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Cell mediated immunity in malaria:

1. Changes in numbers of mononuclear phagocytes during *Plasmodium vinckei petteri* infection in immunized and non-immunized mice.

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ABSTRAK

Supargiyono – *Immunitas yang diperantarai oleh sel pada malaria: Perubahan jumlah sel fagosit mononuklear selama infeksi Plasmodium vinckei petteri pada mencit yang diimunisasi dan tidak diimunisasi*

Penelitian bertahap telah dilakukan untuk mempelajari reaksi-reaksi immunitas selular yang terjadi selama infeksi *Plasmodium vinckei petteri* malaria pada mencit LACA. Fokus kajian pada penelitian tahap pertama ini adalah mempelajari profil perubahan jumlah sel fagosit mononuklear (*mononuclear phagocytes*) dalam sumsum tulang, darah tepi dan dalam limpa, selama infeksi malaria pada mencit yang diimunisasi dan yang tidak diimunisasi. Imunisasi dilakukan dengan penyuntikan secara intravena 10^8 parasit stadium darah yang dibebaskan dari eritrosit dengan larutan saponin dan difiksasi dengan larutan formalin. Kedua kelompok mencit, yang diimunisasi dan yang tidak diimunisasi diinfeksi dengan 10^3 eritrosit terinfeksi, pada hari ke 15 sesudah imunisasi. Jumlah sel fagosit berinti satu beserta sel prekursornya dievaluasi berturut-turut pada hari ke 0, 3, 6, 9 dan 12 sesudah infeksi. Hasil penelitian menunjukkan bahwa selama infeksi *P. v. petteri*, jumlah sel fagosit mononuklear meningkat di dalam sumsum tulang, kemudian di dalam darah tepi dan dalam limpa. Derajat peningkatan jumlah sel fagosit tersebut lebih tinggi pada mencit yang telah diimunisasi sebelumnya dibandingkan dengan pada mencit yang tidak diimunisasi. Tingginya peningkatan jumlah sel fagosit mononuklear pada mencit yang diimunisasi tersebut nampaknya berkaitan dengan keberhasilan sistem kekebalan tubuh hospes dalam menurunkan parasitemia. Peningkatan jumlah sel fagosit tersebut didahului dengan peningkatan jumlah sel prekusornya di dalam sumsum tulang, sehingga bisa diantisipasikan bahwa produksi dan proliferasi dari sel prekursor di dalam sumsum tulang juga meningkat dalam usaha memenuhi kebutuhan sel dewasa di dalam darah dan jaringan selama infeksi malaria.

Key words: *Plasmodium vinckei petteri* – malaria – LACA mice – mononuclear phagocyte – cellular immunity.

(Berkala Ilmu Kedokteran, Vol. 27, No. 1, March 1995)

INTRODUCTION

The mononuclear phagocyte system (MPs), consisting of promonocytes and tissue macrophages, represents the major cellular components of the classical resticulo-endothelial system. These cells are widely distributed in the body, exist in the bone marrow, blood, liver, lymphoid tissues, lungs, connective tissues, nervous tissues and the serous cavities. Their presence in almost all parts of the body suggests that they exert a modulatory role in tissue homeostasis, and immunological and inflammatory responses. These phagocytes are highly active cells, and are able to respond to humoral and cellular signals, and therefore they participate in a variety of physiological events. They possess a number of unique properties for tissue responses to external stimuli. First, they can internalize extracellular particles or micro-organisms, and can degrade them to a presentable forms for T-lymphocytes. Second, mononuclear phagacytes are highly secretory cells.[1] Their secretory products include proteases, complement proteins and IL-1, which are important in inflammatory reactions. Third, mononuclear phagocytes are critically situated in various tissues, usually close to the microvasculature and surrounding epithelial and

mesenchymal cells. Fourth, mononuclear phagocytes interact with T and B lymphocytes, and thereby intervene in immunological responses. Fifth, macrophages have surface receptors for lymphokines, the regulatory proteins released by lymphocytes. Upon an interaction with lymphokines these cells acquire novel properties included under the term "activation". Activated macrophages are highly microbicidal and tumoricidal. Thus, the mononuclear phagocyte system is involved in infectious processes, and in the modulation of immunological responses and inflammation.

