An In Vitro Study of Monocyte Phagocytosis in the Peripheral Blood of Healthy and *Babesia*-Infected Beagles

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Abstract. The monocyte-macrophage cell line is an important marker of the host defense system especially in cell-mediated immunity. This report describes a series of assays that can be applied routinely in canine monocyte function, or phagocytosis and gives some information as to the activation of normal monocytes by sensitized lymphocytes in dogs infected with *Babesia gibsoni*. *Babesia*-immune monocytes were more effective in phagocytosis of the parasitized erythrocytes than normal monocytes. *Babesia*-immune monocytes incubated with *Babesia*-immune fresh serum showed the highest percentage of phagocytosis. When normal monocytes were incubated for 24 hours with *Babesia*-immune lymphokines, the percentage of phagocytic monocytes was approximately three times higher than those with normal cultured supernatant.

The monocyte-macrophage cell line has several important functions. The major functional properties of monocyte-macrophages are adherence to glass, ingestion or engulfment of particles by pinocytosis or by phagocytosis, involvement in antigen processing and presentation in the afferent limb of the immune response and involvement in the interaction with lymphoid cells in the expression of cell-mediated immune reactions [3, 13, 14, 20, 21].

In a series of previous studies by the authors on experimental malaria and toxoplasmosis using rats and mice based on cell-mediated immunity, it was reported that macrophage activation was associated with increased cell size, spreading on glass, adhesiveness to a charged surface, phagocytic activity and digestive capacity, and with increase in contents of some lysosomal enzymes [7, 10, 11, 16–19]. However, despite

the extensive literature on monocyte-macrophage physiology, there is a surprising paucity regarding monocyte function in normal dogs and concerning canine diseases with regards to basic and clinical immunology [4, 5, 9–12].

The authors have undertaken this study to evaluate tests of monocyte function applicable to dogs. This report describes also technics for measuring monocyte phagocytosis and *Babesia*-protozoan killing.

Materials and Methods

***Babesia* species:** The strain of *Babesia gibsoni* [6] used in this study was maintained in the authors' laboratory by beagle passages and kept in a deep freezer at -180°C during the experiments. This was originally obtained from a hunting dog that contracted the parasite in Hyogo Prefecture in Japan.

Experimental animals: Fourteen 90 day-old parasite-free beagles from 3 litters produced by the same parents and maintained in a closed environment

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were used in the present study. None of them were vaccinated against distemper, rabies and canine hepatitis until the end of the experiment. Three healthy adult beagles, previously inoculated with *B. gibsoni*, five times in 2 years, weighing approximately 15 kg and harboring no protozoa in the blood, were used as *Babesia* chronic beagles. They showed IgM and IgG serum antibody titers of 20 and 320 by the indirect immunofluorescent method, respectively. To maintain regular conditions for breeding and feeding, each dog was put in a separate cage and was given a limited volume of standard dog diet everyday. Tap water was supplied ad libitum during the experiment.

Monocyte isolations: The standard Conray-Ficoll cell separation procedure performed in this experiment, yields a band of low density cells comprised mainly of lymphocytes with a small proportion of monocytes [22]. Ten ml of heparinized blood from the cephalic vein of the dogs was diluted three times with physiological saline solution. Five ml of blood was added to 2 ml of Conray-Ficoll (10 vol. of 33.8% Conray plus 24 vol. of 9% Ficoll) and centrifuged at 1,350 rpm for 20 minutes. Numerous lymphocytes mixed with monocytes in the upper layer of the Conray-Ficoll were collected, washed three times with Hanks' balanced salt solution (HBSS) containing 20% heat-inactivated calf serum (CS) and then washed again once with medium TC-199 containing 40% CS. For canine monocyte monolayer, 40% CS is the most suitable concentration which can be added to the medium. Resuspension was made in medium TC-199 with 40% CS to a concentration of 5×10^6 cells per ml. This cell suspension was referred to as "monocyte-lymphocyte" (M-L). To obtain pure monocyte preparations, one ml of the mononuclear cell suspension was allowed to adhere to a 15 mm diameter cover slip contained in a plastic culture multidish tray (FB-16-24-TC, Limbro Chem. Co., Inc.) for 5 days in CO₂ incubator at 37°C. One-third of the medium was changed on the first and second day. This was repeated on the 5th day and every 3 days thereafter. Nonadherent cells were removed by gently washing with warm (37°C) medium TC-199 with 40% CS-heparin up to the 5th day. Over 98% adherent monocytes among the cell monolayers including 1 to 2% neutrophils were counted.

