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Development of basophils in Mongolian gerbil - Formation of basophilic cell clusters in the bone marrow after *Nippostrongylus brasiliensis* infection

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Running title: Basophilic cell cluster in bone marrow

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SUMMARY: Development of basophilic leukocytes was studied in the Mongolian gerbil, *Meriones unguiculatus*, following infection with the nematode *Nippostrongylus brasiliensis* (NB). After infection, peripheral blood basophilia developed and peaked after 2 weeks. In bone marrow sections, numbers of alcian blue$^+$/safranine$^-$ basophilic cells were increased. These cells did not bind berberine sulfate and were clearly distinguishable from the bone marrow-resident mast cells, which were safranine$^+$ and berberine sulfate$^+$. Alcian blue$^+$/safranine$^-$ cells were identified by electron microscopy as basophilic myelocytes in various stages of maturation. In the early period of infection, these cells had round to oval granules with homogenous electron-dense matrix, well-developed Golgi apparatus and rough endoplasmic reticulum (rER), and a non-segmented nucleus. By enzyme cytochemical analysis, intense peroxidase activity was demonstrated in all specific granules, rER, and Golgi apparatus. Two weeks after infection, bone marrow basophilic cells were further increased in number, forming distinct clusters or islands composed of up to 100 cells. On electron micrographs, basophilic cells in the clusters appeared to be late basophilic myelocytes; i.e. increased number of granules, less conspicuous Golgi apparatus and rER, horseshoe-shaped to lobulated nucleus, and reduced peroxidase activity. Eosinophils and mast cells were rarely found in the basophilic cell clusters. Four weeks after infection, these clusters were not found. These results show that gerbil basophilic myelocytes tend to form cell clusters in the bone marrow during their active proliferation. The comparative paucity of other cell lineages in basophilic-cell clusters suggests that basophilia is generated from differentiation/proliferation of pre-committed basophil progenitors independently from cells of other lineages.

Abbreviations: NB: *Nippostrongylus brasiliensis* ; rER: rough endoplasmic reticulum

Key words: basophil, mast cell, peroxidase, bone marrow, Mongolian gerbil, ultrastructure, *Nippostrongylus brasiliensis*

Introduction

Basophils and mast cells are derived from multipotent hematopoietic stem cells (Kitamura, 1989; Galli, 1990; Denburg, 1992). Both types of cells have metachromatic granules that contain histamine, proteoglycans and neutral proteases, and these cells express high affinity IgE receptor, $Fc_\varepsilon RI$, on their cell surface (Metzger et al, 1986; Thompson et al, 1990). Upon activation through $Fc_\varepsilon RI$, basophils and mast cells release a variety of mediators such as histamine, proteases and leukotrienes. Aside from these common features, the two types of cells

differ in various aspects in cell differentiation and maturation as well as in some ultrastructural and cytochemical features. By electron microscopy, basophils can be seen to have fewer cytoplasmic granules and less developed cell-surface projections than mast cells (Dvorak et al, 1982; Dvorak et al, 1983). Although basophils, like other granulocyte, complete their differentiation in the bone marrow and then circulate in the blood, mast cell progenitors leave the bone marrow, enter various peripheral tissues, and differentiate into morphologically identifiable mast cells (Kitamura, 1989; Galli, 1990; Denburg, 1992).

Basophils constitute only a small fraction of the total leukocyte population, and are hardly found in the peripheral blood in normal rats and mice. Infection with intestinal nematodes such as *Nippostrongylus brasiliensis* (NB) and *Trichinella spiralis* has been reported to induce distinct basophilia in the blood (Ogilvie et al, 1978; Ogilvie et al, 1980) as well as mastocytosis in the intestinal mucosa (Miller and Jarrett, 1971). We previously showed that NB infection induced significant increases in the numbers of peripheral blood basophils, not only in normal rats, but also in *Ws/Ws* rats, which have a 12-base deletion in the tyrosine kinase domain of the *c-kit* gene and congenitally lack mast cells (Kasugai et al, 1993). Since these phenomena do not occur in nematode-infected athymic nude rats (Ogilvie et al, 1980; Arizono et al, 1990; Kasugai et al, 1993), the proliferation of basophils and mast cells appears to be dependent on T cell factors. The cytokines produced by T cells such as IL-3, IL-4 and IL-5 have been shown to play essential roles in the in vitro production of basophils and IL-3, IL-4, IL-9 and IL-10 for in vitro mast cell development (Denburg, 1992; Kitamura et al, 1993). In fact, significant increases in the gene expression and production of these cytokines have been observed in nematode-infected mice and rats (Street et al, 1990; Svetic et al, 1993; Matsuda et al, 1995). Further, it was shown that continuous infusion of recombinant human IL-3 induced significant basophilia in rhesus monkeys (Dvorak et al. 1989).

