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Transmission electron microscopic study of renal haemopoietic tissues of *Channa punctatus* (Bloch) experimentally infected with two species of aeromonads

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Abstract: The ultrastructural pathological changes in the renal haemopoietic tissue of *Channa punctatus* (Bloch) experimentally infected with 2 species of aeromonads, *Aeromonas hydrophila* and *Aeromonas salmonicida*, were reported. Adult *C. punctatus* specimens were injected intramuscularly, once at a time, with a chosen sublethal dose (2×10^9 cfu mL^{-1}) of aeromonads at 5 µL g^{-1} body weight of fish and were exposed to 72 h of duration. The leucocytes (especially macrophages and melanomacrophages) and other associated cells from the pronephric head kidney of aeromonad-infected *C. punctatus* were studied by fixed ultrathin sections for transmission electron microscopy. The sinusoidal cells, barrier cells, epithelioid cells, macrophages, and melanomacrophages in particular were infected by the chosen sublethal dose of aeromonads, and tissue destruction was initiated at this primary stage of infection. Other major changes were the increases in the number as well as the granular contents of macrophage and melanomacrophage cells for phagocytic activity against invading pathogens.

Key words: *Channa punctatus*, ultrastructure, head kidney, macrophage, aeromonads, melanomacrophage

Introduction

Fishes encounter a variety of pathogens, parasites, and pollution in the aquatic environment. The histopathological changes in several tissues of fish naturally and experimentally infected with different bacterial strains have been described by many authors (Young and Chapman, 1978; Bruno, 1986; Sami et al., 1992; Takahashi et al., 1992; Flaño et al., 1996; Rodger and Richards, 1998; Magnadóttir et al., 2002; Woo et al., 2006; Altinok et al., 2007; Martins et al., 2008; Hagiwara et al., 2009). The infection caused by aeromonads (members of the genus *Aeromonas*) results in diffused necrosis in the kidney, histopathological alterations in the liver (El-Barbary, 2010), and the presence of melanin-containing macrophages (also termed as melanomacrophages) in the blood (Ventura and Grizzle, 1988). The phagocytic and bactericidal activity of a macrophage, a component of the host defence mechanisms against pathogenic microorganisms, has been extensively studied in mammals (Krahenbuhl, 1994; Rook and Bloom, 1994). However, little information is available on the ultrastructural pathology associated with infection of renal haemopoietic tissue in fish. Internally, the pronephric or head kidney is one of the target organs of an acute septicaemia caused by

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aeromonads, and this organ is apparently attacked by bacterial toxins and loses its structural integrity (Huizinga et al., 1979). The head kidney is also one of the major organs involved in the fish immune defence, and it is a tissue particularly rich in macrophages and melanomacrophages (Secombes, 1990; Meseguer et al., 1994).

The spotted murrel (*Channa punctatus*, family Channidae) is an air-breathing and edible fish known as a rich source of protein. Its population, previously abundant in the shallow wetlands of West Bengal, India, is now dwindling due to pollution and various disease problems in nature. The purpose of the present experiment was to describe the changes observed in the pronephric kidney of *C. punctatus* experimentally infected with a sublethal dose of 2 separate strains of aeromonads (2×10^9 cfu mL^{-1}), with special reference to the ultrastructure of the leucocytes and associated cells forming the haemopoietic microenvironment.

Materials and methods

Specimen collection and maintenance

Channa punctatus is an air-breathing fish and generally a freshwater inhabitant, preferring habitats from stagnant muddy pond water to canals, paddy fields, lakes, rivers, etc. For the present experiment, 40 adult *C. punctatus* specimens were collected during summer from a local fish farm (22°31.115′N, 88°24.086′E) located in Kolkata, West Bengal, India. After collection, fish were transported live to the Department of Zoology, University of Calcutta, and were kept alive for 1 week in glass aquaria (size: 0.6 m × 0.3 m × 0.3 m, 5 fish per aquarium) under controlled laboratory conditions with continuous aeration. The specimen selected for study averaged 79.13 ± 1.42 g in weight and 19.67 ± 0.29 cm in length. Fishes were fed ad libitum with *Tubifex* sp. and larvae of *Culex* sp. during the acclimatisation period only. The water was renewed every day to avoid accumulation of unutilised food or metabolic waste products. Different water parameters like temperature, pH, hardness, dissolved oxygen, free carbon dioxide, and total alkalinity were monitored before the experimental set-up following the standard methods (APHA, 2005). The respective values obtained were as follows: water temperature, 24–26 °C; pH, 7.2–7.4; hardness, 230–250 mg L^{-1}; dissolved oxygen, 3.5–4.5 mg L^{-1}; dissolved free carbon dioxide, trace; and alkalinity, 125–135 mg L^{-1}.