Preparation of parasitized erythrocytes: Parasitized erythrocytes were collected from the blood of a splenectomized dog infected with *B. gibsoni* under anesthesia. More than 30% of the erythrocytes contained the parasites. Blood was collected in sterile Alsever's solution. The erythrocytes were washed 4 times with phosphate-buffered saline

(PBS) and centrifuged at 4,000 g for 15 minutes after each wash. An amount of PBS was added to the erythrocytes to a concentration of 10^7 parasitized erythrocytes per ml. The erythrocytes were examined as to whether their surface membranes were free from humoral antibodies using the indirect immunofluorescent technic.

***Babesia* immune fresh serum (BIFS) and *Babesia*-immune inactivated serum (BIIS):** BIFS and normal fresh serum (NFS) with complement was obtained by venisection into cooled tubes which were immediately centrifuged. The fibrin clot was removed and the serum was stored at -20°C until use. These BIFS and NFS which were incubated in a water bath at 56°C for 30 minutes, were termed as inactivated serum containing no complement, that is, BIIS and normal inactivated serum (NIS).

Preparation of *Babesia* lysate antigen: Three volumes of PBS were added to one packed volume of the parasitized erythrocytes mentioned above. The suspension was homogenized with a glass homogenizer and an ultrasonic vibrator (100 W, Kubota Insonator, Model 200, Tokyo) and stored for at least 48 hours at -80°C. The antigen was thawed, rehomogenized, washed 5 times with PBS and recentrifuged at 17,300 g for 20 minutes. The final sediment was homogenized with 1.5 vol. of PBS and lyophilized in 0.5 ml quantities. When needed, 50 mg of lyophilized antigen was mixed with 2 ml of PBS in a 10 ml conical centrifuge tube and centrifuged at 365 g for 3 minutes. The supernatant fluid was collected and stored at -80°C as the "*Babesia* lysate antigen".

Preparation of Lymphokines (LKs): Lymphoid cells in the spleen were collected from normal and *Babesia*-immune dogs by means of a slight modification of the Conray-Ficoll method (Tsuji, 1971). The cells were washed twice with HBSS. Normal and *Babesia*-immune spleen lymphoid cell suspensions were incubated with 50 µg *Babesia* lysate antigen in a humidified 5% CO₂ incubator at 37°C for 24 hours. These lymphoid cell cultures were centrifuged at 1000 g for 30 minutes. The supernatants thus obtained were filtered through a millipore membrane filter (0.3 µm, type PH, Millipore Co., Mass.) and were used as the normal cultured supernatant and *Babesia*-immune lymphokines (LKs). They were diluted 1:2 with medium TC-199.

Phagocytosis: The monocytes which adhered on the cover slips contained in the plastic culture dishes were incubated with 0.5 ml NFS or BIFS in 1.0 ml medium TC-199 with 40% CS at 37°C for 30 minutes. The monocyte monolayers were incubated with 0.5 ml NIS or BIIS which were pretreated at 56°C for 30 minutes, with 1.0 ml medium TC-199

Table 1. Differential percentages of monocytes and other leukocytes in the peripheral blood of beagle and mongrel dogs using the Conray-Ficoll cell separation method