Several studies have also shown the importance role of MPs during malarial infections. These cells become activated as indicated by increased spreading and phagocytosis of opsonized erythrocytes *in vitro*,[2,3,4] and their capacity to release toxic products such as reactive oxygen intermediates.[5,6] The malaria parasite has a very complex life-cycle: natural infection is initiated by the inoculation of sporozoite forms into the blood stream, when an infected mosquito takes a blood meal. These sporozoites rapidly migrate to the liver and initiate a primary (exo-erythrocytic) cycle in the parenchymal hepatic cells. When the schizogonic cycle of this stage is completed, merozoite forms are released into the blood stream, where they invade erythrocytes and undergo the intra-erythrocytic cycle. During this schizogonic cycle, trophozoites develop into mature schizonts, which subsequently burst, releasing both free invasive merozoites and soluble parasite antigens. It is at this stage of infection that the immunocompetent host may respond to the antigenic stimulus of the parasites. Even when the parasite is developing inside the host erythrocytes, the cell surface is altered in such a way that the parasite is recognized as a "foreign" invader. There are constant opportunities for contact between parasite antigens and mononuclear phagocytes, and other cells which are involved in the immune response. Such contact may occur not only in the blood, but more particularly in organs, such as liver and spleen, where there are fixed populations of such cells. These interactions subsequently initiate a complex cascade of humoral as well as cellular immune responses to encounter the invading parasites.

Beside the elevation of their functional activities during infection, increased in the number of MPs is also important in successful defence against invading parasites. Therefore, several workers have focused their attention on the changes of levels of macrophage and monocyte populations specifically in the spleen, liver and bone marrow during rodent malarial infections and have correlated these levels with the parasitaemia. Wyler and Gallin[7] reported that the number of macrophages in the spleen of *P. berghei* infected mice rose sharply within the first four days of infection. Lelchuk *et al.*,[8] performed similar studies with normal and vaccinated mice during *P. yoelii* and *P. berghei* infection and they found that the number of mature macrophages increased in parallel with the parasitaemia during non-lethal *P. yoelii* infection. These investigators concluded that the extent of splenomegaly was greater in mice that had recovered. Playfair *et al.*[9] highlighted the role of phagocytic influx into the liver of mice vaccinated against *P. yoelii*. Histological studies revealed a large increase in monocyte and eosinophil populations in the liver but without significant increase of these cells in the spleen. More recently, Gross *et al.*[10] investigated the lymphocyte and macrophage dynamics in the spleen of BALB/c during the course of *P. berghei* infections and after rechallenge of cured mice, and correlated these changes with the failure of normal BALB/c mice to overcome the infection. They concluded that the failure of the immune system during a lethal infection may be due to preferential increase of the immature, functionally defective macrophages and possibly T suppressor lymphocytes. A similar study was also performed by Villeval *et al.*[11] in *P. berghei* and *P. chabaudi adami*. They reported that during fatal infections there was a rapid lymphocyte depletion in the marrow with a compensating rise in spleen lymphocytes. Monocyte number increased in the bone marrow and later in the spleen, and these increases were found to be two-to three-fold higher in nonfatal infections.

Overall, those studies indicate that there is a remarkable increase in the macrophage population during the course of lethal and nonlethal infections, with an indication of an increase in their production in the bone marrow.

This study is therefore, aimed to investigate the profile of mononuclear phagocytes,

comparing such changes that occurred in normal and in immunized animals, using a *P. v. petteri* in LACA mice model system. The changes in the number of bone marrow, circulating blood and splenic mononuclear phagocytes were analysed and correlated to the ability of the host to overcome the infection.

MATERIALS AND METHODS

Parasites:

Plasmodium vinckei petteri (2CR) were kindly provided by Professor F.E.G. Cox, King's College London, University of London.

Experimental animals:

Female outbred, 6-8 week old LACA mice, purchased from Tuck and Son, Buttlebridge, Essex, England, were used. They were free from haemoprotozoa, kept in groups of six, fed and watered *ad libitum*.

Immunization and infection procedures:

Infection and immunization procedures against *P. v. petteri* infection were performed as described in the previous paper.[12] To study the changes in the number of bone marrow, circulating blood and splenic mononuclear phagocytes during the course of *P. v. petteri* infection and following immunization, 5 groups of 3 immunized and 3 non-immunized mice were infected with *P. v. petteri* on day 0. The parasitaemias were monitored daily by examining the Giemsa stained thin blood smears prepared from the tail blood. At each time point: days 0, 3, 6, 9 and 12 post infection, 3 immunized and 3 non immunized mice were sacrificed, and the number of bone marrow mononuclear phagocytes, circulating blood monocytes, and spleen macrophages were assessed.

Assessment of bone marrow mononuclear phagocyte numbers:

To assess the changes in bone marrow mononuclear phagocyte number during the course of *P. v. petteri* infection and following immunization, bone marrow cells were extracted from 2 femurs of 3 immunized and 3 non-immunized animals by enzymatic digestion, and the promonocytes, monocytes and resident bone marrow macrophages were isolated from the other cells and counted under a microscope.

Extraction of bone marrow cells by enzymatic digestion :

The procedure used to extract and isolate bone marrow mononuclear phagocytes was modified from that of Crocker and Gordon.[13] Mice were sacrificed, both femurs were excised, the epiphysis removed and the marrow plugs extruded by inserting a 23 g needle into one of the cut ends, teasing gently and flushing with RPMI 1640 containing 0.05% w/v collaginase (Boehringer Mannheim, East Sussex, England), and 0.001% w/v deoxyribonuclease (DN-ase) type I (Sigma Chem. Co). The marrow plugs from 3 mice (6 femurs) were pooled and suspended in 10 ml of the same enzyme solution, and digested at 37°C by tumbling at 1 revolution/second for 1 h. Digestion was stopped by adding fetal calf serum (FCS) to a final concentration of 1% v/v. Cells were washed twice, resuspended in 5 ml RPMI, and the clusters were separated from single cells for promonocyte, monocyte and resident macrophage preparations.

