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Morphologic, cytochemical and ultrastructural features of grey eosinophils in 9 cats

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Manuscripts

1   **Morphologic, cytochemical and ultrastructural features of grey eosinophils in 9 cats**

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3   **Short title: Feline grey eosinophils**

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22   **Abstract**

23   **Background**: Grey eosinophils, resembling those in sighthound breeds have never previously been

24   reported in cats.

25   **Objectives**: To provide a morphological, cytochemical and ultrastructural description of grey

26   eosinophils in cats.

27   **Methods**: Blood films examined as part of routine hematology profiles in cats from May 2015 to July

28   2018 were evaluated for the presence of grey eosinophils. When identified with modified Wright

29   stain, cells were morphologically assessed and additionally stained with Diff Quik, ALP, Luna and

30   Luxol-fast blue and compared with feline controls. Two cases were prepared for transmission

31   electron microscopy (TEM) and compared with a feline control.

32   **Results**: Grey eosinophils were identified in 9 out of 2641 cats during the study period. In

33   comparison with typical feline eosinophils these cells contained abundant round granules instead of

34   the characteristic rod-shaped specific granules. These granules lacked the characteristic intense

35   pink/red staining with Romanowsky stains and did not stain with ALP, Luna or Luxol-fast blue stains.

36   On TEM the classical electron-dense core of these granules was replaced by a core with fragmented

37   or amorphous internal material. Typical eosinophils were not identified in any cat in which grey

38   eosinophils were identified.

39   **Conclusions**: The distinct morphological, cytochemical and ultrastructural changes in feline grey

40   eosinophils may be associated with a reduction or lack of major basic protein (MBP) in specific

41   granule cores. Similar to canine grey eosinophils, accurate recognition of these cells is essential to

42   prevent their misclassification as toxic neutrophils.

43

44   **Keywords**: grey eosinophil, feline, major basic protein, specific granule, secondary granule

45

46   Grey or 'vacuolated' eosinophils (GE) are commonly reported in the Greyhound and other sight-

47   hound breeds, occasionally in non-sighthound breeds but they have not been reported in the cat[5, 7].

48   In the dog, the term 'grey eosinophil' reflects the presence of variably-sized, unstained granules

49   resembling clear vacuoles[7].

50   Ultrastructural characterisation of canine grey eosinophils has shown a decrease, not only in the

51   overall secondary granule size, but also a reduction in the dense matrix and outer rim width which

52   resembles changes observed in humans with eosinophil peroxidase deficiency[1]. Functional

53   abnormalities have not yet been found and their distinctive cytologic appearance is thought to be

54   due to a difference in granule staining properties, which appears more prominent with aqueous

55   Romanowsky stains such as Diff Quik[5, 7]. Accurate recognition of these cells is important in peripheral

56   blood film examination where they may be mistaken for toxic neutrophils, especially in sight-hounds

57   breeds which may have a concurrently lower WBC or neutrophil concentration compared with other

58   canine breeds[15]. Inaccurate classification (underestimation) by haematology analysers is also

59   reported, although for dogs with eosinophil concentrations within reference interval, this is unlikely

60   to be clinically significant[5].

61   Following identification of suspected grey eosinophils in a routine feline hematology sample from

62   the initial case, the aim of this project was to morphologically, cytochemically and ultrastructurally

63   describe these cells, never previously reported in the cat and determine their clinical significance.

64

65   Ethical approval for the project was attained (URN 2019 1928-2). A total of 4257 blood films from

66   2641 cases, stained with modified Wright stain (automated stainer; Hema-tek 2000, Bayer) from

67   routine feline hematology profiles were evaluated between May 2015 and July 2018 for the

68   presence of grey eosinophils. When grey eosinophils were identified, additional blood smears were

69   made from EDTA blood for further cytochemical staining and analysis. Two cases and a feline control

70   were selected for transmission electron microscopy (TEM) evaluation.