Bacterial culture collection and maintenance

Microbial cultures of 2 bacterial species of aeromonads, *Aeromonas salmonicida* (MTCC 1522) and *Aeromonas hydrophila* (MTCC 646), were collected from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The aeromonads were received as lyophilised cultures and subsequently revived by adding nutrient broth and transferring the rehydrated culture to a fresh nutrient agar medium. Consequently, the streak plate method was followed to get isolated bacterial colonies on a large part of the agar surface.

Experimental inoculation of aeromonads on *C. punctatus*

Both strains of aeromonads (*A. salmonicida* and *A. hydrophila*) were cultured in nutrient broth and incubated at 25 °C and 37 °C, respectively, 24 h prior to infectivity testing. Bacterial cells were harvested by centrifugation at 5000 × *g* for 5 min and washed in physiological saline (PS; 0.85% NaCl).The strains were enumerated by correlating the optical density (OD) values (600 nm) of the growing culture with the corresponding colony forming units (cfu) obtained by the spread plate dilution method (OD 600_{nm} 1 = 2×10^9 cfu mL^{-1}). Different concentrations of bacteria ranging from 1×10^5 to 1×10^{11} cfu mL^{-1} were established in PS (working volume: 5 μL g^{-1} body weight of fish) and injected intramuscularly into different groups of *C. punctatus*, each containing 15 fish. The fish were observed for changes in their behavioural patterns as well as development of haemorrhagic ulcers and tissue necrosis. The viability of the infected fish was checked for 72 h and the corresponding LD_{50} doses were determined for both strains (Reed and Muench, 1938).

For the present experiment, 2 groups of fish (each containing 15 individuals) were intramuscularly injected with the same sublethal and single dose (2×10^9 cfu mL^{-1}) of bacterial cells. One group was inoculated with *A. salmonicida* (MTCC 1522) and the other with *A. hydrophila* (MTCC 646), based in physiological saline solution. The remaining 10 *C. punctatus* were injected only with sterile PS and used as sham-injected controls.

TEM analysis

After 72 h of exposure to aeromonads, 10 fish samples each from *A. hydrophila*- and *A. salmonicida*-infected stock and 3 sham-injected controls were euthanised by anaesthetic overdose using a 200 mg L^{-1} solution of MS-222. The fish were then bled by severing the tail, and the kidney (chiefly the pronephros) was aseptically removed. For TEM analysis, small pieces (1 mm^3) of head kidney tissue were washed in 0.1 M phosphate buffer solution (pH 7.2) and fixed in a freshly prepared solution of fixative for 12 h at 4 °C, followed by washing with freshly prepared buffer (Karnovsky, 1965). The composition of fixative (100 mL) was as follows: 4% paraformaldehyde solution, 45 mL; 0.1 M phosphate buffer solution (pH 7.2), 45 mL, and 25% glutaraldehyde (stock solution), 10 mL. The tissues were postfixed in 1% aqueous osmium tetroxide (OsO_4) solution for 2 h at 4 °C. After repeated washing in 0.1 M phosphate buffer, tissues were dehydrated in ascending grades of ethanol up to 100%, infiltrated, and embedded in Araldite CY 212 (TAAB, UK). Ultrathin sections (60–80 nm) were cut on an ultramicrotome (Ultracut E, Reichert–Jung, Austria). The sections were mounted on meshed copper grids and double-stained with aqueous uranyl acetate (10 min) and lead citrate (10 min), respectively (Reynolds, 1963). The sections were examined on a Morgagni 268 D transmission electron microscope (FEI Electron Optics, the Netherlands) at an accelerating voltage of 80 kV at the All India Institute of Medical Sciences (AIIMS), New Delhi.