Preparation of bone marrow promonocytes, monocytes and resident macrophages:

Clusters were separated from single cells by unit gravity velocity sedimentation in RPMI containing 30% FCS. Five ml of tissue digest was layered over 10 ml of RPMI containing 30% FCS in a 50 ml Falcon centrifuge tube .(Becton Dickinson & Co., Oxnard CA). After 1 h at room temperature, 14 ml of medium was carefully aspirated with a sterile Pasteur pipette for bone marrow promonocytes and monocytes preparation. After washed in RPMI twice, the cells were resuspended in complete medium, nucleated cells were counted in a haemocytometer chamber and the cell suspension was adjusted to a density of $5x10^6$ cells/ml. 300 µl of this cell suspension was plated as a drop on triplicate cover slips in 3 wells of a 24 well tissue culture plate, and was

incubated at 37°C and 5% CO_2 in a humidified incubator. The cells/clusters in the remaining 1 ml of the column were washed twice in RPMI by centrifuging at 100 g for 10 minutes and re-suspended in 3 ml RPMI containing 10% FCS. After cell/cluster counts, 300 µl cluster suspensions were plated as a drop on triplicate cover slips in 3 wells of a 24 well tissue culture plate and incubated at 37°C and 5% CO_2 in a humidified incubator. After 30 minutes incubation, 0.5 ml complete medium was carefully added into each well, and incubated further for a total incubation of 2 h for promonocyte and monocyte preparations, and 4 h for resident macrophage preparations. The cover slips plated with single cell suspensions were washed 3 times in RPMI, and the adherent cells were incubated further for a total incubation of 6 h in complete medium. After being washed, the cover slips were fived with methanol and stained with Giemsa and after being air dried they were mounted on glass slides for microscopic examination. The cover slips containing clusters were rinsed 5 times with PBS and left in calcium and-magnesium free PBS for 30 minutes at room temperature. Clustering cells were than detached by repeated and direct gentle flushing with PBS. The remaining adherent cells were then methanol fixed and Giemsa stained, and after being air dried the cover slips were mounted on glass slides. The slides were examined under a microscope using ×400 magnification, the number of promonocytes, monocytes and resident bone marrow macrophages that had adhered to cover slips were counted, and the total number of these cells/2 femurs were calculated. Since bone marrow cells from two femurs represent 11.8% of total bone marrow cells,[14] the number of bone marrow promonocytes, monocytes and resident bone marrow macrophages/mouse could be computed.

Assessment of the circulating blood monocyte numbers

The number of circulating blood monocyte was calculated from the total number of blood leucocytes and the proportion of monocytes to leucocytes in Giemsa stained thin blood smears.

a. Blood leucocyte count

One volume of blood taken by cardiac puncture was diluted in 20 volumes of white blood cell diluting fluid containing 6% glacial acetic acid, and the number of nucleated cells was counted in a haemocytometer chamber using a Leitz microscope.

b. Differential cell count:

Differential cell counts were done with Giemsa stained thin blood smears prepared from blood taken by cardiac puncture. Blood monocytes were differentiated morphologically, and the ratio of monocytes to total leucocytes was determined by counting at least 200 leucocytes. The number of monocytes/ml blood was calculated from the monocytes to leucocyte ratios and the total number of leucocytes. The total numbers of blood monocytes per mouse was calculated, based on mouses body weight; the absolute mouse blood volume = 8.33% body weight.[15]

Assessment of splenic macrophage number:

To assess the number of spleen macrophages from each mouse, spleen leucocyte suspensions from whole spleen were prepared and the number of leucocytes was counted in haemocytometer chamber. Splenic macrophages were isolated by their adherent property on glass coverslip and counted under a microscope.

a. Spleen cell preparation:

Spleen of sacrificed animals was perfused by slow injection of 10 ml of 0.6 mM EDTA through the heart while the vena cava in the abdomen was cut. They were removed and put into a 50 mm diameter petri dish (Sterilin, Hounslow, Middlesex, England) containing 5 ml of 0.05% collaginase in RPMI. They were cut into pieces, and incubated at 37°C and 5% CO_2 for 30 minutes, then teased gently with sterile forceps to release a single cell suspension. Cell suspensions were transfered into 10 ml centrifuge tubes. The cells were washed in RPMI, and pelleted by centrifuging at 1200 rpm at 4°C for 10 minutes.