71   Aqueous Romanowsky and cytochemical stains for alkaline phosphatase (ALP), Luna and Luxol-fast

72   blue were performed as previously described for blood films from cats identified with grey

73   eosinophils and feline controls[9]. Buffy coat preparations from two cats with grey eosinophils and one

74   control cat were fixed using 3% glutaraldehyde for TEM analysis. In brief, samples were post-fixed in

75   1% osmium tetroxide and following dehydration in ascending concentrations of ethanol, were

76   infiltrated with and embedded in Agar 100 resin (Agar Scientific Ltd). The samples were then cured

77   for at least 20hrs at 60°C. Subsequently, ultrathin sections (90nm) were cut and then stained with

78   0.5% uranyl acetate and 3% lead citrate. The sections were evaluated in a Hitachi H7500

79   transmission electron microscope at an accelerating voltage of 80kV. Grey eosinophils were

80   identified based on size, shape and granule structure after exclusion of neutrophils and basophils,

81   and visually compared with feline control eosinophils.

82

83   Over the ~~three year~~three-year study period, grey eosinophils were identified in a total of nine (of

84   2641) cats presenting to the Queen Mother Hospital for small animals (QMHA) at the Royal

85   Veterinary College (RVC). Cats comprised 5 males (4 neutered; 1 entire) and 4 females (1 neutered; 3

86   entire) with ages ranging from 2 months to 12.5yrs. Domestic shorthaired (4/9) cats were most

87   commonly represented, with British shorthairs (BSH) accounting for one third of cases (3/9) with one

88   Ragdoll (1/9) and one domestic longhair (1/9). Presenting clinical signs were related to the

89   underlying clinical disease which included congenital extrahepatic portosystemic shunts (3/9),

90   neoplasia (2/9; lymphoma and haemangiosarcoma), gastrointestinal disease (IBD; 1/9), peripheral

91   vestibular disease (1/9) with two cases lacking a definitive diagnosis. Hematological changes were

92   mild in the majority of cases and included mild lymphopaenia (3/9) attributed to a stress/steroid

93   response, microcytosis (2/9) associated with portosystemic shunts (PSS), mild thrombocytopenia

94   (1/9) or were unremarkable (2/9). The remaining cat had a mild to moderate Heinz body hemolytic

95   anemia.

96   In cats with grey eosinophils, in both methanolic and aqueous Romanowsky stains, the same

97   eosinophil morphology was identified in 100% of eosinophils observed, which was repeatable across

98   follow up samples in two cases. These cells measured approx. 9-10 μm in diameter, had a band to

99   segmented nucleus (average 2-3 lobes) with clumped mature chromatin. Cells had pale blue/grey

100   cytoplasm which contained abundant small (approx. 0.2 – 0.5 μm diameter), round, non-staining

101   granules (Figure 1). Eosinophils with the typical feline morphology were not identified in any of

102   these cases. Granules also lacked Luna and Luxol-fast blue staining (Figure 2). Eosinophil

103   concentrations in all nine cats were within reference interval ($0 – 1.5$ x10^9/L) and ranged from 0.09

104   to 0.79 x10^9/L.

105   TEM analysis of grey eosinophils identified ultrastructural abnormalities of the specific granules

106   (Figure 3). In comparison to feline control eosinophils, the characteristic electron dense core of

107   specific granules was not present in either cat with grey eosinophils. Instead granules had a

108   fragmented core with homogenous peripheral cortex and thin limiting membrane. Neutrophil and

109   basophil ultrastructure were similar to controls.

110

111   This study describes the morphologic, cytochemical and ultrastructural features of specific granules

112   in a population of cats with grey eosinophils, not previously described in this species.

113   Eosinophils in most species are identified on light microscopy by the 'eosin-loving' properties of their

114   granules, the characteristic staining pattern a result of the high cationic protein content. The most

115   numerous and cationic of these proteins is major basic protein (MBP), which forms the crystalline

116   lattice structure of the core of eosinophil (specific/secondary) granules. It is this core which gives the

117   eosinophil its unique ultrastructural feature[2, 8, 11, 12].

