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Research Article

Cytogenetics of Reactive Bone Marrow Associated with a Fungal Infection (*Hemomycetes avium*) of Ducklings

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Abstract

The manuscript describes the consequences of fungal infection (*Hemomycetes avium,* Ha*)* in Bone Marrow (BM) and blood of lame ducklings, and demonstrates some of the anatomical characteristics of this fungus. Microscopic evidence suggests that multiple atypical cells appear when Ha is present in BM and blood. This atypia involves members of all series; leukocytes, granulocytes, erythrocytes, and other (stromal) cells. Binuclear and tri-nucleate Plasmacytes (PC) either with equal or unequal size nuclei are found. These suggest the parent cells divide by both mitosis and amitosis. Many such cells are giants (cell area, A_C>300 μm^2) and likely polyploid. Cytogenetic atypia includes sticky and vagrant chromosomes, haphazard metaphase arrangements, and micronuclei. Many of the cytogenetic atypia resemble types commonly seen in the *Allium* system used to detect plant toxins. Guidance from the *Allium* system was used to classify some cytogenetic anomalies, and guidance from a system used to classify Multiple Myeloma (MM) was applied to atypical plasmacytes. Bacteria, either free-swimming or attached to the surface of various cells Cell-Associated Bacteria (CAB), are common in fields also containing Ha. These are likely responsible for toxin(s) contributing to the cytology and cytogenetic anomalies.

Keywords: Cell-associated bacteria; Cytogenetics; Cytology; Ducklings; *Hemomycetes avium*; Reactive bone marrow

Abbreviations

C: Conidia; H: Hyphae; M: Mycelium; S: Shards; Lb: Lymphoblast; LM: Medium Lymphocyte; LS: Small Lymphocyte; C_X: Chromosome #x; CAB: Cell-Associated Bacteria; CM: Cell Membrane; HC: Classic Heterophil; HT: Typical Heterophil; HSC: Hematopoietic Stem Cell; A_C(μm^2): Area of the Cell; A_N(μm^2): Area of the Nucleus; P_C(μm): Perimeter of the Cell; MI: Mitotic Index; MM: Multiple Myeloma; PC: Plasmacyte; PMB: Polymicrobial Bacteremia; pRBC: polychromatic RBC; R: Phagocytic Reticular Cell

Introduction

Avian Bone Marrow (BM) like its mammalian counterpart is composed of cells of several series. All developmental stages of erythrocytes, lymphocytes, myelocytes, and thrombocytes are present, along with bone cells (osteoclasts and osteoblasts) and reticular cells (stroma). During homeostasis the frequency of dividing cells (mitotic index, MI) is low. Lucas and Jamroz, Table 10, p200, [1] give values of <1% dividing cells over a range spanning embryonic to advanced post-hatch stages of chickens.

The transition from homeostasis to a pathologic state, as a consequence of toxin exposure, alters normal BM cytology, cytogenetics, and MI. Some of the atypical cells can make their way into circulation.

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Sensitive assay systems capable of detecting toxins are available in plants. These methods involve exposing growing cells to varying levels of a candidate substance and then enumerating the frequency and types of cytogenetic aberrations. The meristematic root-tip cells of *Allium* (onion) provide a convenient means. An animal system that mimics *Allium* may occur naturally.

Hemomycetes avium (Ha), a blood-borne fungus, occurs in a wide variety of poultry, Cotter [2]. Positive samples identified in Wright-Giemsa-stained blood films, and other tissues, often come from samples also with evidence of Polymicrobial Bacteremia (PMB); Cotter [3]. Ha was invariably accompanied by a complex hemogram, and leukocytosis, often exceeding 200k WBC/Ml, Cotter [4]. Atypia involving cells of all lymphoid and granulocyte series, associated with PMB, have been described Cotter and Heller [5], Cotter [6], Cotter and Bakst [7], Cotter [8,9]. Ha was also a component in many of these studies.

The purpose of this manuscript is to illustrate the variety of cytogenetic anomalies, chromosomal aberrations, and atypical cell division, accompanying combined infection with Ha and bacteria. Some anomalies are classified using guidance from the *Allium* system developed for plants; others (plasmacytes) are adapted from a system designed for MM cells. Selected examples of atypia, present in all leukocyte series of lame duckling BM, are the subject.

Materials and Methods

Ducks

White Pekin ducklings were grown in floor pens covered with wood shavings. Lame ducklings were identified by trained service personnel who prepared slides on site. Lame ducklings were removed from pens to prevent further harm. Welfare was monitored under the Maple Leaf Farms Trident Stewardship Program for Duck Well Being and procedures were reviewed by a PAACO-certified auditor and licensed Veterinarian.

Staining

Slides prepared on-site, were later immersed in 95% ethanol and postfixed for 10 min to 15 min. Films were stained by Wright's method followed by brief exposure to Giemsa following recommendations by Hewitt [10].