The cell pellets were resuspended in 2 ml Tris buffered ammonium chloride to lyse the erythrocytes. After being washed with RPMI, the leucocytes were resuspended in 10 ml complete medium, and the number of leucocytes was counted in a haemocytometer chamber.

b. Splenic macrophage isolation

Three hundred µl spleen leucocyte suspension from each mouse was plated as a drop on triplicate cover slips in 24 well tissue culture plates. After 30 minutes at 37^0C and 5% CO_2, 0.5 ml of the same medium was added very gently into each well and further incubated for 2 h. Non adherent cells were washed off with RPMI, and the adherent cells retained on cover slips were fixed with methanol. Cover slips from each mouse were then Giemsa stained, and after being washed and air dried, they were mounted on glass slides for mononuclear phagocyte counts.

c. Splenic macrophage count

The cover slips were examined using a Leitz microscope under ×400 magnification. Spleen macrophages that had adhered on to cover slips were counted as the number of splenic macrophages in 100 µl spleen cell suspension. The total number of spleen macrophages/mouse was calculated from the number of spleen macrophages in 100 µl spleen cell suspension and the total volume of spleen cell suspension.

RESULTS

Changes in the number of bone marrow promonocytes and monocytes

Bone marrow promonocytes and monocytes were isolated from the bone marrow and separated from the other cells by their adherent property on glass cover slips. This method was modified from that of van Furth *et al*,.[16] Microscopic examination of the cover slips indicated that after 6 h incubation in complete medium the majority of adherent cells were mononuclear phagocytes, contaminated with some granulocytes and other cells. The promonocytes and monocytes were differentiated morphologically, and the number of these cells/cover slip was counted. The changes in the number of bone marrow promonocytes and monocytes during *P. v. petteri* infection are shown in FIGURE 1. The average number of

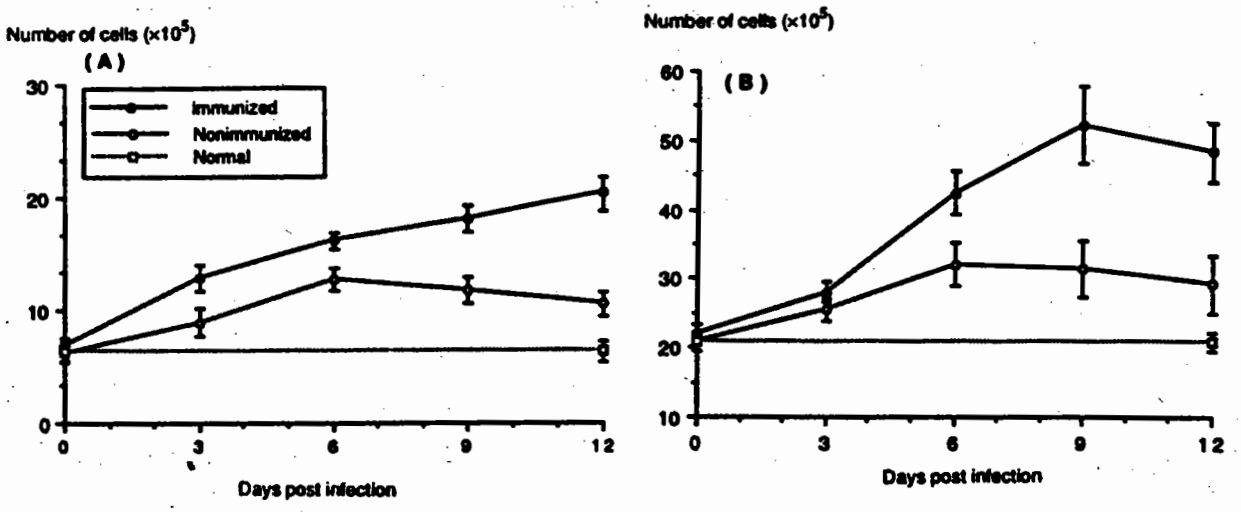

FIGURE 1. Changes in number of bone marrow promonocytes (A) and monocytes (B) during *P. v. petteri* infection. Two groups of 15 immunized and 15 non-immunized mice were infected with 10^3 parasitized red blood cells (PRBC) 15 days after immunization. At each time point, 3 mice from each group were sacrificed (except in the non-immunized group only 2 mice survived on day 12), and the numbers of promonocytes and monocytes were assessed. Each point represents the mean and SD of the number of cells/mouse. Dotted line is the value for normal non-infected mice.

bone marrow promonocytes in normal non-infected control mice and in immunized as well as non-immunized mice on day 0 (before infection) were similar, around 6×10^5 cells/mouse. By day 3 post infection, the number of promonocytes in immunized mice increased up to around 13×10^5 cells/mouse, and this was significantly higher than that in the non-immunized group ($P < 0.05$), which increased only up to around 8×10^5 cells/mouse. The number of cells in immunized mice continued to rise until the parasitaemia went down. On day 12 this peak was calculated to be about 20×10^5 cells/mouse. In non-immunized mice the number of bone marrow promonocytes increased to a lesser degree, and reached a peak of about 14×10^5 on day 6, followed by a decline until the mice died. In this group, one mouse died on day 11, and the other two were very ill before being sacrificed on day 12 post infection. Meanwhile, the average number of bone marrow monocytes also increased but to a different degree from that of promonocytes. During day 3 post infection the number of monocytes in immunized mice increased to around 1.4-fold, and in non-immunized mice around 1.2-fold, of the normal value. The former is significantly higher than the later ($P < 0.05$). The number of these cells were even higher during the later stages: in immunized mice they reached a peak of about 2.5-fold on day 9, while in the non-immunized group reached about 1.6-fold the normal level on day 6, and in both cases this was followed by a decline.