118   Eosinophil specific granule abnormalities are occasionally described in the literature and are noted in

119   association with eosinophil peroxidase (EPO) deficiency (Presentey's anomaly). This is characterised

120   in humans and mice by a reduction in the volume of electron-lucent matrix with maintenance of the

121   electron dense granule core[3]. Whilst this is suspected to be the case abnormality present in canine

122　grey eosinophils, ~~this~~ EPO deficiency would ~~does~~ not explain the striking ~~difference in comparison~~

123　~~with the~~ ultrastructural changes noted in the cats in this study[1]. Eosinophil secondary granules in

124　both MBP-1 ~~The lack of MBP in MBP-1~~ knock out mice (MBP-1$^{-/-}$) and cystatin F deficient mice (CF

125　null) have a staining pattern and ultrastructural appearance with similarities to those detected in the

126　eosinophil secondary granules in this study's cats. MBP-1 $^{-/-}$ mice ~~has been found to have distinct~~

127　~~staining and ultrastructural effects on~~ eosinophil ~~specific~~ secondary granules. ~~Whilst granule~~

128　~~number, size and cross-sectional area appeared unaffected, granules~~ lack~~ed~~ the characteristic

129　pink/red staining with Wright-Giemsa, and at the ultrastructural level, the electron dense core is

130　absent. This indicates that the core structure in mice eosinophils is dependent on the presence of

131　MBP. MBP is also significantly reduced in cystatin F deficient mice (CF null) compared with wild-type

132　(WT) counterparts, and accordingly, they have eosinophil secondary granules with an extensive

133　electron lucent periphery and cores with internal material which is often amorphous or lucent. Given

134　the similar changes to the electron dense cores within eosinophil secondary granules in this study's

135　cats, ~~suggesting a lack of the cationic nature needed for eosin binding. Subsequent ultrastructural~~

136　~~analysis revealed that MBP-1 deficient mice lacked the characteristic electron dense core, indicating~~

137　~~the core structure in mouse eosinophils is dependent on the presence of MBP-1. Interestingly,~~

138　~~electron-dense cores were still present in heterozygotes of MBP-1 knock out mice similar to wild~~

139　~~type mice, suggesting that core formation is not susceptible to an approx. 50% decrease in MBP[2].~~

140　~~Ultrastructural analysis of eosinophil granules in cystatin F deficient mice (CF null) also show striking~~

141　~~differences to the wild-type (WT) counterparts with granules having an extensive electron lucent~~

142　~~periphery and cores with internal material which was often amorphous or lucent. Consistent with~~

143　~~these core abnormalities MBP was much reduced in comparison to WT mice[10]. Given the similar~~

144　~~changes to the electron dense core within eosinophils in this study's group of cats,~~ a complete lack

145　or significant decrease ~~or lack~~ in the MBP content of the eosinophil secondary granules is considered

146　likely.

147   Luna staining is commonly used for histological identification of eosinophils in many species and is

148   described as highly specific[6]. Biebrich scarlet, the chromatophore utilised within the Luna staining

149   protocol has a high affinity for basic proteins and readily highlights eosinophil cytoplasmic granules

150   as a result of their basic nature[2, 4]. The suspected lack or marked reduction in MBP content in the

151   granules of these cats likely accounts for the negative lack of Luna staining compared with control

152   cats. To identify a reduction or lack of MBP in these cats, further work will focus on validation and

153   optimisation of techniques for use of an anti-major basic protein antibody to stain for MBP, with

154   genetic analysis and sequencing of the proteoglycan 2 (PRG2) gene in these cats in comparison with

155   feline controls.

156   Other differentials considered for the atypical findings on ultrastructural analysis included granule

157   deterioration due to eosinophil activation and granule content release or granule deterioration due

158   to improper fixation. Three different mechanisms of granule content release are recognised in

159   eosinophils; exocytosis, piecemeal degranulation (PMD) and eosinophil cytolysis (ECL). Granule

160   proteins may be secreted by classical exocytosis, in which a single granule fuses with the plasma

161   membrane, or compound exocytosis, in which multiple granules may fuse together before fusing

162   with the plasma membrane to release their contents. Specific granule components are released

163   through membrane-bound secretory vesicles in PMD with empty granule chambers retained within

164   the cell. In ECL there is loss of cell cytoplasm with rupture of the cell membrane and release of

165   membrane-bound specific granules, with chromatolysis of the cell nucleus[12, 13, 14]. Partially empty

166   specific granules or free extracellular specific granules with signs of cell chromatolysis were not

167   identified on TEM analysis of feline grey eosinophils, suggesting that granule release is not the cause

168   for the atypical granule appearance. Improper fixation method also appears unlikely, given the

169   repeatability of the findings and structural integrity of other leukocytes. Eosinophil nuclei were

170   intact without evidence of fragmentation or pyknosis, and limiting membranes of specific granules

171   remained well preserved.

