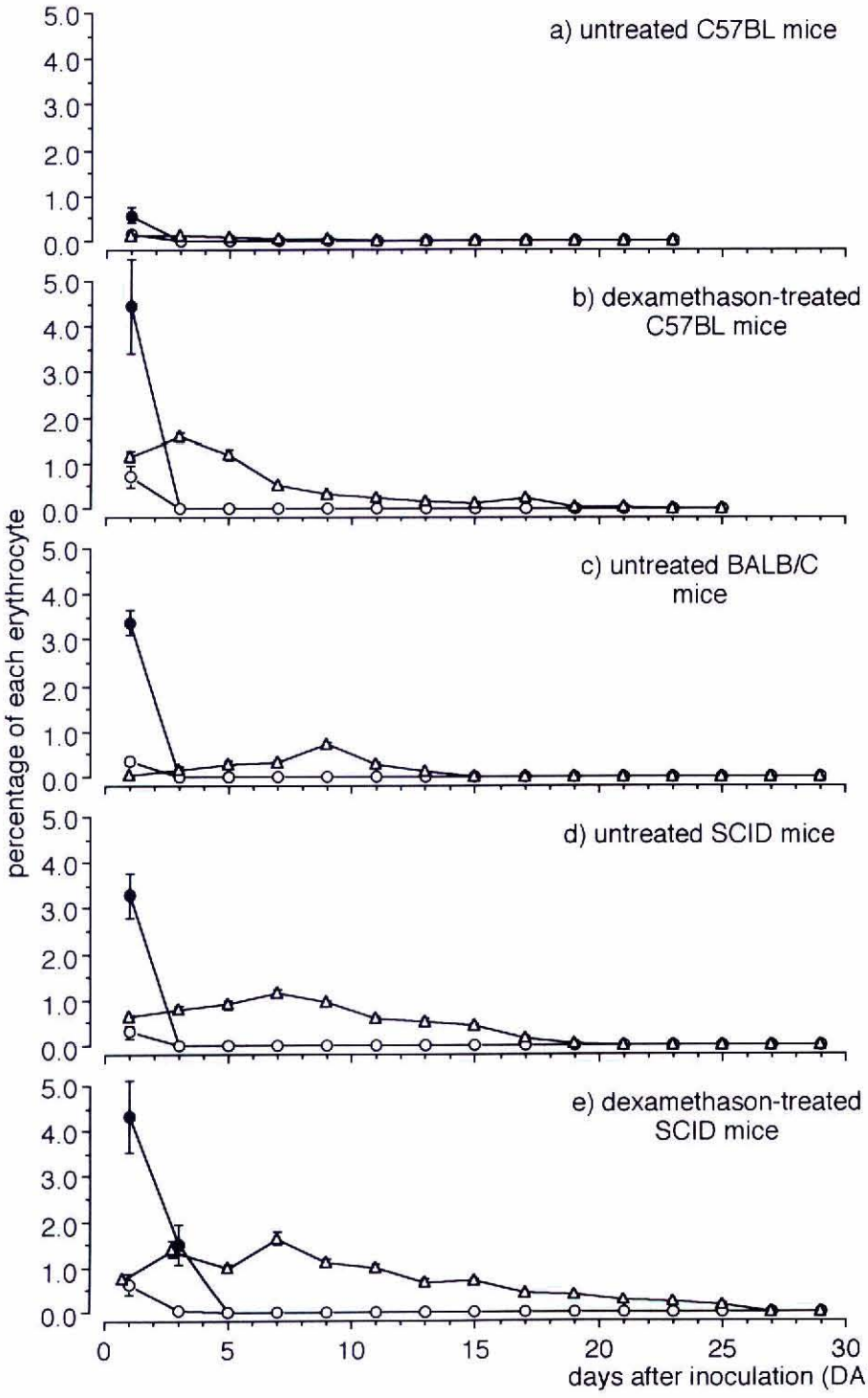

Figure 3. Changes of average of percentage of each erythrocyte type after inoculation with parasitized dog erythrocytes. Each symbol shows the average of parasitized mouse erythrocytes (PME, —△—), parasitized dog erythrocytes (PDE, —○—), and non-parasitized dog erythrocytes (NDE, —●—) respectively with standard error (5 mice in each group).

In the untreated C57BL mouse group, NDE and PDE were observed only the first day after inoculation. The peak value of PME was observed at 3 DAI; PME continued to decrease until it completely disappeared at 11 DAI (Fig. 3a). In the dexamethason-treated C57BL mouse group (Fig. 3b), changes in ratio of each erythrocyte class showed basically the same tendency as the untreated C57BL group, though the initial values of NDE and PDE and the peak value of PME were higher than those in the untreated ones, and also the existent period of PME was apparently extended (21 days) than that of the untreated ones (9 days). In the untreated BALB/c mice (Fig. 3c), NDE and PDE disappeared completely from peripheral blood smears by 3 DAI. However, PME continued to increase and reached a peak value of 0.72% at 9 DAI. In the untreated SCID mice (Fig. 3d), the dynamics of respective classified erythrocytes were almost the same as the BALB/c mouse group, except that the peak value and the period in which PME were observed were 1.14% (on 7 DAI) and 17 days respectively. In the dexamethason-treated SCID mice (Fig. 3e), NDE and PDE were observed till 3 DAI, and the peak value and the period in which PME were observed were 1.64% (on 7 DAI) and 25 days respectively.

PME were observed in all groups in the present study. These result suggests that *B. gibsoni* organisms released from PDE were able to invade into the mouse erythrocytes. However, in C57BL mice, which were used as representative of ordinary mice, the peak value of PME was observed on the day dog erythrocytes had completely disappeared and then the PME value decreased gradually, suggesting that organisms released from destroyed dog erythrocytes could only invade into mouse erythrocytes but not proliferate or re-invade and were destined only to disappear.

In contrast, in both SCID and BALB/c mice (which were used as the genetic normals of SCID mice), peak values of PME were observed at several days after complete disappearance of dog erythrocytes. Although the erythrocytes holding some alian substances have to be removed from the circulation more quickly in general, PME increased temporarily rather than decreased after the disappearance of PDE as the source of parasite. From these finding, it was considered that the invaded organisms into mouse erythrocytes could not only invade and stay there but also proliferate and reinvade into other mouse erythrocytes even if it was temporary. It is known that erythrocyte metabolism in dogs is different from most other animals, including mice (Bernstein 1954; Chan et al. 1964). The lack of Na^+-K^+-activated adenosine triphosphatase (Na^+-K^+ ATPase) in dog erythrocytes leads to lower concentrations of some cytoplasmic substances, of which uptake depends on the gradient of Na^+ concentration across the erythrocyte membrane, than those in erythrocytes of almost other animals having Na^+-K^+ ATPase in their

erythrocytes (Maede et al. 1983). Therefore, *B. gibsoni* may not need the stringently same internal environment of the dog erythrocyte to maintain themselves.

Dexamethason treatment and/or using the genetic character of SCID prolonged the lifespan of PME in all the mice examined. However, no evidence of increase of PME was observed after disappearance of PDE in any C57BL mouse. Therefore, proliferation and re-invasion might depend not on the dexamethason treatment or the genetic character of SCID, but on the strain of mouse. To establish a new infection model of *B. gibsoni*, optimal results might be produced by focusing on the strain of mouse used rather than on immunosuppression. The staining method described here would provide a new approach to the study of the invasion and proliferation of *B. gibsoni* organisms in non-specific host animals, and it may be possible that the mouse, a very convenient and useful laboratory animal, is a candidate to substitute for dogs in the study of canine babesiosis.

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