Changes in number of resident bone marrow macrophages

Isolation of resident bone marrow macrophages from the bone marrow stoma using gentle enzymatic dispersion was used, because techniques based on physical dispersion (vigorous pipetting or passage through fine gouge needles) result in damage to the delicate resident bone marrow macrophage plasma membrane[17]. After digestion with collaginase, the majority of marrow plug fragments had been dispersed into a homogenous suspension of cells, consisting of nucleated cells mostly in the form of single cells, and a number of clusters of more than 5 cells. The clusters were then separated from single cells by velocity sedimentation on a column of 30% FCS. Using these methods, the changes in the number of clusters recovered from the bone marrow of mice during *P. v. petteri* infection was monitored, and the results are shown in FIGURE 2.

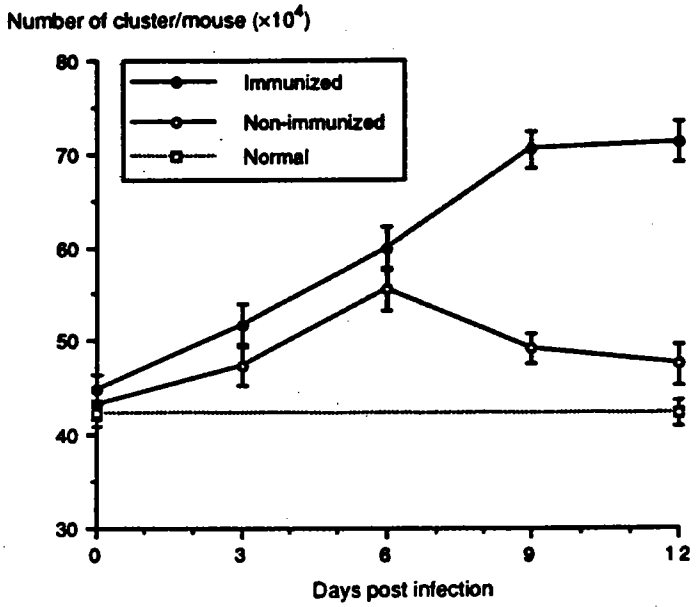

FIGURE 2. Changes in number of bone marrow haematopoietic clusters in mice during *P.v.petteri* infection. Two groups of mice: 15 immunized and 15 non-immunized were infected with 10^3 PRBC 15 days after immunization. At each group were sacrificed (except in the non-immunized group only 2 mice survived on day 12), bone marrow cells from 3 or 2 mice (6 or 4 femurs) were pooled and the numbers of bone marrow haematopoeitic clusters were assessed. Each value represents the mean and SD of the number of clusters calculated per mouse from 3 readings. Dotted line is the value of normal non-infected mice.

In normal non-infected control mice, the numbers of clusters recovered were calculated to be about 43×10^4 clusters/mouse. Microscopic examination of the clusters before detaching the clustering cells showed that the size of clusters varied between 5-88 cells/clusters. In non-immunized infected mice on day 0 (before infection) the average number of clusters was the same as those in the normal control group, but increased to around 46×10^4 on day 3 post infection. Number of clusters then continued to rise, reached a peak of about 58×10^4 on day 6, then declined afterwards. In the immunized group, the average number of clusters on day 3 post infection was 51.7×10^4/mouse, and this was significantly higher than the normal value ($P < 0.05$). The rise persisted until day 12 post infection, when it reached around 71×10^4 clusters which is about 1.6-fold the normal level.

To analyse the changes in the number of resident bone marrow macrophages, clustering non- adherent cells were detached by direct gentle flushing with PBS and the number of resident bone marrow macrophages that had adhered to coverslips were counted. The changes of the number of this cell during *P. v. petteri* infection is shown in FIGURE 3. In normal non-infected mice, the average number of resident bone marrow macrophages was calculated as 80.4×10^4 cells/ mouse. On day 3 post infection, the number of resident bone marrow macrophages in immunized mice increased to around 91×10^4 cells/mouse, which was significantly higher than the normal values ($P < 0.05$), while in the non-immunized group, the number of resident bone marrow macrophages calculated was still within the normal range. The numbers of resident bone marrow macrophages in immunized mice continued to rise, and reached nearly 2-fold the normal levels on day 9 and 12 post infection. In non-immunized infected mice, the number of resident bone marrow macrophages also increased but to a lesser degree, and reached a peak of about 120×10^4 cells/mouse on day 9 post infection followed by a decrease toward normal levels.

