Journal of Cell and Animal Biology Vol. 5(8), pp. 169-175, August 2011
Available online at http://www.academicjournals.org/JCAB
ISSN 1996-0867 ©2011 Academic Journals

Full Length Research Paper

A comparative *in vitro* study of morphology and phagocytic capacity of the free surface respiratory macrophages in the duck and the rabbit

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Accepted 13 July, 2011

Quantitative and qualitative characteristics of the free surface respiratory macrophages (SM) of the domestic duck (*Anas platyrhynchos*) were compared with those of the domestic rabbit (*Oryctologus cuniculus*) under similar experimental conditions. The duck had significantly fewer SM compared to those of rabbit. In the duck, there was flux of SM as depicted by increase of SM during the first three progressive lavages before the number started to decline during the fourth and fifth lavages. In the rabbits, there was decline in number of the SM during the five progressive lavages. Morphologically, the cells were similar at light and electron microscopy levels. Ultra structurally, the SM had filopodial extensions and variable vesicular cytoplasmic bodies. The diameters were comparable with duck SM mean diameter measuring about 12 µm while that of the rabbit SM mean diameter being 13 µm. The mean volume density of polystyrene particles ingested by the SM, that is volume of particles per unit volume of SM, revealed that the duck SM had significantly higher mean phagocytic capacity at 20% than rabbit SM whose mean phagocytic capacity was 9%. The assertion that domestic birds are prone to respiratory diseases due to dearth of SM alone may not be true. The higher phagocytic capacity observed in the duck SM probably compensates for the few resident SM. Vulnerability to respiratory diseases by the domestic birds may be due to other factors such as poor husbandry and management strategies and, severe genetic manipulations for fast growth and productivity that may have weakened cellular immunological defenses.

Key words: Avian, cellular defense, lung, phagocytosis, rabbit, surface macrophages.

INTRODUCTION

The different designs of the vertebrate respiratory systems were imposed by factors such as the physio-chemical characteristics of the respiratory media used, the nature of the habitat occupied, and the lifestyle pursued (Maina, 1994). To promote gas exchange by diffusion, extensive surface areas and thin tissue barriers were contrived. In the avian parabronchial lung, the blood–gas barrier is 56 to 67% thinner than that of a mammal of equivalent body mass while the respiratory surface area is approximately 15% greater (Maina, 1989).

These anatomical properties of the avian lung–air sac system render it as the most efficient gas exchanger respiratory system among the vertebrates. Although evolution of thin and vast blood–gas barrier enhanced the flux of respiratory gases between the body and the environment, the capacity of the body to physically ward off harmful biological pathogens and ameliorate the harmful effects of toxic environmental pollutants at respiratory surfaces were innately compromised. Without developing an efficient defense mechanism, the respiratory organs would offer easily assailable areas during the incessant assaults by pathogens. Evolution of gas exchangers was therefore accompanied by formation of apt defense devices. Surface respiratory macrophages are the lung tissuecomponent of the mononuclear phagocytic system that arise from the emigrating monocytes and apparently differentiate in response to local conditions and factors (Furth et al., 1980). The SM exist at the tissue air interphase where they phagocytose particles

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and microorganisms bound to opsonins or through opsonon independent mechanisms (Hunninghake, 1984). In mammals, the SM constitutes 93% of the lung macrophage pool with interstitial macrophages constituting 7% (Blusse and Furth, 1979). Numerous environmental agents can alter the number of SM in the lung as well as their function. Smokers have increased numbers of SM and the diameter of the SM harvested by lavage has been found to be slightly larger (Hof et al., 1990).

Susceptibility of domestic birds to respiratory diseases has been attributed to few residents SM in the lung–air sac system (Toth and Sigel, 1986; Fulton et al., 1990). In the chicken and the rock dove, values of SM harvested by lavage have been reported to be 2.5×10^5 and 1.6×10^5 respectively (Kiama et al., 2008; Maina and Cowley, 1998). Lack of SM has been remarked by Klika et al. (1996) and Lorz and Lopez (1997). In mammals, values of SM recovered by pulmonary lavage that range from 0.55 to 1.55×10^6; 2.88 to 4.46×10^6, and 1.08 to 1.77×10^7 have been reported in the mouse, the black rat and the guinea-pig respectively (Holt, 1979).