Species	No. of cases tested	Conray-Ficoll method	Mean percentage of the differentiated leukocytes			
			Monocytes Mean±SD range	Lymphocytes Mean±SD range	Neutrophils Mean±SD range	Others Mean±SD range
Beagle	10	Before	4.3±1.3 (2-6)	18.4±1.6 (15-20)	74.7±2.4 (71-78)	2.6±0.7 (2-4)
	10	After	5.6±1.5 (4-8)	85.3±4.3 (81-93)	6.8±2.9 (2-10)	2.3±1.1 (1-4)
Mongrel	10	Before	4.2±1.0 (3-6)	33.4±4.1 (27-37)	57.4±3.8 (53-63)	5.0±1.4 (3-7)
	10	After	2.8±1.1 (1-5)	91.1±5.6 (81-96)	2.6±3.2 (0-9)	3.5±3.1 (0-8)

Table 2. Phagocytic percentages of the parasitized erythrocytes in monocytes from normal and *Babesia* chronically infected beagles

Minutes after addition of erythrocytes	Phagocytic percentages of the parasitized erythrocytes in*					
	Normal monocytes			<i>Babesia</i> -immune monocytes		
	30	60	120	30	60	120
Incubation with						
Inactivated normal serum**	9.4	10.0	16.0	26.0	29.0	28.8
Fresh normal serum	13.6	13.0	37.8	18.6	26.2	29.7
Inactivated immune serum**	18.0	24.0	28.5	35.7	43.7	33.4
Fresh immune serum	21.4	24.6	30.9	56.6	41.4	59.7

Remarks.

* Average were calculated from 5 independent experiments.

** Fresh serum was inactivated at 56°C for 30 minutes.

with 40% CS at 37°C for 30 minutes. One ml of the parasitized erythrocyte suspension was added to each of these monocyte monolayers. The cover slips were taken from the culture dishes and stained with May-Grünwald and Giemsa at 30, 60 and 120 minutes after incubation [15]. Phagocytized erythrocytes ingested by 500 monocytes were counted to get the mean phagocytized parasites per monocyte.

Results

The results of monocyte isolation, as shown in Table I, were collected from 10 ml of heparinized blood. This volume was sufficient to perform the full battery of the assays described with M-L suspension making monocyte monolayers and lymphocyte products or LKs.

Before Conray-Ficoll cell separation, the mongrel dog blood contained 3 to 6% monocytes (4.2±1.0), 27 to 37% lymphocytes (33.4±4.1), 53 to 63% neutrophils (57.4±

3.8) and 3 to 7% other cells (5.0±1.4). After separation, the separated M-L suspension from the same mongrel dog contained 1 to 5% monocytes (2.8±1.1), 81 to 96% lymphocytes (91.1±5.6), 0 to 9% neutrophils (2.6±3.2) and 0 to 8% other cells (3.5±3.1). Before separation, the beagle dogs had a range and mean percentage of 2 to 6% monocytes (4.3±1.3), 15 to 20% lymphocytes (18.4±1.6), 71 to 78% neutrophils (74.7±2.4) and 2 to 4% other cells (2.6±0.7). The separated M-L suspension from the same beagles contained 4 to 8% monocytes (5.6±1.5), 81 to 93% lymphocytes (85.3±4.3), 2 to 10% neutrophils (6.8±2.9) and 1 to 4% other cells (2.3±1.1). There was a significant difference in the percentage of lymphocytes and neutrophils in the separated suspension and non-separated blood of both mongrel and beagle dogs.

As shown in Table 2, phagocytic percen-

Table 3. Phagocytic percentages of the auto- and iso-erythrocytes in monocytes from normal and *Babesia* chronically infected beagles

Minutes after addition of non-parasitized erythrocytes	Percentages of erythrocyte phagocytosis*	
	Normal monocytes 30	<i>Babesia</i> -immune monocytes 30
Iso-erythrocytes with		
inactivated normal serum**	9.4	14.1
fresh <i>Babesia</i> -immune serum	8.2	11.7
Auto-erythrocytes with		
inactivated normal serum**	10.0	
fresh <i>Babesia</i> -immune serum		8.2

Remarks.