Results

Ultrastructural changes observed in leucocytes and other associated cell types of the renal haemopoietic tissue of *C. punctatus* were recorded from 72 h postinfection with 2 species of aeromonads and are demonstrated in Figures 1–8.

A marked dilation of the sinusoids of the kidney (KS) was found in fish injected with *A. hydrophila*. Infected sinusoidal-lining cells (S) showed degenerative changes of the nucleus as well as of the cytoplasm (Figure 1). The endothelial cells contained many large lysosome-like granules (L) loaded with bacteria (BAC). On the other hand, a few barrier cells (BC) often appeared fused together in syncytial networks in the necrotic areas infected by *A. salmonicida* (Figure 2).

The number of macrophages (MAC) and melanomacrophages (MMAC) increased considerably in the kidneys of both strains of aeromonad-injected *C. punctatus* compared to the sham-injected control.

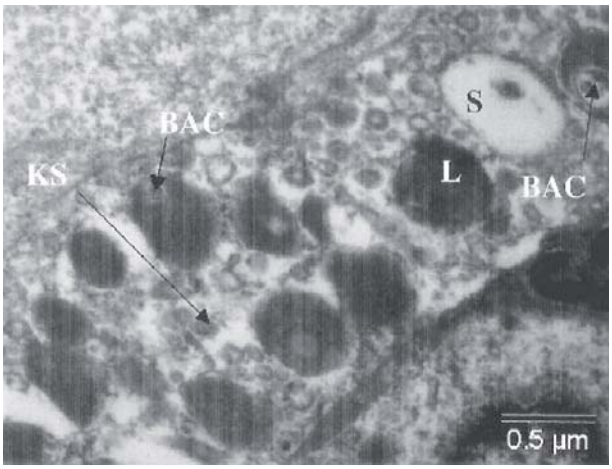

Figure 1. Transmission electron micrograph of kidney sinusoid (KS) of *Aeromonas hydrophila*-infected *Channa punctatus* at 3 days postinfection showing a degenerated sinusoidal cell (S). The endothelial cells contained many large lysosomes (L) loaded with bacteria (BAC). Scale bar = 0.5 µm.

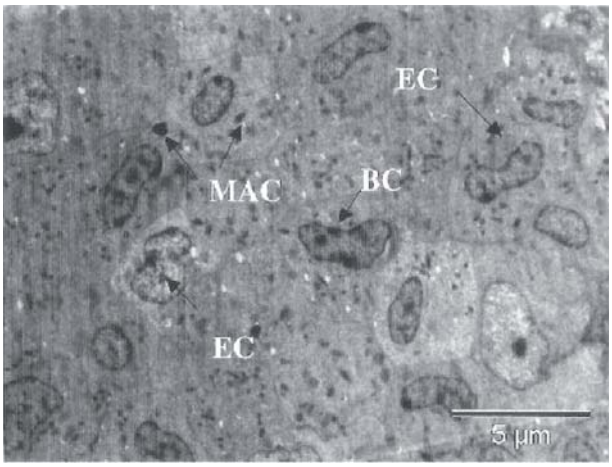

Figure 2. Transmission electron micrograph of the pronephros of *Aeromonas salmonicida*-infected *Channa punctatus* at 3 days postinfection showing aggregation of barrier cells (BC), macrophages (MAC), and epithelioid cells (EC) forming a syncytial network. Scale bar = 5 µm.

Macrophages (Figure 3) were characterised by their abundant cytoplasm, eccentric nucleus, prominent Golgi apparatus, numerous mitochondria, and intracytoplasmic granules (G) and moderately abundant rough endoplasmic reticulum (RER). The morphology of the macrophage sometimes changed after engulfment of the aeromonads. Some of the macrophages infected with *A. hydrophila* were monopodic, while others were multipodic with appendices gathering together to form a foot structure (F). Numerous moderate electron-dense vesicles (EDV) were present in the cytoplasm of the macrophages along with large clusters of dense granules resembling lysosomes (Figure 4). The lysosomes had a single limiting membrane.

The epithelioid cells (EC) present in the *A. hydrophila*-infected renal haemopoietic tissue had monocyte-like features. Loss of nuclear chromatin (NC) and disintegrated mitochondrial cristae (DM) were observed in epithelioid cells (Figures 5 and 6).