Since phagocytosis is the most important defense mechanism in all phyla of the animal kingdom (Nicoid, 2005), it is particularly important that the contribution of the SM in the clearance of particles and neutralization of pathogens in the avian lung should be well understood. This study therefore took the inquiry further. The SM in the duck and the rabbit harvested by lavage of the respiratory systems were counted and the viabilities assessed. Measurement of diameters of the SM was done and their morphology determined at light and electron microscopy levels. In addition, the phagocytic capacities of the SM were assed using polystyrene particles.

MATERIALS AND METHODS

Pulmonary lavage of the avian respiratory system

Surface respiratory macrophages (SM) were obtained from the lung-air sac system of seven mature specimens of the domestic duck, *Anas platyrhynchos* as described by Nganpiep and Maina (2002) and Kiama et al. (2008). Briefly, ducks were killed by intravenous injection of an overdose of pentobarbitone sodium (Euthanase ®) into the brachial vein. The trachea was then exposed and cannulated with sterile cannula attached to a funnel. Sterile pre-warmed (40°C) phosphate buffered saline (PBS) was poured down the respiratory system from a height of 30 cm. During instillation, the coelomic cavity was gently massaged to expel air in order to ensure penetration of PBS into all air spaces, including the air sacs. The instilled fluid was left in the respiratory system for 5 min and thereafter aspirated with a 50 ml syringe. The recovered lavage fluid was centrifuged and pelleted respiratory surface macrophages re-suspended in sterile RPMI-1640 cell-culture medium. Five washes were performed per duck.

Bronchoalveloar lavage of the rabbit

Bronchoalveolar lavage of the rabbits was executed as described by Kiama et al. (2008). Briefly, seven mature domestic rabbits; *Oryctologus cuniculus*, were anaesthetized with intraperitoneal injection of ketamine hydrochloride (65 mg/kg) followed by intraperitoneal injection of pentobarbitone. The dosage was adjusted to reach deep anesthesia. The trachea was exposed, tracheotomy performed, and a cannula introduced and tightly fixed with a thread. A pneumothorax was created before the bronchoalvelor lavage procedure was started. The procedure consisted of five washes (25 ml per wash) of the lungs with Ca^{2+} and Mg^{2+} free PBS. The total recovered lavage fluid was centrifuged and the pellet re-suspended in sterile cell culture medium (RPMI-1640).

Counting of the surface respiratory macrophages

Counting of the SM was done as described by Maina and Cowley (1998). Briefly, the hemocytometer chambers were charged by drawing 200 µl from the concentrated lavage fluids using a micropipette. Counting of SM was restricted to the cells that were identified as morphologically round or slightly amorphous, refractile, frequently granular cells.

In vitro viability of the surface respiratory macrophages

Viability of the harvested respiratory surface macrophages from the duck and the rabbit were determined by using trypan blue exclusion test (Kiama et al., 2008; Maina and Cowley, 1998). For every animal, a mixture of 50 µl of cell suspension, 270 µl of PBS and 180 µl of trypan blue (0.5% in physiological saline) were prepared in sterile microtube and 200 µl drawn from the mixture and charged into a hemocytometer. All dead cells contained blue dye in their cytoplasm.

Phagocytosis of polystyrene particles

The duck and rabbit SM were washed twice in PBS and re-suspended in fresh RPMI-1640 medium at a concentration of 1×10^6/ml. The cells were co-cultured with polystyrene microspheres of 6 µm diameter in sterile microtube at a concentration of about 100 microspheres per cell for 5 h. The tubes were shaken regularly to avoid sedimentation of the cells and particles (Ficken et al., 1986).