172   The significance of grey eosinophils in cats is not fully understood. Functional abnormalities were not

173   suspected, given that affected cats did not appear to have clinical signs associated with dysfunction

174   or deficiency of eosinophils. However, if the lack of MBP is confirmed, functional abnormalities such

175   as reduced parasite immunity similar to CF null mice, could be considered[10].

176

177   In summary, we describe the morphological, cytochemical and ultrastructural features of grey

178   eosinophils in a series of cats. Whilst not currently expected to be of clinical concern, in contrast to

179   grey eosinophils in sighthounds, cells exhibit distinct cytochemical and ultrastructural changes. The

180   absence of the characteristic electron dense core in specific granules is proposed as the cause of the

181   features identified, with a decrease in or complete lack of MBP content suspected. Similarly to

182   sighthounds, accurate recognition of these cells is important, to prevent their classification as toxic

183   neutrophils and an association with inflammation.

184

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191   organisation or entity with a financial interest in, or in financial competition with, the subject matter

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240   **Legends for figures**:

241   **Figure 1**: Population of granulocytes with small, round, non-staining granules and pale blue

242   cytoplasm; peripheral blood smear; modified Wright stain; 100x objective. Bar 10μm.

243   **Figure 2**: A-E: feline grey eosinophils, F-J: feline control eosinophils, 100x objective.

244   Non-staining clear, round granules with methanolic Romanowsky (modified Wright stain; A),

245   aqueous Romanowsky stains (Diff-Quik; B). and Luna stain (C). Granules remain negative but are

246   ~~highlighted with pale blue cytoplasmic staining with~~with Luna (C), ~~~~Luxol fast blue (D) ~~with~~

247   ~~negative~~and alkaline phosphatase (BCIP-NBT) staining ~~of granules~~ (E). Classic ~~R~~rod-shaped, red/pink

248   staining granules in control eosinophils with methanolic Romanowsky (modified Wright stain; F) and

249   aqueous Romanowsky stain (Diff-Quik; G). Control eosinophils granules ~~have~~ show intense red

250   staining with Luna (H) and mid-blue staining with Luxol fast blue (I). Alkaline phosphatase (BCIP-NBT)

251   staining of control feline eosinophils demonstrated stain uptake by granules (J). Bar 10μm.

252   **Figure 3**: Transmission electron micrographs of ~~normal~~ control (A-B) and grey (C-F) eosinophils

253   in the cat. ~~Normal~~ Control feline eosinophil with characteristic ~~heterogeneous bicompartmental~~

254   specific granules containing a central electron dense core 15,000x (A); higher magnification of

255   specific granules in control cat 60,000x (B); Grey eosinophil granules with fragmented pattern ~~to~~

256   ~~specific granules~~ and absence of electron dense core in two ~~separate~~ cats 15,000x (C and E);

257   higher magnification of abnormal specific granules, lacking characteristic electron dense core
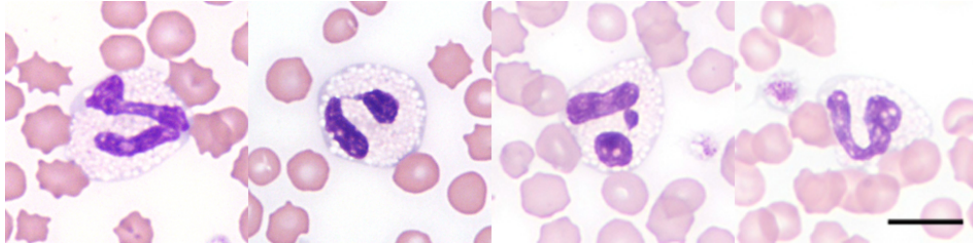
258   60,000x (D and F).