Changes in number of blood monocytes

In Giemsa stained thin blood smears prepared from blood taken by cardiac puncture, the

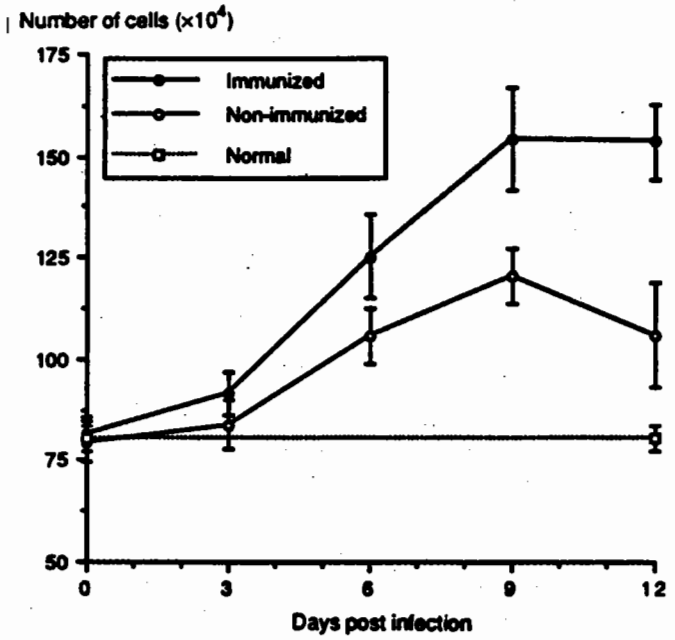

FIGURE 3. Changes in number of resident bone marrow macrophages during *P.v.petteri* infection. Two groups of mice: 15 immunized and 15 non-immunized were infected with 10^3 PRBC on day 0. At each time point, 3 mice from each group were sacrificed (except in the non-immunized group only 2 mice survived on day 12), and the number of resident bone marrow macrophages isolated by enzymatic digestion of the marrow plugs were measured. Each value represents the mean and SD of the number of cells from 3 cover slips calculated per mouse. Dotted line is the value of normal non-infected mice.

monocytes are clearly distinguished by their size, which are about 12-16 μm, and their indented or lobulated nucleus and greyish blue, finely granulated cytoplasm. In the smears taken during the late stage of infection, some of them contained parasite pigment. The number of blood mono- cytes in each mouse was calculated from the ratio of monocytes to leucocytes and the total number of blood leucocytes.

The changes in the number of blood monocytes during *P. v. petteri* infection are shown in FIGURE 4. In normal mice, the number of blood monocytes was calculated to be about $1.4x10^6$ cells/mouse. On day 0 (before infection) the number of these cells in immunized and non-immunized mice was within normal level. On day 3 post infection a significant increase was already observed in immunized ($P < 0.05$) but not in non-immunized mice. In non-immunized mice, the number of these cells reached the peak of about $3.4x10^6$ cells/mouse on day 6 post infection, then it declined until their death. In two mice which still survived on day 12, the average number of blood monocytes was $1.9x10^6$ cells/ mouse. In immunized mice, the number of these cells was greater as compared to the non-immunized group, and reached a peak of about $5.5x10^6$ cells/mouse on day 9 and declined to around $4.8x10^6$ on day 12 post infection. The average number of blood monocytes in immunized mice on day 9 and 12 post infection was significantly higher than in the non-immunized groups ($P < 0.01$).

Changes in the number of spleen macrophages

Cell yield: the weight of the spleen for normal control, immunized and non-immunized mice before infection averaged 133 mg and differed considerably between mice (range 82-184 mg). The average number of white cells collected was $1.4x10^8$ cells/spleen (range, $1.0\text{-}2.15x10^8$ cells/ spleen).

After 2 h incubation of spleen cells, the majority of adherent cells retained on glass cover slips were macrophages (± 85%) as judged by morphological criteria and esterase staining. Some immature forms (promonocytes) were also observed, and became more numerous during the later stages of infection. The spleen adherent