Processing SM for transmission electron microscopy

Processing of SM was as described by Maina (1989). Briefly, surface respiratory macrophages were washed three times in PBS and re-suspended in 2.5% phosphate buffered glutaraldehyde solution. The glutaraldehyde fixed cells were centrifuged and post-fixed in 1% osmium tetroxide in 0.1 M sodium-cacodylate buffer. This was followed by dehydration in graded series of ethanol (70, 80, 90, and 100% twice) and gradual replacement of ethanol with propylene oxide before infiltrating and embedding the cells in epoxy resin.

Semithin and ultrathin sections were cut using a Reichert® ultramicrotome. The ultrathin sections were picked on 200-wire mesh copper grids, stained with uranyl acetate, counterstained with lead citrate, and observed with a Philip 201C transmission electron microscope under an accelerating voltage of 60 kV. The semithin sections were collected on glass slides, stained with 0.5% toluidine blue, viewed, and photographed under a light microscope.

Table 1. Volume of the phosphate – buffered saline (PBS) instilled in the respiratory systems of the rabbits and the ducks, the volume recovered, and the recovery rate.

Animal	Body mass (Kg)	Instilled fluid (ml)	Recovered fluid (ml)	Recovery rate (%)
Rabbit 1	2	125	100	80
Rabbit 2	2	125	110	88
Rabbit 3	1.8	125	98	78
Rabbit 4	1.8	125	114	91
Rabbit 5	2.2	125	112	90
Rabbit 6	1.8	125	96	77
Rabbit 7	2.5	125	110	88
Mean	2	125	106	85
SD	0.22		7.4	6.0
Duck 1	2	2050	1950	95
Duck 2	2	1670	1500	90
Duck 3	1.8	2070	1800	87
Duck 4	2	1960	1725	88
Duck 5	2.2	1870	1610	86
Duck 6	2.5	2320	2040	88
Duck 7	2.2	2205	2010	91
Mean	2.1	2021	1805	89
SD	0.22	215	207	3

Estimation of the diameters of the SM and the volume density of the phagocytized particles

The diameters of the SM of the duck and the rabbit were determined under an ocular graticule with a linear scale at a magnification of ×100. In each field, to avoid bias, the cells were singled out at random. Only the diameters of the cells at the four corners of the fields and one at the middle were measured.

Volume density of the phagocytised particles in the SM [Vv (p, c)] was determined as the ratio of the total volume of phagocytised particles to the cell volume. The ratio was estimated by point counting on the plane sections as described by Kiama et al. (2001, 2008). Briefly, one ultrathin section was randomly sampled from each processed block of cell pellets and 35 fields systematically sub-sampled from each section. The corresponding micrographs of the sampled fields were recorded on a 35 mm electron microscope film of which the individual negatives were projected onto a screen at a final magnification of × 14,000. A quadratic lattice grid was superimposed at random position onto each projected image. The total number of points falling onto profiles of the phagocytised particles [P (p)] and the entire cell [P (c)] were counted. An estimator of the volume density of the phagocytised particles [Vv (p, c)] was then calculated as follows:

$Vv\ (p, c) = P\ (p) \times P\ (c)^{-1}$ (Kiama et al., 2008).

Statistical analysis of the data

The student t-test was used to compare the values on the duck and rabbit in the various experiments. The level of significance was set at $p \leq 0.05$.

RESULTS

The average volume of PBS instilled and recovered from the respiratory tract of a rabbit was 125 and 106 ± 7 ml, respectively. This represents a recovery rate of 85 ± 5%. In ducks, the average volume of PBS instilled and recovered from the lung-air-sac system was 2021 ± 215 ml and 1805 ± 207 ml (Table 1). This represents a recovery rate of 89 ± 3%. The animals have comparable body mass ($p \leq 0.05$) with a mean body mass of 2 ± 0.3 and 2.1 ± 0.22 kg for the rabbit and duck respectively (Table 1). The lavage fluids recovered from the respiratory systems of the ducks and rabbits contained surface respiratory macrophages, and epithelial cells presumed to have desquamated from the lining of the respiratory tracts. The epithelial cells were either ciliated or non ciliated (Figure 1). Ultra structurally, the duck and rabbit SM were similar. Typically, they had filopodial extensions and variably electron dense cytoplsamic vesicular bodies (Figure 1).