* Averages were calculated from 4 independent experiments.

** Fresh serum was incubated at 56°C for 30 minutes.

Table 4. Induction of phagocytic activity in normal peripheral monocytes following incubation for 24 hours with lymphokines

Non-immune monocytes were cultured for 24 hours with supernatants from	Mean percentage of monocytes* with parasitized erythrocytes (2 hours after addition)
Normal beagle spleen cells + <i>Babesia</i> antigen	5.6 ± 1.3
<i>Babesia</i> -immune spleen cells + <i>Babesia</i> antigen	14.1 ± 2.2

Remarks.

* The mean ± S.D. are within representative experiments done independently 4 times.

tages of the parasitized erythrocytes in normal monocyte monolayers incubated with NIS at 30, 60 and 120 minutes incubation were 9.4, 10.0 and 16.0, respectively. In normal monocytes incubated with NFS, the percentages of phagocytosis were 13.6, 13.0 and 37.8 at 30, 60 and 120 minutes incubation. The positive rate of normal monocytes ingesting parasitized erythrocytes increased to 18.0, 24.0 and 28.5 per cent, 30, 60 and 120 minutes after adding BIIS. By addition of BIFS to normal monocytes at 30, 60 and 120 minutes incubation, the phagocytosis in the monocytes increased remarkably to 21.4, 24.6 and 30.9 per cent as compared with those in NIS. When immune monocytes were added to NIS, however, they engulfed parasitized erythrocytes by phagocytosis with percentages of 26.0,

29.0 and 28.8 at 30, 60 and 120 minutes incubation. With the addition of BIFS, the phagocytic ability of immune monocytes increased remarkably to 56.6 per cent 30 minutes after incubation. Immune monocytes incubated with BIFS for 120 minutes had the highest percentage (59.7%) of ingestion of parasitized erythrocytes.

To determine the phagocytosis of non-parasitized erythrocytes by normal or immune monocytes as shown in Table 3, NFS or BIFS with iso-erythrocytes and auto-erythrocytes were added to normal and immune monocyte monolayers. In normal monocytes incubated with either NIS or BIFS, the percentage of monocytes ingesting iso-erythrocytes or auto-erythrocytes were 9.4, 8.2 or 10.0, thirty minutes after addition of the erythrocytes. Immune monocytes in-

cubated with the same conditions as in normal monocytes phagocytosed 14.1% iso-erythrocytes and 8.2% auto-erythrocytes.

The data in Table 4 showed the enhancement effect of the LKs on phagocytosis of parasitized erythrocytes by normal monocytes two hours after the introduction of the infected erythrocytes. When normal monocytes were incubated for 24 hours with the *Babesia*-immune LKs which were produced by the immune lymphocytes and the specific antigen, the percentage of phagocytic monocytes was approximately 14, as compared with that of normal cultured supernatant showing 5.6.

Discussion

The major functional properties of monocytes are adherence to glass with surface receptors on the plasma membrane of the cells, ingestion and engulfment of foreign bodies by pinocytosis and phagocytosis, cytotoxicity and metabolic activity. Phagocytosis and Babesiacidal killing properties were chosen because these functions give a basic insight into the host defense status and to the degree of activation of the monocytes. Both are crucial elements in the mammalian host defense system.

Monocytes obtained from immune dogs exhibited enhanced function when cultured in vitro. They appeared more spread out on slide cover glass and more phagocytic showing enhanced Babesiacidal activity. The in vivo state of activation of monocytes obtained from dogs undergoing an active infection can be simulated in vitro by exposing monocytes from normal individuals to lymphocyte mediators, LKs. It was shown in mice and rats that normal macrophages could be activated after 24 hours incubation with *Toxoplasma* or *Plasmodium* immune LKs [7, 10, 11, 17–19]. In previous reports by the authors on protozoan im-

munity of *Toxoplasma*, non-immune macrophages infected with *T. gondii* in vitro, possessed the ability to inhibit the multiplication of intracellular *Toxoplasma* either by co-cultivation with immune lymphocytes or by the addition of the LKs present in the supernatant fluids from immune lymphocytes cultured with *Toxoplasma* specific antigen or non-specific mitogens [10, 11, 16–19].