In macrophages and melanomacrophages, distinct stages of phagosome (P) formation were also evident during the early infective stage of *A. salmonicida* along with an increase in the extent of phagosome–lysosome fusion (Figure 7), and bacteria that appeared to adhere tightly to the surface of macrophages showed more advanced stages of phagosome formation. Some phagosomes appeared to be surrounded by lysosomes while others appeared to have fused with the lysosomes to form phagolysosomes (PLs) and contained degenerated or lysed bacteria (Figure 8). An irregular electron-transparent zone (ETZ) was found to separate the BAC from the phagosome wall (PW). Some bacteria had lost their transparent zone. Apart from the above cited cells, the renal haemopoietic tissue of infected *C. punctatus* was severely depleted of other types of leucocytes and erythrocytes at this stage of infection.

Discussion

Appropriate identification of fish leucocytes is a foremost requisite for histopathological investigations. The micromorphometry, relative distribution, and ultrastructural details of leucocytes from the pronephric kidney of healthy *C. punctatus* were described by Ghosh and Homechaudhuri (2007). In the present experiment, ultrastructural observations in the pronephric kidney during

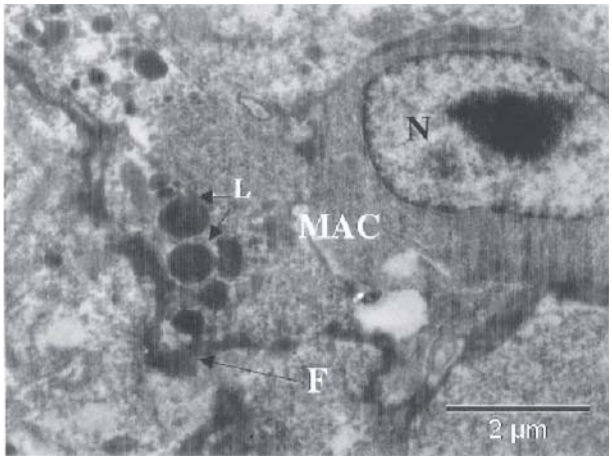

Figure 3. Transmission electron micrograph of a macrophage (MAC) from haemopoietic tissue of the pronephros of *A. hydrophila*-infected *Channa punctatus* at 3 days postinfection. The plasma membrane showed a much indented, multipodic outline with appendices gathering together to form a foot structure (F). One pole of the cell contained the nucleus (N), while the other pole contained many lysosomes (L). Scale bar = 2 µm.

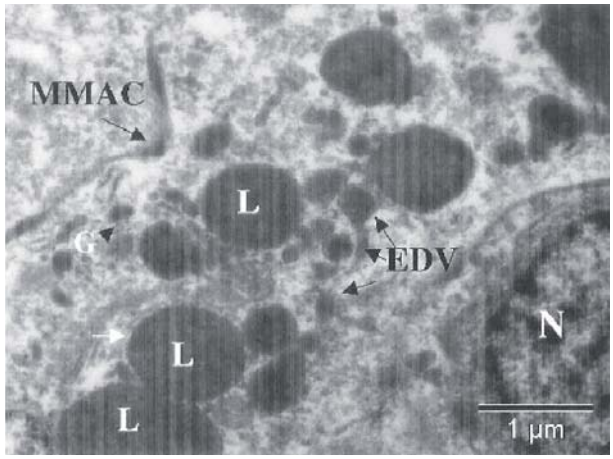

Figure 4. Transmission electron micrograph of a melanomacrophage (MMAC) from the pronephros of *A. hydrophila*-infected *Channa punctatus* at 3 days postinfection showing an eccentric nucleus (N) and intracytoplasmic granules (G) and many electron-dense vesicles (EDV). Secondary lysosomes (L) were abundant, varying both in size and in content density, and were surrounded by a single limiting membrane (white arrow). Scale bar = 1 µm.