The rabbit SM exhibited a higher *in vitro* viability ($p \leq 0.05$) than the duck SM. The mean *in vitro* viability of the rabbit SM was 97 ± 1% while that of the duck SM was 86 ± 4% for the same period. Despite having equivalent body masses, ducks had significantly ($p \leq 0.05$) fewer SM harvested by pulmonary lavage than the rabbits. Ducks had a mean number of $1.1 \times 10^6 \pm 148780$ SM while the rabbits had a mean number of $1.5 \times 10^7 \pm 911362$ SM (Table 2 and Figure 2). In the rabbits, the number of SM decreased steadily with progressive washes but in the ducks, there was progressive increase in the number of SM during the first three lavages (Figure 2). The mean diameter of the duck SM (11 ± 1.1 µm) was not significantly different ($p \leq 0.05$) from that of

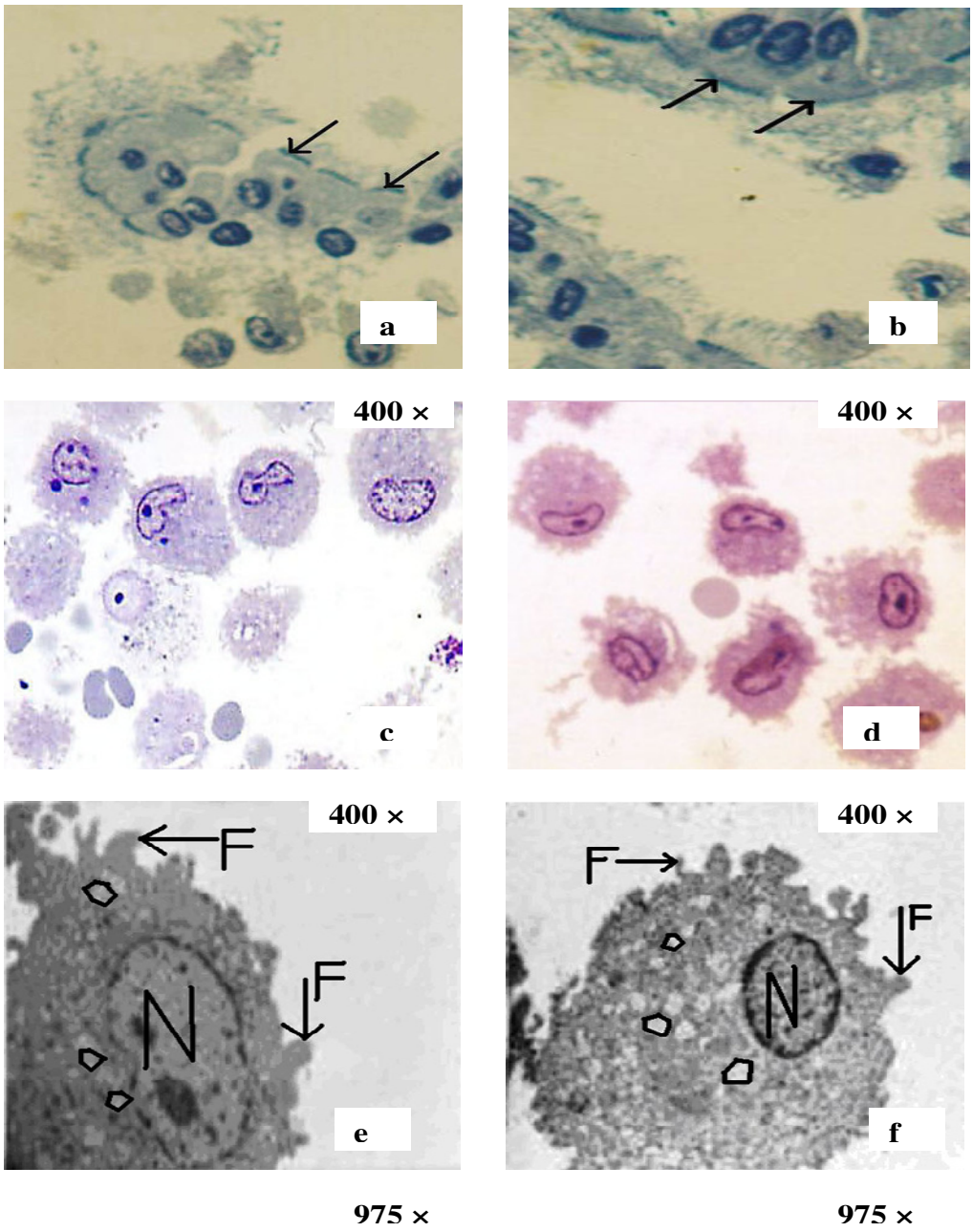

Figure 1. Top, a-b are light micrographs of the rabbit and duck epithelial cells, cilia (arrows). Middle, c-d are light micrographs of the rabbit and the duck SM recovered by pulmonary lavage of the rabbit and duck respiratory systems. In e-f (bottom), are electron micrographs of the rabbit and the duck SM with eccentric nucleus (N), filopodia (F), and cytoplasmic vesicles (encircled).

the rabbits SM (13 ± 1.3 µm) (Table 3). Quantitative estimation of the loading of the SM with polystyrene particles showed that the duck SM, of which the phagocytised particles formed a volume density of 20% of the volume of a SM, took up significantly ($p \leq 0.05$) more particles than the rabbit SM in which the volume density of the phagocytised particles formed 9% (Table 3 and Figure 3).

DISCUSSION