The erythrocyte phagocytic activity of normal monocytes, 30 to 120 minutes after the addition of the parasitized erythrocytes was shown to be in the range of 5.6 to 16.0 in this experiments. The difference of the phagocytic percentage in the normal monocytes might be caused by using the parasitized erythrocytes in the different status and also by some mechanical damages which were due to thorough washing with PBS. However, the erythrocyte phagocytic activity of normal monocytes was very weak in every experiment as compared with those of activated monocytes which were done in the same condition. *Babesia*-immune monocytes with BIFS were the most efficient in the ingestion of parasitized erythrocytes. As for the phagocytosis of *Babesia*-parasitized erythrocytes by normal beagle monocytes with the addition of LKs incubated for 24 hours, the phagocytic activity in the monocyte was more enhanced as compared with that of normal cultured supernatant. In the study conducted by Rosse [13, 14], the reactions of monocytes with erythrocytes covered with complement only or with antibody only were investigated. When erythrocytes were coated with complement, they readily adhered to the surface of the phagocytic cells faster than that with antibody only. These phenomena were also compatible with the observations of Criswell [2] and Makimura [7] in experimental malaria. Brown [1] observed also in vitro

that macrophages from BCG-infected mice phagocytosed *Plasmodium knowlesi* infected erythrocytes more readily than macrophages from normal mice in the presence of specific antibody. The cooperative mechanism to the monocytes between LKs and BIFS has not been clarified yet. However, taken together with the present data, it is clear that monocyte phagocytic activity in the status of activated monocytes by the addition of *Babesia*-immune LKs was more enhanced by the adherence of the parasitized erythrocytes with addition of BIFS. The phagocytic phenomena in the canine monocytes in vitro system from the observed results are very likely similar to the mechanism for ingestion by phagocytes, generally called opsonization.

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要 約

健康およびバベシア原虫感染ビーグル末梢血液中単球の貪食性について：石嶺 毅（大阪大学微生物病研究所原虫部門），長沢秀行・鈴木直義（帯広畜産大学家畜生理学教室）——細胞性免疫現象は特異的に感作されたリンパ球と，これによって活性化されたマクロファージあるいは感作リンパ球産生物質（リンホカイン）などが相互に関与しているものと考えられている．これらは，*in vitro* で解析する研究方法が確立されだして，その機序が徐々に明らかになりつつある．しかしながら，犬においての報告は殆んど見当らない．そこで，*in vitro* 培養によるビーグルの末梢血液中単球を用いて，バベシア原虫感作リンパ球産生物質，リンホカインが単球におよぼす影響を貪食能の面から検討を加えた．犬末梢血液中単球の単層培養作成には，40% 仔牛血清添加培養液を用いて，5 日間前培養をおこない，試験に供した．バベシア原虫感染慢性犬から採取した単球（バベシア免疫単球）と健康ビーグルからの単球（正常単球）にバベシア含有赤血球を添加して30分後の貪食率を求めると，それぞれ 26% および 9.4% であった．これらの培養層中に，バベシア免疫新鮮血清を投与すると，それぞれの貪食率は 56.6% および 21.4% に増加した．正常単球がバベシア免疫リンホカインあるいは正常リンパ球培養上清と共に24 時間培養されて後，バベシア原虫含有赤血球を単球培養層に添加した．添加後 2 時間での単球の赤血球貪食率は，それぞれ 14.1% および 5.6% で，正常単球の貪食性は免疫リンホカイン添加培養により約 3 倍亢進した．