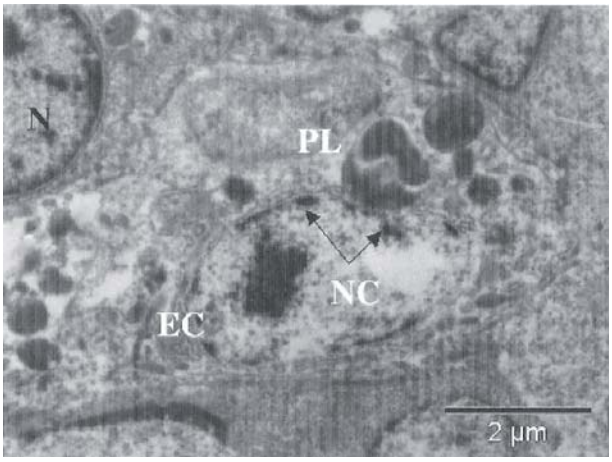

Figure 5. Transmission electron micrograph of epithelioid cells (EC) from the pronephros of *A. hydrophila*-infected *Channa punctatus* at 3 days postinfection showing loss of nuclear chromatin (NC) and initiation of phagolysosome formation (PL). Scale bar = 2 µm.

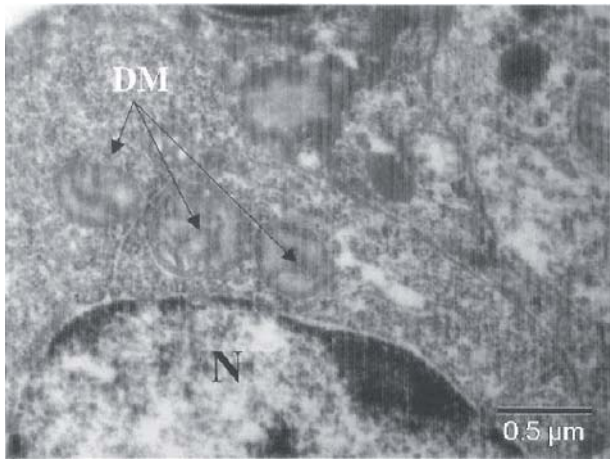

Figure 6. Transmission electron micrograph of epithelioid cells (EC) from the pronephros of *A. hydrophila*-infected *Channa punctatus* at 3 days postinfection showing the nucleus (N) and cristae of disintegrated mitochondria (DM). Scale bar = 0.5 µm.

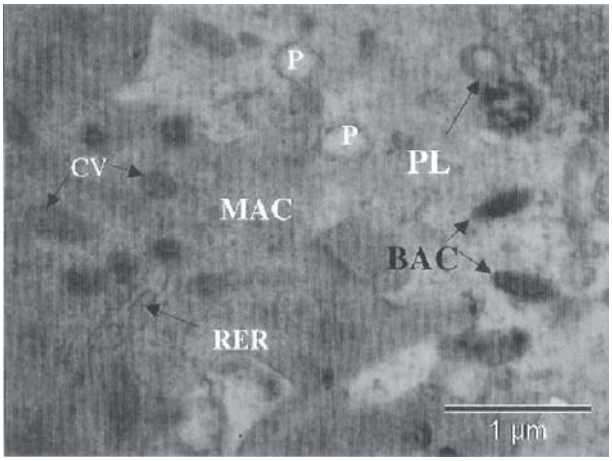

Figure 7. Transmission electron micrograph of a macrophage (MAC) from the pronephros of *A. salmonicida*-infected *Channa punctatus* at 3 days postinfection showing moderate rough endoplasmic reticulum (RER) and cytoplasmic vesicles (CV) in the cytoplasm. A lysosome has fused with a phagosome (P) to form a phagolysosome (PL). Bacteria (BAC) that appeared to adhere tightly to the surface of macrophages showed more advanced stages of phagosome formation. Scale bar = 1 µm.