The avian blood–gas barrier is 56 to 67% thinner than that of a mammal of equivalent body mass and the respiratory surface area is 15% more (Maina, 1989). All other prevailing factors being the same, from the perspective of the basic structural parameters, that is a vast and attenuated separation between air and blood,

Table 2. Number of surface respiratory macrophages harvested by pulmonary lavage of the duck and the rabbit respiratory systems.

Animal	Body mass (Kg)	Total number of SM ($\times 10^6$)
Rabbit 1	2	16.425
Rabbit 2	2	15.38
Rabbit 3	1.8	14.5
Rabbit 4	1.8	15.62
Rabbit 5	2.2	13.6125
Rabbit 6	1.8	15.125
Rabbit 7	2.5	14.5125
Mean	2.0	15.025
SD	0.26	0.911362
Duck 1	2	1.275
Duck 2	2	1.15
Duck 3	1.8	1.1125
Duck 4	2	0.9
Duck 5	2.2	0.9375
Duck 6	2.5	1.05
Duck 7	2.2	1.275
Mean	2.1	1.1
SD	0.22	0.14878

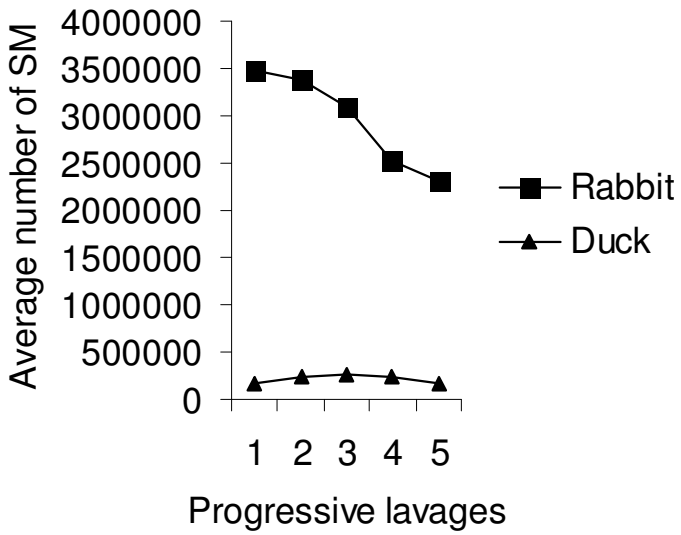

Figure 2. Comparison of the average number of SM recovered from the rabbit and duck respiratory systems by pulmonary lavage during five progressive lavages.

the avian lung-air sac system should be more assailable by pathogens than the mammalian one. Applying a similar corollary, for similar defense competence, more SM may occur on the surface of the avian lung. Paradoxically, dearth of SM (Ficken et al., 1986; Maina and Cowely, 1998) and even lack of them (Klika et al., 1996; Lorz and Lopez, 1997) have been reported in domestic birds. In chicken, a value of SM of 2.5×10^5 has been reported (Kiama et al., 2008) and 1.6×10^5 SM in rock doves (Maina and Cowely, 1998). This study authenticates the previous accounts that, in comparison to mammals, a dearth of SM exists in the avian respiratory system.