The Mongolian gerbil is a suitable experimental model for studying neuronal and cerebrovascular diseases because of the animal's high susceptibility to cerebral infarction (Bertorelli et al, 1995; Vincent et al, 1979). Although basophilis were reported to be present at less than 0.5 % both in peripheral blood as well as in the bone marrow of this animal when observed by conventional staining methods (Weeks and Glomski, 1978; Termer and Glomski, 1978), the ultrastructure and the development of basophils in the bone marrow has not yet been studied. Recently, we reported that mucosal mast cells in Mongolian gerbils showed different cytochemical features from those in rats and mice; intestinal mucosal mast cells in mongolian gerbils were formalin-resistant and exhibited positive staining with safranine and berberine sulfate similarly to dermal mast cells, although the mucosal and connective-tissue mast cells were distinguishable by their ultrastructural features as well as by the types of proteases in the granules (Horii et al, 1992; Nawa et al, 1994). In the present study, we examined the proliferation/differentiation of basophilic leukocytes in the bone marrow of mongolian gerbils following NB infection. Our results showed that the Mongolian gerbil exhibits a strong basophil response and forms basophilic cell aggregates or islands in the bone marrow after NB infection.

Results and Discussion

1. Peripheral blood basophilic cells in the Mongolian gerbil

It has been shown that significant mucosal mastocytosis is induced in the Mongolian gerbil 2 weeks after NB infection (Horii et al, 1992). In the present study, the numbers of peripheral blood total leukocytes, eosinophils and basophils were counted before and after NB infection. Numbers of eosinophils and basophils increased and reached a peak 2 weeks after

infection, and then decreased gradually (Fig. 1). Numbers of basophils at 2 weeks after infection were 18.7-fold greater than those in uninfected animals. The level of peripheral blood basophilia in the gerbil was comparable to that in NB-infected rats (Kasugai et al, 1993).

Buffy coat was obtained from peripheral blood of gerbils 2 weeks after infection, and the ultrastructure of circulating basophils was examined. Basophils had large cytoplasmic granules, 0.5-1.0 μm in diameter, which were larger than those of eosinophils (Fig. 2A, B). Basophil cytoplasmic granules were round to oval membrane-bound structures with homogeneous electron-dense matrix and no crystalloid structures. The nucleus was segmented into multiple lobes with chromatin heavily condensed beneath the nuclear membrane. The plasma membrane had short thick folds. The ultrastructural architecture of gerbil basophilic granules, which were homogenous and electron dense, was the same as those reported in mice and rats (Dvorak et al, 1982; Kasugai et al, 1993), while they differed from guinea pig basophilic granules which display parallel crystalline arrays (Terry et al, 1969; Dvorak and Monahan, 1985), or from human basophilic granules which contain electron-dense particles and/or membranous structures (Zucker, 1967; Hastie, 1974; Dvorak et al, 1983).

2. Formation of basophilic cell clusters in the bone marrow of the Mongolian gerbil

To identify basophilic cells in the bone marrow, sections of bone marrow were cut and stained with alcian blue and safranine. In uninfected gerbil marrow, alcian blue$^+$/safranine$^-$ cells were sparsely distributed (Fig. 3A). After infection, the numbers of alcian blue$^+$/safranine$^-$ cells increased significantly (Fig. 3B), and then these cells formed large clusters or islands 2 weeks after infection (Fig. 3C). These cells were shown to have metachromatic granules in their cytoplasm with toluidine blue-O as well as with Giemsa staining. . The alcian blue$^+$/safranine$^-$ cells which formed the clusters were not stained with berberine sulfate, which specifically binds to heparin (Fig. 4).

Basophilic cell clusters were examined by electron microscopy. To locate basophilic cell clusters, 1-μm Epon-embedded sections were cut and stained with toluidine blue-O. The Epon blocks were trimmed so that a basophilic cell cluster was properly placed in the center of field, and ultrathin sections were cut. As shown in Fig. 5, the clusters consisted mainly of cells with large cytoplasmic granules similar to those found in peripheral blood basophils. A few macrophages, neutrophilic cells, and eosinophilic cells were also seen in the clusters. However, because of the paucity of these cells, it was presumed that the majority of these cells may have been contaminants mixed in from the periphery of the clusters during the process of bone marrow sampling.