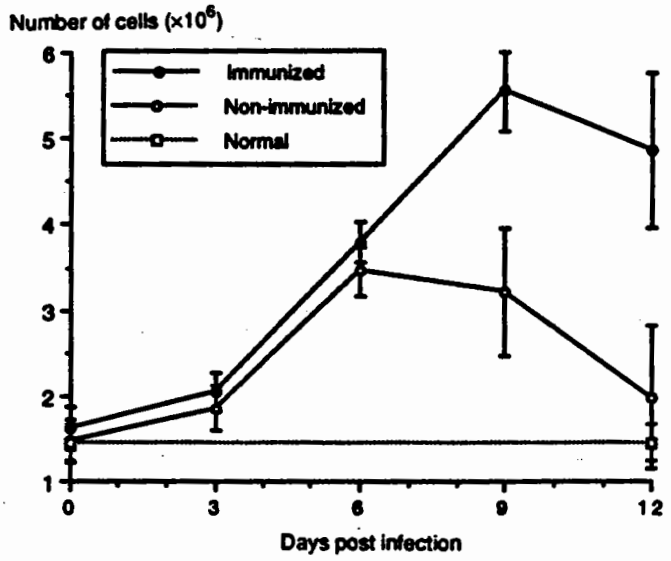

FIGURE 4. Changes in the number of circulating blood monocytes during *P. v. petteri* infection. Two groups of mice: 15 immunized and 15 non-immunized were infected with 10^3 PRBC 15 day after immunization. At each time point, 3 mice from each group were sacrificed (except in the non-immunized group only 2 mice survived on day 12), and number of circulating blood monocytes were measured. Each value represents the mean and SD of the number of cells per mouse. Dotted line is the value of normal non-infected mice.

macrophages are fairly homogenous in size and shape at 2 h after plating, with diameters ranging from 11.2-16.8 μm. The nucleus is usually round or oval and the cytoplasm is greyish blue and often contains refractile inclusions or some debris. In infected mice, the cytoplasm of the spleen macrophages was more extended than that from normal mice, and during later stages of infection it was mostly loaded with parasite pigment.

The changes in the number of spleen macrophages during *P. v. petteri* infection is shown in FIGURE 5. In normal non-infected control mice, the average number of spleen adherent macrophages was about 4.5×10^6 cells. The number of these cells in immunized and non-immunized mice increased gradually with the course of infection. In the non-immunized group this increase reached a peak of about 28.5×10^6 cells, which was around 6.3-fold the normal value, on day 9 post infection and declined afterwards. In immunized mice, the increase of the number of spleen macrophage was greater, and by day 12 the average number of spleen macrophages was about 12 fold the normal level.

DISCUSSION

The quantification of mature mononuclear phagocytes in the bone marrow, circulating blood and spleen of LACA mice during the stages of *P. v. petteri* infection indicated that there is a substantial increase in numbers of these cell subpopulations during the early stages, followed by a decline toward normal levels during the later stages until the death of the host. Similar observations had also been reported in acute infections caused by *P. berghei*.[7,8,10,11] In this immunization model, the increase of these cells is exacerbated and persistent up to the later stages until their recovery. Greater increases in numbers of mononuclear phagocytes were also observed in mice infected with non lethal *P. yoelii*[8,18,19] and *P. chabaudi adami*[11] compared to lethal *P. berghei* infection.

In human[20] as in rodents,[21,22] macrophage hyperplasia associated with splenomegaly is a prominent feature of the immunological defence of the host against malarial infection. The present result shows that the increase in numbers of spleen macrophages as well as circulating blood

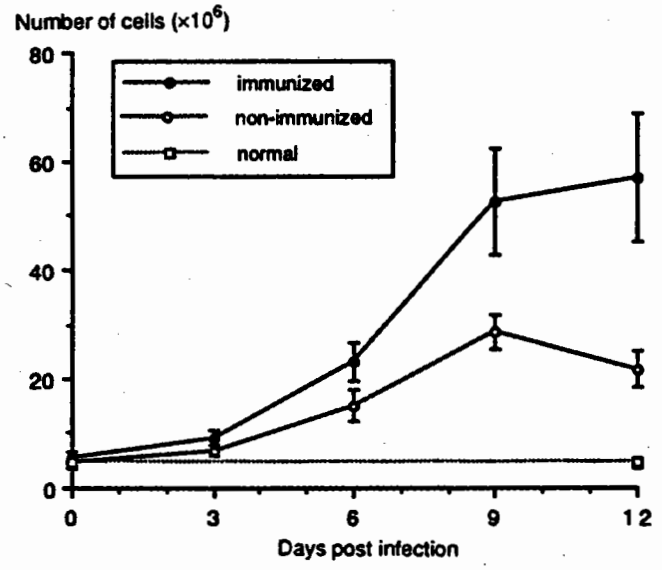

FIGURE 5. Changes in the number of spleen macrophages during *P. v. petteri* infection. Two groups of mice: 15 immunized and 15 non-immunized were infected with 10^3 PRBC 15 days after immunization. At each time point, 3 mice from each group were sacrificed (except in the non-immunized group only 2 mice survived on day 12), and the number of spleen macrophages were measured. Each value represent the mean and SD of the number of cells per spleen. Dotted line is the value for normal non-infected mice.

and bone marrow mononuclear phagocytes closely related to the reduction of parasitaemia in immunized mice, suggesting that these cells may contribute to the parasite elimination. Spleen macrophages in immunized mice were significantly higher than in non-immunized groups from day 6 post infection and even more expanded, up to about 12-fold the normal values, on day 9 and 12, which coincided with the decline of parasitaemia. In non-immunized mice this increase was only up to 6-fold on day 9 followed by a decline until death. Gross *et al*,[10] in their investigation, using *P. berghei* infected BALB/c mice, concluded that the increase of splenic macrophages, which were predominantly immature forms and functionally defective, likely contributed to the failure of the immune system of the host to overcome the disease during lethal infection.