259

Figure 1: Population of granulocytes with small, round, non-staining granules and pale blue cytoplasm; peripheral blood smear; modified Wright stain; 100x objective. Bar 10μm.
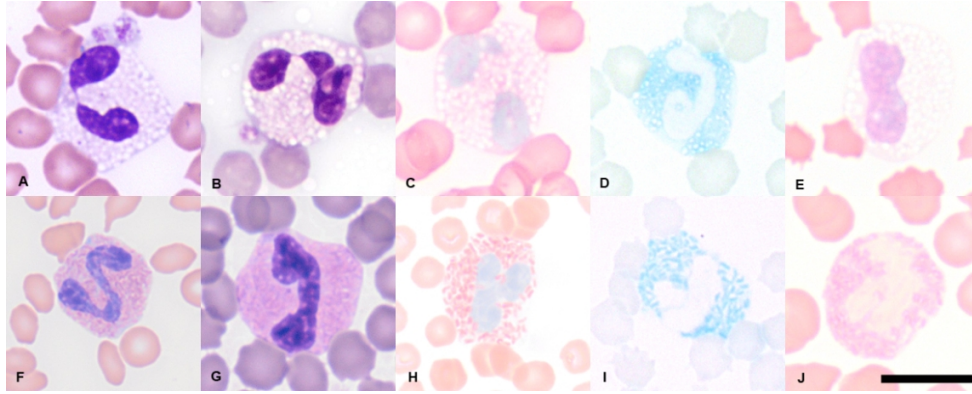
80x19mm (300 x 300 DPI)

Figure 2: A-E: feline grey eosinophils, F-J: feline control eosinophils, 100x objective.
Non-staining clear, round granules with methanolic Romanowsky (modified Wright stain; A), aqueous
Romanowsky stains (Diff-Quik; B). Granules remain negative with Luna (C), Luxol fast blue (D) and alkaline
phosphatase (BCIP-NBT) staining (E). Classic rod-shaped, red/pink staining granules in control eosinophils
with methanolic Romanowsky (modified Wright stain; F) and aqueous Romanowsky stain (Diff-Quik; G).
Control eosinophils granules show intense red staining with Luna (H) and mid-blue staining with Luxol fast
blue (I). Alkaline phosphatase (BCIP-NBT) staining of control feline eosinophils demonstrated stain uptake
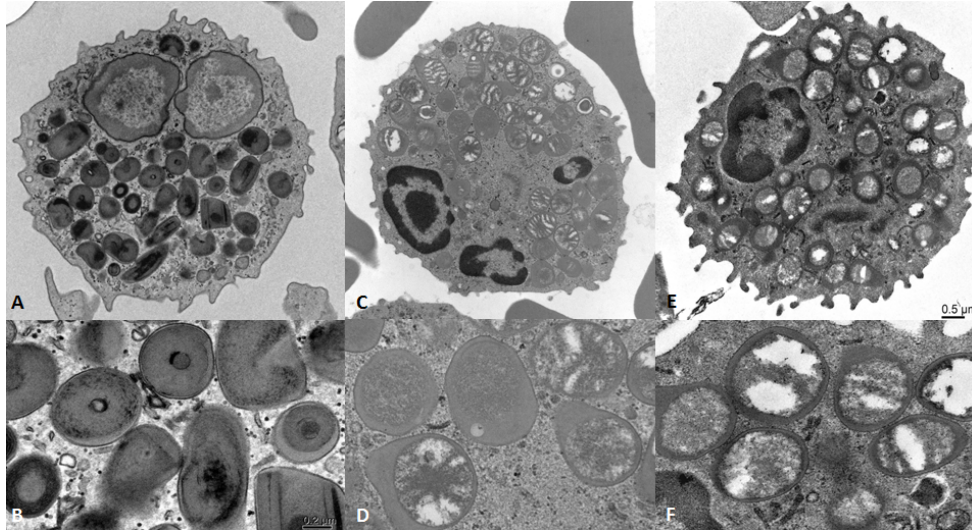by granules (J). Bar 10µm.

99x39mm (300 x 300 DPI)

Figure 3: Transmission electron micrographs of control (A-B) and grey (C-F) eosinophils in the cat. Control feline eosinophil with characteristic specific granules containing a central electron dense core 15,000x (A); higher magnification of specific granules in control cat 60,000x (B); Grey eosinophil granules with fragmented pattern and absence of electron dense core in two cats 15,000x (C and E); higher magnification of abnormal specific granules, lacking characteristic electron dense core 60,000x (D and F).

80x44mm (300 x 300 DPI)