Previously, we reported that both connective tissue and gut mucosa gerbil mast cells stained positively for safranine and berberine (Nawa et al, 1994). In the bone marrow sections in the present study, small numbers of safranine$^+$ cells were also observed (Fig. 3A, B), and these cells were found to bind berberine sulfate (Fig. 4). With toluidine blue-O as well as with Giemsa staining, these cells were seen to have numerous metachromatic granules. From these features, the safranine$^+$ berberine$^+$ cells were considered to be bone marrow-resident mast cells. Bone marrow mast cells did not appear within the basophilic cell clusters and their numbers did not increase after infection. These results support the generally accepted view that basophils and mast cells develop from different progenitors (Denburg, 1992; Galli et al, 1984; Galli, 1993).

The sizes of clusters and the numbers of basophilic cells in each cluster were analyzed in 4 μm sections obtained 2 weeks after infection. As shown in Fig. 6A and B, the mean area of the clusters was 1548 μm^2 and each

cluster contained up to 100 basophilic cells. The clusters were significantly reduced in size and number at 3 weeks after infection and disappeared at 4 weeks when alcian blue$^+$/safranine$^-$ cells were scattered at a density comparable to that in uninfected animals. This indicates that after completion of differentiation in the bone marrow, basophils left the bone marrow and entered the circulation.

3. Ultrastructure and peroxidase activity of bone marrow basophilic cells in the Mongolian gerbil

Development of bone marrow basophilic cells was studied 1 and 2 weeks after nematode infection. One week after infection, immature basophilic cells were frequently observed. These cells had several large membrane-bound granules, prominent Golgi apparatus and rER, and oval-shaped nuclei with dispersed chromatin (Fig. 7A). We considered them to be basophilic myelocytes. High levels of peroxidase activity were found in all granules, rER, Golgi apparatus and perinuclear cisternae by enzyme cytochemistry (Fig. 7B).

As described above, basophilic cell clusters appeared 2 weeks after infection. The majority of cells in the clusters appeared to be more differentiated than those found 1 week after infection. The nucleus was horseshoe-shaped or roughly lobulated, and the chromatin became more condensed (Fig. 7C). Cytoplasmic granules were more abundant than those found 1 week after infection, and Golgi apparatus and rER became less conspicuous. These cells were considered to be late basophilic myelocytes. Almost fully mature basophils were also observed in the clusters. Peroxidase activity of granules in late basophilic myelocytes and mature basophils varied from cell to cell and among granules of the same cell, usually localizing only to some specific granules (Fig. 7D). No reaction product was present in the rER or Golgi apparatus. Gerbil basophilic myelocytes showed intense endogenous peroxidase activity, while peroxidase synthesis appeared to cease after maturation. These results are consistent with those in rat basophilic myelocytes (Kasugai et al, 1993), but not with guinea pig and human basophilic cells which are negative for endogenous peroxidase activity (Dvorak et al, 1972; Dvorak et al, 1985).

Bone marrow mast cells were clearly distinguishable from basophilic cells by their ultrastructure. Mast cells had well-developed elongated processes on the cell surface and more uniform and round-shaped cytoplasmic granules than those of basophils (Fig. 7E). Some of the mast cell granules were positive for peroxidase activity (Fig. 7F).

An important finding in the present study was that gerbil basophilic myelocytes formed distinct cellular clusters in the bone marrow in response to nematode infection. To our knowledge, such clusters or aggregates of basophilic cells have not been reported in the bone marrow of other mammals in non-neoplastic states. In the present study, gerbil bone marrow was recovered *en bloc* and was cut into paraffin- or Epon-embedded sections without dispersing the cells, allowing the bone marrow to maintain the original cellular association at least to some degree. On the other hand, in many previously reported ultrastructural studies, bone marrow cells were obtained by flushing from the bone marrow, which might have dispersed the cells in basophilic cell clusters. We studied sections of NB-infected rat bone marrow obtained *en bloc*, but definite clusters of basophilic cells were not found even though basophilic myelocyte numbers were significantly increased (Kasugai et al, 1993). Thus, gerbil basophilic myelocytes might exhibit more significant cell-cell adherence than those in other mammals.