Assuming the defense capacities of the SM in mammals and birds are comparable, this would mean that the scarce SM in birds are either quantitatively or qualitatively deficient or have higher phagocytic capacity than those of mammals. This study confirms that the domestic duck SM were not only similar in morphology in comparison to those of the rabbit, but that the SM in both animals had equivalent diameters. However, the duck SM exhibited a significantly higher phagocytic capacity than the rabbit SM. This is a useful property of avian SM that probably compensates for the fewer resident SM.

In this study, there was increase of SM recovered during the first three progressive lavages in the duck. The number, however, declined during the fourth and fifth lavages. In the rabbit, the number of SM declined steadily during the five progressive lavages. Influx of avian SM has been reported in other studies. Ficken et al. (1986) and Toth and Sigel (1986) demonstrated influx of SM onto surface of the chicken lung–air sac system.

Various investigators have reported paucity and even lack of SM in domestic birds. Without unequivocal empirical evidence, the condition has been used to explain a purported high susceptibility of birds, especially poultry to respiratory diseases. The dearth of SM in the avian lung should not, however, mean *ipso facto* that the avian pulmonary cellular defenses are inadequate or

Table 3. Diameters of the SM and volume density of phagocytised particles in SM of the rabbit and the duck.

No. of sample	Diameter of the rabbit SM (µm)	Diameter of the duck SM (µm)	Volume density of particle in the duck SM (%)	Volume density of particle in the rabbit SM (%)
1	12	9	23	9
2	15	11	20	10
3	12	10	19	9
4	14	11	22	8
5	13	12	18	7
Mean	13	11	20	9
SD	1.3	1.1	2.1	1.1