Spleen macrophages originated from their progenitors in the bone marrow, after differentiation and maturation processes, migrated via the blood circulation into the spleen. Van Furth and Diesselhoff-den Dulk[23] suggested that some spleen macrophages are produced locally and determined a dual origin of spleen macrophages in the present study, the increase in number of bone marrow promonocytes resulted in the increase of bone marrow and blood monocytes and subsequently spleen macrophages. The observation of some splenic promonocytes on the cover slips suggests the increase of local monocyte production since these cells were not observed in the blood smears. However, the possibility of migratory origin from the bone marrow still cannot be excluded, since the culture of peripheral blood nucleated cells from mice during *P. berghei* infection can also produce granulocytes and macrophage colonies.[19]

Previous studies have demonstrated the important role of mononuclear phagocytes during malarial infections. Besides increases in the number, these cells are more activated, as indicated by the increase of the cell size, Fc and complement receptor expressions,[2] and the ability to release parasiticidal molecules.[5,6] In addition, the important of the spleen in protection against blood stage malarial infections has also been well documented. In most cases removal of the spleen results in the recurrence or relapse of an otherwise latent infection.[24] Furthermore, it has been shown that an architecturally intact spleen is necessary to control non-lethal infections.[25] The importance of this organ can also be attributed to the function of splenic mononuclear phagocyte populations.

In the present study, the majority of resident bone marrow macrophages from infected mice were overloaded with parasite pigment. Erythrophagocytosis and hyperplasia of resident bone marrow macrophages have also been reported in patients with *P. falciparum* infection.[26] However, the role of resident bone marrow macrophages in protection against malarial infections is still not fully understood. Crocker and his colleagues suggested that these cells play a role in the modulation of haematopoietic proliferation. After their isolation, the majority of these cells expressed the sheep erythrocyte receptor (SER), which recognized sialylated glycoconjugate on the surface of erythrocytes.[27,28] Within the bone marrow stroma they formed clusters with some erythroid and myeloid cells including those of the granulocytic and monocytic lineage. Therefore it is likely that these cells also play a role in the modulation of mononuclear phagocyte proliferation, probably through the release of specific macrophage growth factors as shown in mouse blood monocytes after stimulation with phorbol myristate acetate (PMA) and phytohaemagglutinin (PHA)[29] or with granulocytes macrophage colony stimulating factor (GM-CSF) and lipopolysaccharide (LPS)[30] and, in human blood monocytes, after stimulation with LPS and PMA.[31] In the present study, the changes in number of haematopoietic clusters and resident bone marrow macrophages isolated from the bone marrow during the course of infection indicated the haematopoietic activities which are probably related to the haemopoetic demand, including monocytic requirement, to overcome the infection. The average number of resident bone marrow macrophages/cluster, which increased from 1.8 in normal controls to 2.2 and 2.1 in immunized and non-immunized mice during day 9 post infection, probably also indicates an increase in the effectivity of clusters in haemopoetic production. During the late stage of infection, the majority of resident bone marrow macrophages contained parasite pigment indicate

that these cells also play a role in the clearance and possibly in killing the parasites. The functional activities of resident bone marrow macrophages as effector cells and as growth factor producers will be discussed in the other publication.

CONCLUSION

During the course of *P. v. petteri* infection the number of bone marrow promonocyte in immunized mice increased from 6×10^5 cells/ mouse to 13×10^5 cells/mouse on day 3 post infection and continued to increased up to 20×10^5 cells/ mouse on day 12, when the parasitemia went down. In non-immunized mice the number of cells increased only up to 14×10^5 cells/ mouse on day 6 post infection followed by decline until the mice died. The number of bone marrow monocyte in immunized mice increased 2.5 fold by day 9 and in non-immunized mice increased 1.6 fold by day 6, both were followed by decline. The number of blood monocyte also increased from 1.4×10^6 cells/mouse to 5.5×10^6 cells/mouse in immunized mice on day 9 post-infection, and this was significantly higher than the number in non-immunized mice. The number of splenic macrophage of immunized mice increased up to 12 fold on day 12 post infection, while in non-immunized mice increased 6.3 fold on day 9 then declined afterward. The changes in the number of mature mononuclear phagocytes in the bone marrow, circulating blood and spleen during *P. v. petteri* infection represent an effort of the immune system to overcome the infection. The number of these cells increased more in immune than in non-immune mice. The increase in the number of these cell populations indicates the increase in the production and proliferation activities of their progenitors which mostly occur in the bone marrow[29] and the migration of mature cells into the circulation and subsequently into organs.

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