The gerbil basophilic cell clusters in 4-μm sections consisted of up to 100 cells, indicating that the actual numbers of basophilic cells in a cluster would be much larger. The clonal origin of the cluster-forming cells was not confirmed in the present

study. However, since the maturation of basophilic myelocytes in the clusters appeared to proceed rather synchronously, it is presumed that clusters were each derived from the same colony-forming progenitor in the bone marrow. It has been reported that not only pure basophil colonies but also mixed eosinophil/basophil colonies were generated in methylcellulose cultures of human peripheral blood mononuclear cells from normal as well as from atopic subjects in the presence of various conditioned media or recombinant cytokines (Leary and Ogawa, 1984; Denburg et al, 1985; Gibson et al, 1991). In the gerbil, on the other hand, no substantial coexistence of eosinophilic cells was observed in the basophilic cell clusters. Thus, it is suggested that the development of basophils induced by nematode infection in the Mongolian gerbil might be derived largely from basophil-committed progenitors.

Methods

Animals and nematode infection

An inbred strain (MSG/Sea) of Mongolian gerbil, *Meriones unguiculatus*, was purchased from Seiwa Experimental Animals (Fukuoka, Japan). Gerbils received a subcutaneous injection of 1000 infective-stage larvae of NB at 8 weeks of age as described previously (Horii et al, 1992).

Counting of peripheral blood leukocytes

Blood samples were obtained from the tail vein. Numbers of total leukocytes were counted with an improved Neubauer hemocytometer. Numbers of eosinophils and basophils were directly counted. Basophil counts were performed according to the method using staining medium containing alcian blue, originally described by Gilbert et al. (1975) and slightly modified by Kasugai et al. (1993). Eosinophils were stained with Hinkelmann's solution and their numbers were counted as described previously (Kasugai et al, 1993).

Staining of bone marrow basophilic leukocytes

The bone marrow was carefully removed *en bloc* from the femurs through a wide crack in the bone made using pliers. The samples were fixed in Carnoy's fluid, dehydrated and embedded in paraffin. Sections (4 μm thick) were then cut and stained with toluidine blue-O, Giemsa, or with alcian blue and safranine according to the method described by Enerbäck (1966). Some sections were stained with berberine sulfate as described by Enerbäck (1974) and photographed under a fluorescence microscope. These sections were washed in distilled water, and subsequently stained with alcian blue and safranine to examine the same field under a light microscope.

Measurements of basophilic-cell clusters

All the alcian blue$^+$/safranine$^-$ basophilic-cell clusters in 4 μm paraffin-embedded bone-marrow sections were photographed, and the numbers of alcian blue$^+$/safranine$^-$ basophilic cells in each cluster were counted on the photographs. The area of each cluster was determined using Nikon Cosmozone 1S electric digitizer (Nikon, Tokyo, Japan).

Electron microscopic analysis of peripheral blood basophils

Blood samples were obtained from the tail vein and drawn into acrylic capillary tubes coated with heparin (0.9 mm inner diameter, 75 mm in length, Kayagaki Co. Ltd., Tokyo, Japan). The samples were centrifuged for 10 minutes at 350 g, and the capillary tubes were each cut into a 2 mm section at the level where the buffy coat was formed. The cut tube containing the buffy coat was immersed in a mixture of 2.5% glutaraldehyde, 2% paraformaldehyde, 0.2% $CaCl_2$ and 0.1 M cacodylate buffer, pH 7.4, for 2 hours at 4°C. The buffy coat was then removed carefully from the tube. After washing with 0.1 M cacodylate buffer, the buffy coat was quickly mixed with

warmed 2% agar in a test tube (No. 72.700, Sarstedt Co. Ltd., Nümbrecht, Germany), centrifuged, and placed in an ice bath for 30 minutes. The buffy coat in agar was removed by cutting the bottom of the tube using a razor blade, post-fixed for 1 hour in 1% osmium tetroxide, and immersed in 1% tannic acid for 30 minutes at 4°C. The samples were washed three times in 1% Na_2SO_4 and 1% CH_3COONa at 4°C to remove phosphate, stained *en bloc* at 4°C overnight with 0.5% uranyl acetate, and embedded in Epon 812 (Nacalai Tesque Co. Ltd., Kyoto, Japan). Ultrathin sections were cut, stained with lead citrate, and observed under a JEOL JEM-100 CX II electron microscope.

Electron microscopic analysis of bone marrow cells

The bone marrow was carefully removed *en bloc* from the femurs as described above and fixed in a mixture of 2.5% glutaraldehyde, 2% paraformaldehyde, 0.2% $CaCl_2$ and 0.1 M cacodylate buffer, pH 7.4, for 2 hours at 4°C. After washing with 0.1 M cacodylate buffer, the samples were post-fixed for 1 hour in 1% osmium tetroxide, and embedded in Epon 812.

To locate basophilic cell clusters, sections 1 μm thick were cut and stained with toluidine blue-O. Epon blocks were trimmed so that a cluster was placed in the center. Serial ultrathin sections were cut, stained with lead citrate and uranyl acetate, and observed under an electron microscope.