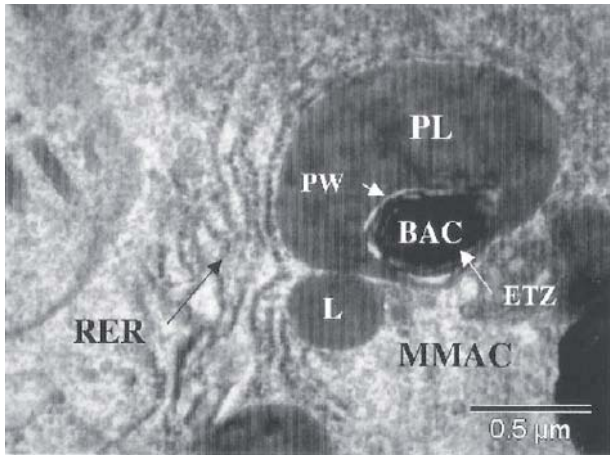

Figure 8. Transmission electron micrograph of a melanomacrophage (MMAC) from the pronephros of *A. salmonicida*-infected *Channa punctatus* at 3 days postinfection showing a distinct phagolysosome (PL), which contained a lysed bacteria (BAC), and lysosome (L) around the phagolysosome and abundant rough endoplasmic reticulum (RER) in the cytoplasm. An irregular electron-transparent zone (ETZ) was found to separate the bacterium from the phagosome wall (PW). Scale bar = 0.5 µm.

the early stage of experimental infection of adult *C. punctatus* with 2 species of aeromonads were recorded. The study revealed that sinusoidal cells, macrophages, melanomacrophages, epithelioid cells, and barrier cells were infected by experimental inoculation of aeromonads. The sinusoidal-lining cells proved actively phagocytic, in agreement with the reports of Alvarez et al. (1988) and Dannevig et al. (1994) for salmonids, and were probably the earliest cells to contain intracytoplasmic bacteria (due to the formation of the first physical barrier to circulating bacteria in kidney). The most prominent effects were

an increase in the abundance of macrophage cells among the leucocytes and a reduction in the number of haemoblasts and other types of leucocytes such as lymphocytes in the renal haemopoietic tissues (Peters and Schwarzen, 1985; Flaño et al., 1996). A few days after injection of aeromonads, macrophages also showed an increase in the number of lysosome-like granules and vesicles that contained medium electron-dense materials inside. Different types of phagosomes were also found within the macrophages examined in this study. In some cases, phagosomes had fused with the lysosome, and bacterial debris appeared within the phagolysosome. The scavenging functions and migration of the macrophages and melanomacrophages in the kidney were observed in various species of fish injected with pathogenic microorganisms (Wolke, 1975; Vogelbein et al., 1987; Chen, 1992; Meseguer et al., 1994; Chinabut, 1999; Roberts, 2001). An increase in the number of circulating monocytic cells (Bruno and Munro, 1986) and of phagocytic cells in the kidney was observed in rainbow trout and Atlantic salmon experimentally infected with *Renibacterium salmoninarum* (Bruno, 1986). An increase in the number and size of melanomacrophages was also observed in goldfish, *Carassius auratus* L., injected with phenylhydrazine, which caused a fast and intense haemolysis (Herráez and Zapata, 1986) and confirmed the role of these cells in the degradation of erythrocytes and other cells as reported by many authors (Graff and Schlüns, 1979; Fulop and McMillan, 1984). Lamas et al. (1994) observed an increase in the number of macrophages and melanomacrophages containing many peroxidase-positive granules of different sizes from the kidney and spleen of both bacteria-injected and extracellular product-injected rainbow trout. The phagocytic capability of fish neutrophils has been demonstrated by various authors (Finn and Nielson, 1971; Suzuki, 1984; Lehmann et al., 1987). However, phagocytosis of aeromonads by neutrophils in the pronephros of *C. punctatus* could not be detected in the present study. Flaño et al. (1996) reported the ultrastructural histopathological changes occurring in the renal and splenic haemopoietic tissues of coho salmon *Oncorhynchus kisutch* experimentally infected with *Renibacterium salmoninarum*. Electron microscopic studies (both in vitro and in vivo) of the phagocytosis of *Mycobacterium* sp. by rainbow trout head kidney macrophages were conducted by Chen et al. (1998). Transmission electron microscopic studies by Graham et al. (2002) revealed primarily membrane-bound aggregates of nonenveloped virus particles with an electron dense core in the spleen, kidney, gill, and erythrocytes of Atlantic salmon, *Salmo salar*, during a disease outbreak with high mortality.

The present experiment showed that artificial inoculation with aeromonads initiated destruction of the tissues of the pronephric kidney of *C. punctatus* at short-time exposure. The tissue alteration involved the destruction of the haemopoietic microenvironments, the appearance of epithelioid cells and barrier cells forming a network, and an increase in the number as well as the granular contents of macrophages and melanomacrophages for the phagocytosis of the invading pathogens.

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