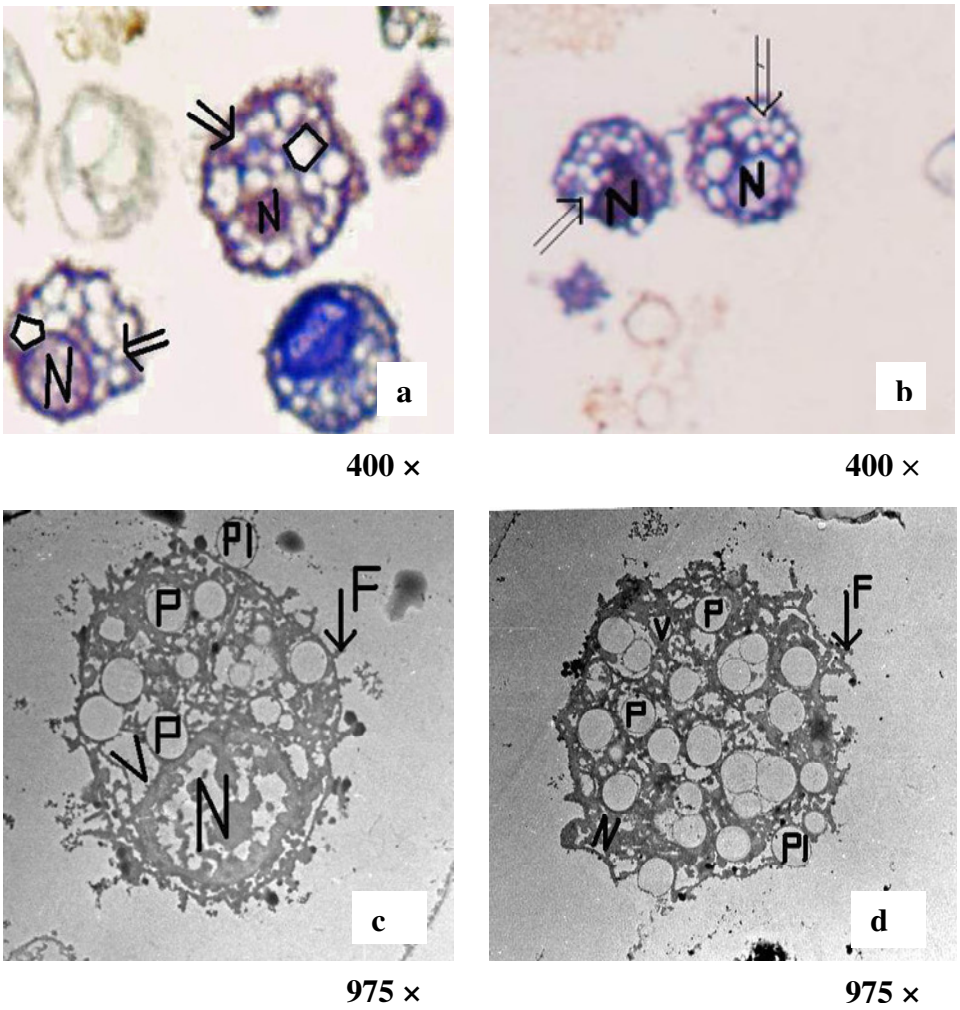

Figure 3. Top, a-b are light micrographs of the rabbit and the duck SM with internalized particles (arrows), encircled areas show vacuoles while N show eccentric nucleus. The bottom, c-d are electron micrographs of the rabbit and duck SM with P-internalized particles, N- the nucleus, V- vacuoles, and F-the filopodia. The particles on the surface of the cell membrane are shown as PI and these particles have thick dark rim on their surface which is lacking in the internalized particles.

even compromised. As shown in this study, the domestic duck SM not only had higher phagocytic capacity, but there was influx of the cells, these properties of the avian SM probably compensates for the fewer resident SM in the bird lung compared to the number of resident SM in mammals. The intense genetic manipulations for fast

growth and productivity may have compromised the robustness of SM to mount effective immune responses against respiratory diseases in the domestic birds.

Conclusion

Paucity of avian respiratory macrophages in the respiretory systems of domestic birds has, without unequivocal empirical evidence, been used to explain a purported high susceptibility of domestic bird to respiratory diseases.

The dearth of SM on the avian lung should not however mean *ipso facto* that the avian pulmonary cellular defenses are inadequate or even compromised. As reported in this study, despite having equivalent body masses, the duck SM are quantitatively fewer than the rabbit SM. The average number of SM recovered by lavage was 1.1×10^6 and 1.5×10^7 in the ducks and rabbits respectively. The implication of this finding is that, in a normal steady avian lung, there are fewer resident SM than in a mammalian respiratory system. However, while there was decline of the SM recovered from the rabbits' lungs with progressive lavages, in the ducks, there was substantial increase of SM during the first three lavages. This efficient influx of avian SM probably compensates for the few residents SM on the avian lung.

In both species, the SM exhibited similar morphology. The plasma membrane was ruffled with filopodial extensions. Filopodia are crucial in motility of SM as well as in engulfing pathogens and particulates. The eccentric nuclei and numerous cytoplasmic vacuoles in the SM show common ontogeny and adaptation to perform similar functions in the respiratory systems.

Morphometric observations revealed that both the duck and the rabbit SM had equivalent diameters of 12 and 13 µm, respectively. Despite having equivalent diameters, the duck SM phagocytosed more particles than the rabbit SM. On average, the mean volume of particles internalized by the duck SM was 20% while the mean volume of particles internalized by the rabbit SM was 9%. The duck SM, therefore, exhibited a higher phagocytic capacity than the rabbit SM. This could imply a higher ability of domestic birds' SM to internalize particles more efficiently as a means of compensating for the fewer resident SM on the avian respiratory lung.

ACKNOWLEDGEMENTS

We are grateful to Louise Bowditch, Boston USA for providing funding for this study. We are also grateful to the technical staff at Chiromo Campus, University of Nairobi for their assistance.

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