Light Microscopy and photomicrographs

Olympus CX-41(Olympus America, Center Valley, PA 18034-0610) is equipped with Plan N 40x, 0.65 numerical aperture dry, and Plan N, 1.25 numerical aperture 100x oil objectives. All images were captured at 100x with an Infinity-2 1.4-megapixel charge-coupled device Universal Serial Bus 2.0 Camera, and processed with Infinity Analyze software (Release 5.0.3) (Lumenera, Inc., Ottawa, ON, Canada).

Cell sorting

Cells are classified as lymphoid or granulocytic using guidance developed for embryos and post-hatch chickens [1]. Histiocytes are classified as granular or non-granular based on cytoplasm composition [8]. The declaration of a cell as polyploid (giant cell) is size based; determined by nuclear area (A_N, using the diameter (D/2) of the longest axis as input. Nuclear/Cytoplasmic ratios (N/C) are computed by dividing the nucleus area by the area of its cell (A_N/A_C).

Cytoplasm

Classification of lymphoid cytoplasm as primitive, intermediate, or derived, is based on its hue. Violet (basophilic cytoplasm) cells are considered primitive; those with a blue-gray hue are intermediate, and those with light-blue cytoplasmic hues are derived types. The classification is based on the assumption these differences represent progressive developmental stages [11]. It parallels the system used for erythrocytes (RBC) where the gray cytoplasm of cells not yet fully hemoglobinized (polychromatic, pRBC) is differentiated from the yellow-orange color of a mature RBC. Additional guidance came from a scheme used in classifying neoplastic MM cells [12].

Chromosomal aberrations and atypical mitosis

Atypical chromosomes were classified by extrapolation of the observations of Fiskesjö [13] developed for the *Allium* toxicology test procedure. Chromosomal atypia are: fragmented metaphase and anaphase, chromosomal bridges (intact and/or broken) bi and multi-nuclear cells, polyploidy, C-metaphase, micronuclei, stickiness, and vagrant chromosomes. Cellular atypia are: lobed, irregular, and extruded nuclei, arrested segregation, giant size, and irregular shapes. Due to space limitations all categories are not shown here.

Results

The strategy for presentation will begin with a description of *Hemomycetes avium* (Ha), a presumed toxin source, as it appears in BM and tissues of ducklings [2]. Atypical cells demonstrating various anomalies associated with Ha infection will follow.

Hemomycetes avium (Ha), not yet cultivated *in vitro*, is presumed to be a member of the Ascomycete family until its definitive taxonomic position has been established. It can present in blood and tissues as a Mycelium (M) in Figures 1 and 2A. More often it presents as mycelial fragments, called "shards" (s) in Figures 1 and 2B. Shards may be free or phagocytosed; Hyphae (H) are relatively uncommon. Ha is often found in blood partly because these samples are easily available. It has also been detected in assorted tissues such as BM, bursa of Fabricius (Figure 2B); hock joint fluid (not shown), and thymus (Figure 3B). It is common to detect bacteria, either free-swimming or attached Cell-Associated Bacteria, (CAB) in the company of Ha.

Multinuclear cells of the Plasmacyte series

As a working hypothesis, binuclear cells arise by a failed effort at cytokinesis. If both daughter nuclei are of similar size and not themselves polyploid, the defect will solely be cytoplasmic. If daughter nuclei are of unequal size the defect extends to nuclear phenomena. Examples are in Figure 3, Panel A. A giant trinuclear cell with heterogeneous nuclei (size and shape) is likely a member of the plasmacyte series (A_C 316 μm²; P_C 76 μm). N_1 the largest nucleus (A_{N1} 50 μm²) is elliptical with unequal-sized nucleoli. N_2 is a circular nucleus (A_{N2} 45 μm²) with 2 equal nucleoli. The smaller elliptical nucleus N_3 (A_{N3} 32 μm²) has a rectangular Dutcher body at its left base, possibly representing a crystallized immunoglobulin (Ig). The nuclear sizes indicate polyploidy, possibly as high as 6C (N_1). The adjacent cell, a giant mesomyelocyte (a developmental granulocyte; A_C 181 μm²) at metaphase, is also a polyploid. Its largest chromosomes are sticky and appear as projections from the rest of the aggregated chromosomal mass. The arrow is assumed to locate multiple copies of the closely paired arms of C_1 (polyteny). A Ha shard is encircled. Panel B. A giant (A_C 181 μm²) plasmacytoid cell residing in the thymus is at anaphase; and displays the consequence of sticky chromosomes; N/S locates the presumptive poles.