Cytochemical analysis of endogenous peroxidase

The bone marrow tissue obtained as described above was fixed in a mixture of 1.25% glutaraldehyde, 1% paraformaldehyde, 0.025% $CaCl_2$ and 0.1 M cacodylate buffer, pH 7.4 for 2 hours at 4°C. After washing with 0.05 M Tris-HCl buffer, pH 7.6, the samples were incubated for 1 hour at room temperature in Graham and Karnovsky's medium (0.05% 3-3' diaminobenzidine tetrachloride and 0.01% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6) as descried by Dvorak et al. (1981). Samples were then washed three times in Tris buffer, pH 7.6, postfixed in 1% osmium tetroxide for 1 hour, and embedded in Epon 812. Ultrathin sections were cut, stained lightly with lead citrate, and observed under an electron microscope.

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Figure Legends

Fig. 1.
Numbers of total leukocytes (A), eosinophils (B) and basophils (C) in the peripheral blood of Mongolian gerbils after infection with *N. brasiliensis*. Data shown are means ± SE of 6 animals. *Significantly different from the preinfection values ($P < 0.05$).

Fig. 2.
Ultrastructural features of a basophil (A) or eosinophil (B) leukocyte in the peripheral blood of Mongolian gerbil 2 weeks after *N. brasiliensis* infection. The basophil has a segmented nucleus with condensed chromatin, and large oval cytoplasmic granules. The short surface processes of the basophil are comparable to those of the eosinophil. X 8,600.

Fig. 3.
Basophilic cells in the bone marrow of gerbils before (A), 1 week (B), and 2 weeks (C) after infection with *N. brasiliensis*. Bone marrow sections were cut and stained with alcian blue-

safranine. Arrowheads indicate safranine$^+$ mast cells. Alcian blue$^+$/safranine$^-$ basophilic cells were increased in number after infection, and formed large clusters after 2 weeks (C). X380.

Fig. 4.
Successive staining of basophilic cells with berberine sulfate and alcian blue-safranine in the bone marrow of gerbils 2 weeks after *N. brasiliensis* infection. Section were first stained with berberine sulfate and photographed under a fluorescence microscope (A). The sections were then washed and stained with alcian blue and safranine (B). Alcian blue$^+$/safranine$^-$ basophilic cells did not bind berberine sulfate, whereas safranine$^+$ mast cells (arrow) were stained with berberine sulfate. X380.

Fig. 5.
Ultrastructure of a part of basophilic cell cluster in the Mongolian gerbil bone marrow 2 weeks after *N. brasiliensis* infection. The cluster consists mainly of immature or mature basophils. Basophilic cells have a horseshoe-shaped or lobulated nucleus and are filled with many round or oval granules. X3,400.

Fig. 6.
Size of basophilic cell clusters (A) and the numbers of cells in each cluster (B) in the bone marrow of Mongolian gerbils 2 weeks after *N. brasiliensis* infection. A total of 35 clusters were analyzed in alcian blue-safranine-stained 4 μm sections.

Fig. 7.
Ultrastructural features of basophilic myelocytes (A, B), late basophilic myelocytes (C, D) and mast cells (E, F) in the bone marrow of Mongolian gerbils. Peroxidase activities are shown in B, D and F. The basophilic myelocyte observed 1 week after infection contains basophil-specific large granules and numerous rER and has slightly concave nucleus (A). A mast cell (M) can be seen adjacent to the basophilic myelocyte (A). In the basophilic myelocyte, the rER, perinuclear cisternae, all cisternae of the Golgi apparatus and all cytoplasmic granules are peroxidase$^+$ (B). A late basophilic myelocyte observed 2 weeks after infection; this cell has a lobulated nucleus with slightly condensed chromatin (C). In the late basophilic myelocyte, peroxidase activity is localized only in some granules (D). A mast cell showing thin, elongated surface processes, a round nucleus, and numerous cytoplasmic granules (E). Some of the mast cell granules show peroxidase activity (F). X7,500.

Fig 1

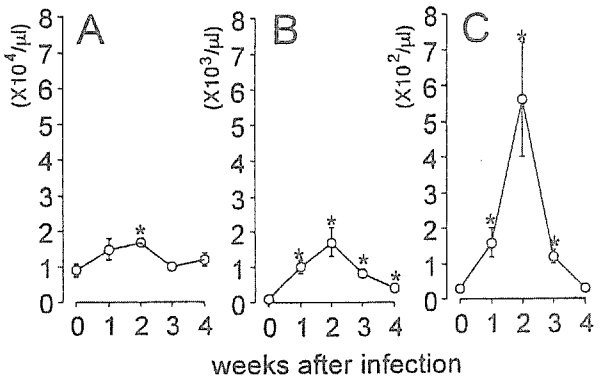
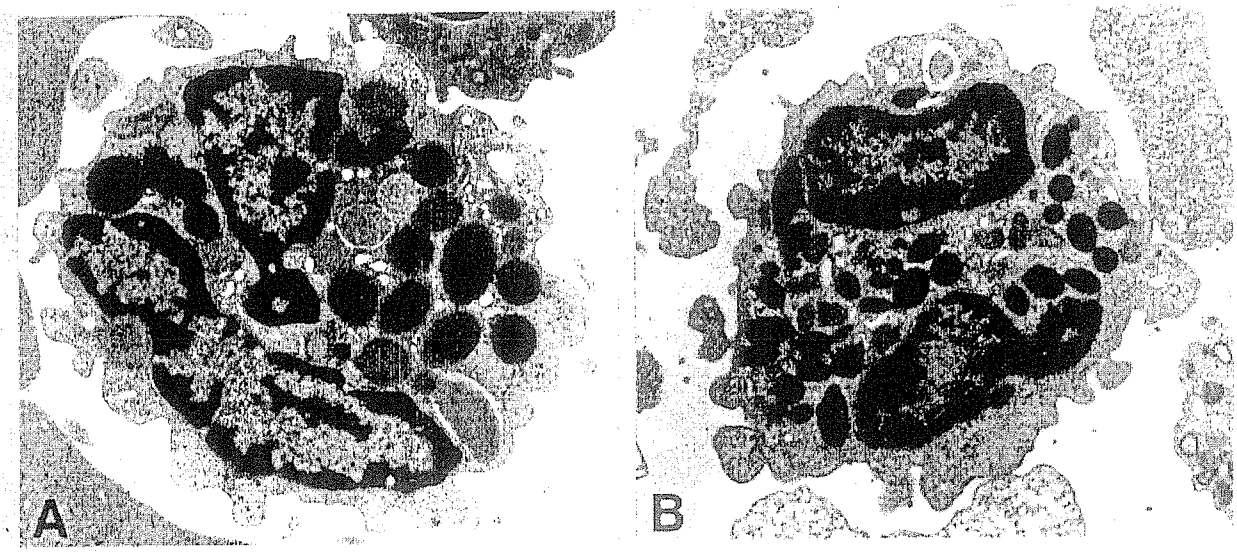

A
B
C
(X10⁴/μl)
(X10³/μl)
(X10²/μl)
0 1 2 3 4 5 6 7 8
0 1 2 3 4
weeks after infection

Fig 2 x2400



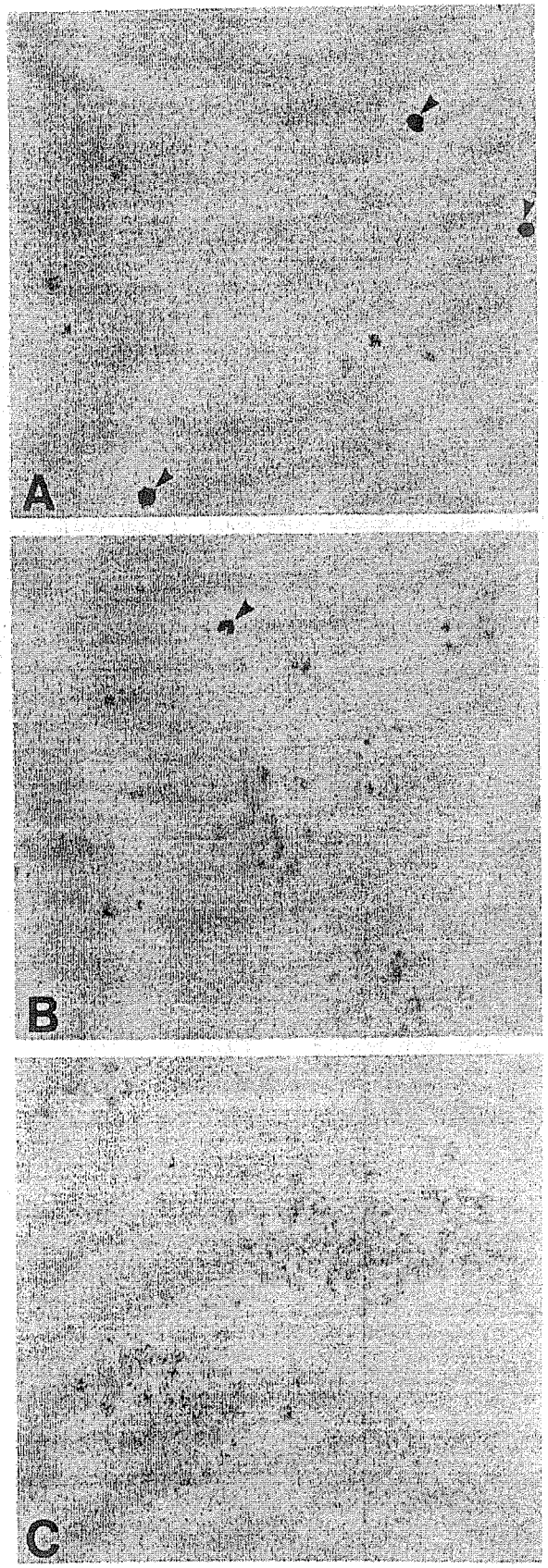

A
B
C

Fig 4 x 350

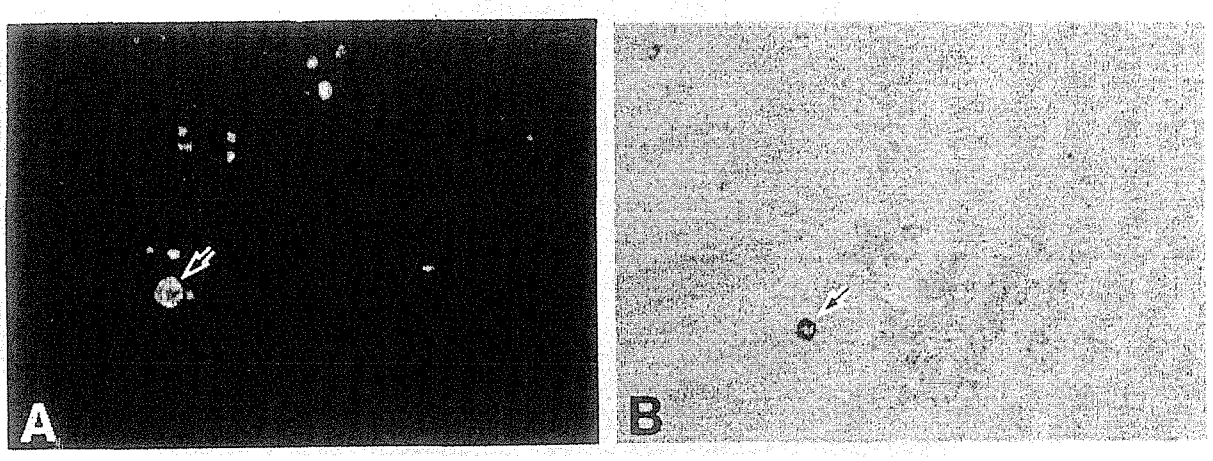


Fig 5

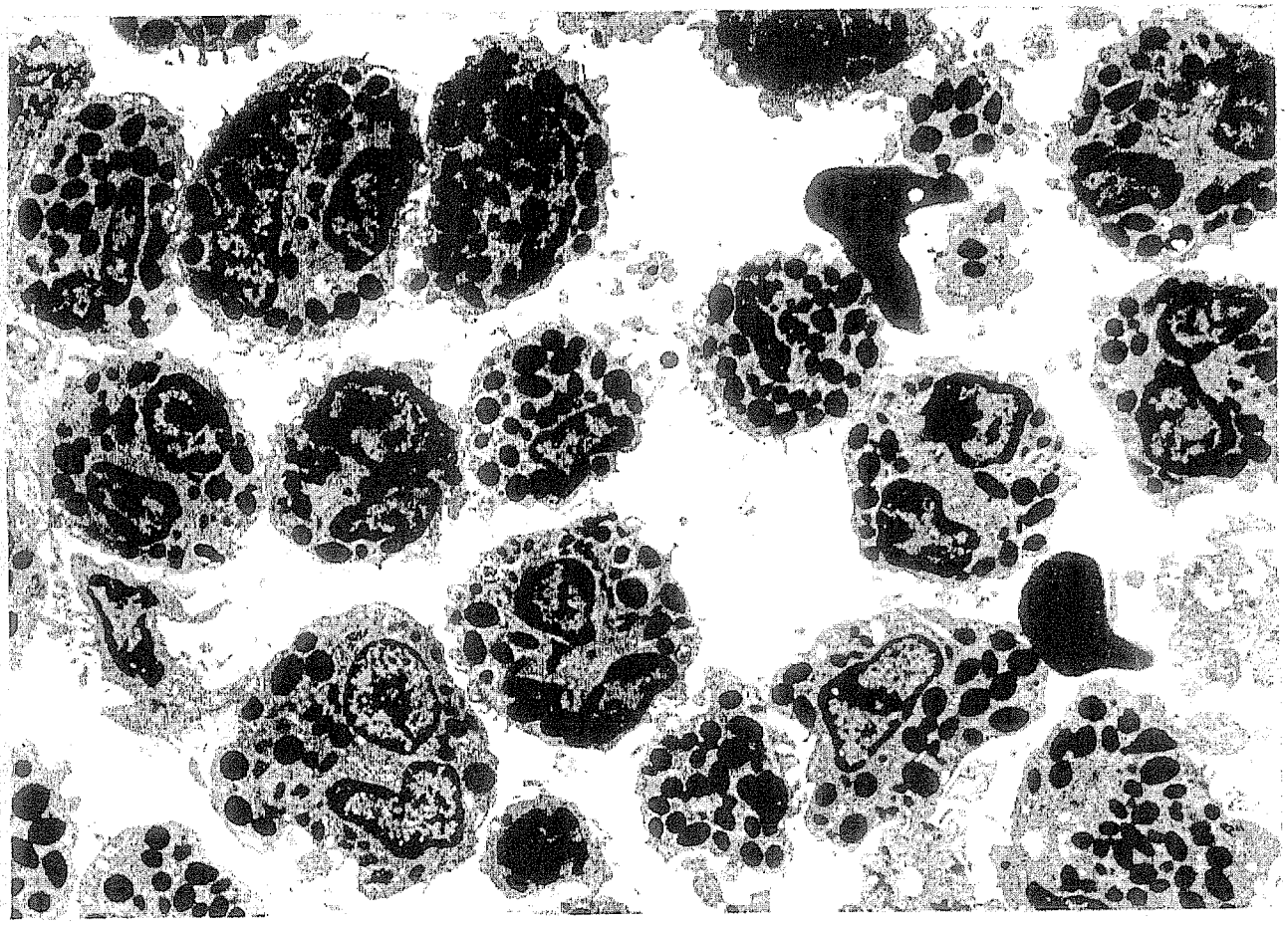


Fig 6

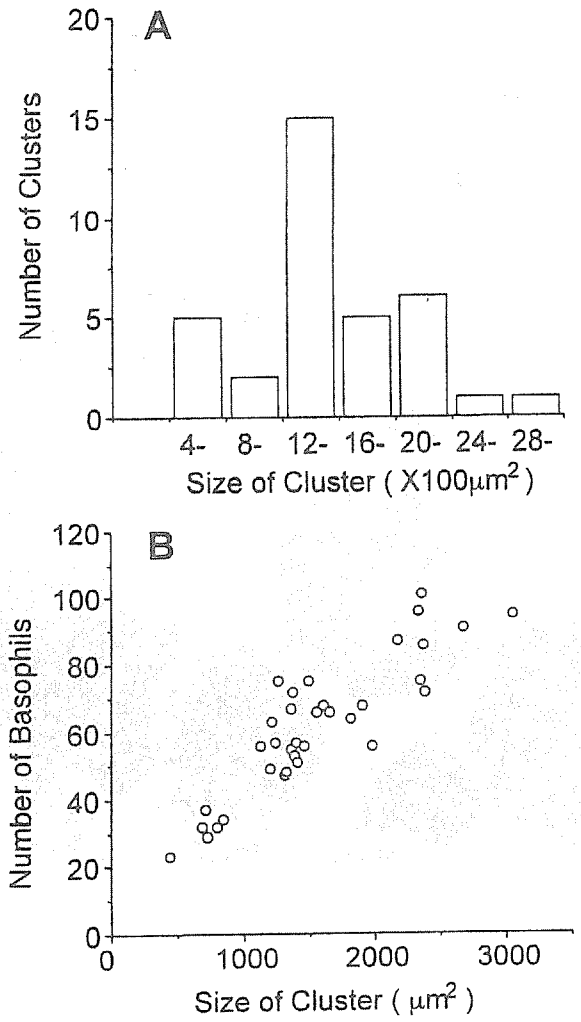

A
Number of Clusters
20
15
10
5
0
4-
8-
12-
16-
20-
24-
28-
Size of Cluster (X100μm2)
B
Number of Basophils
120
100
80
60
40
20
0
0
1000
2000
3000
Size of Cluster (μm2)

Fig 7