Figure 4, Panel A Three standard sized lymphoid cells are members of the plasmacyte series; cell 1, lymphoblast (Lb; D 11.8 μm, A_C 34 μm², N/C 0.62) cell 2, a reactive lymphocyte, (Lm; D 8.3 μm, A_C 50 μm², N/C 0.62). Cell 3, is a Plasmacyte (PC) with a characteristic eccentric nucleus and condensed chromatin in a "cart-wheel" configuration; a paranuclear H of (Golgi) is also seen (D 6.2 μm; A_C 30 μm², N/C 0.30). The cytoplasm is fenestrated by small vacuoles, but these are too small and too few to represent a true Mott PC [12]. Cell 4 is a lysed histiocyte, and cells 5 and 6 are nuclear remnants of uncertain origin. Panel B. A binuclear PC with primitive (blue-violet) cytoplasm rests in between a primitive Ls and a reactive Lm. The Cell Membrane (CM) folds displayed by the latter illustrate zeiosis, a prodromal stage of apoptosis. A central Hof appears to serve both nuclei whose chromatin is condensed into cart-wheel arrangements. A_N are approximately equal, at ~25 μm²; A_C ~117 μm², giving an N/C 0.44; using $A_{N1}+A_{N2}$ for the computation. As no evidence of a cleavage furrow exists, the defect is purely cytoplasmic. Panel C. A binuclear plasmacyte with an irregular edge. Two micronuclei (small arrows)

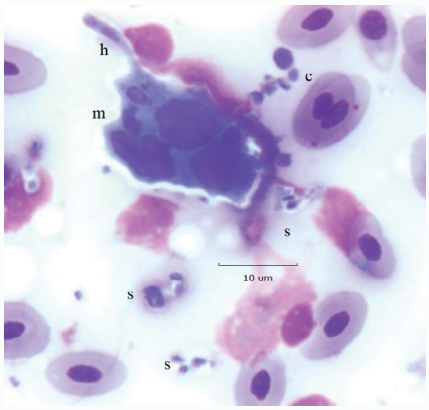

Figure 1: *Hemomycetes avium* (Ha) as it appears in BM of a lame duckling. Mycelium (m) hyphae (h) conidia (c) and fragmented mycelium (s shards) are as labeled.

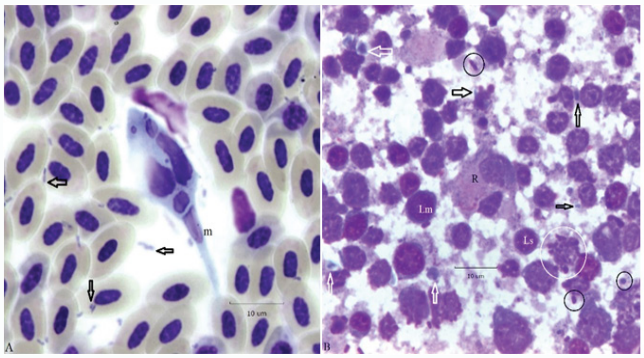

Figure 2: *H. avium* as it appears in tissues. Panel A) Mycelium (M) in duckling blood in the company of free and CAB (arrows). Panel B. Ha shards (arrows) embedded within the matrix of the bursa of Fabricius: R, phagocytic reticular cell; LM, Medium Lymphocyte; Ls: Small. Additional descriptions of cells are in the text.

are associated with the irregular top nucleus whose contents are partially extruded. Two clear Dutcher bodies, nuclear equivalents to Russell bodies, representing retained immunoglobulin, are also seen. The presence of micronuclei, and the nuclear membrane irregularity, indicates the defect (a binuclear cell) is a result of the failure of both nuclear and cytoplasmic events. A phagocytosed bacillus containing an endospore is at the left edge (long arrow); its presence suggests a source contributing to the atypical cytology. Panel D, A mitotic PC at the anaphase/telophase transition has chromosomal bridges. Bridge 1 remains intact and is presumed to represent Chromosome #1 (C_1); the largest of the duck karyotype; bridges 2 and 3; representing C_2 and C_3, have broken. The cytoplasm has differentiated into ecto/endoplasmic regions. The cell above (Lm) shows sufficient characteristics to be classified as a PC. A Classic Heterophil (HC) and a Typical Heterophil (HT) are nearby [9].

Giant cells with sticky chromosomes

A polyploid (4C) progranulocyte (pG; A_C 254 μm^2, A_N 141 μm^2; N/C 0.55) at the prometaphase stage has condensed chromosomes and a retained nuclear membrane is in Figure 5. The arrow locates the nuclear membrane edge where a clear distinction between the nucleoplasm and cytoplasm is found. Other nearby cells is an Ls and an Lm both with primitive cytoplasm. pRBC were slightly shorter than nearby RBC (Ave. 9.8 μm *vs.* 12.8 μm) suggesting they are

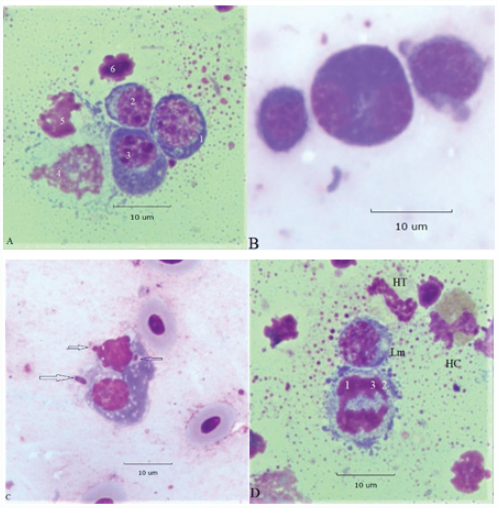

Figure 4: Panel A. Three lymphoid series cells; 1, Lymphoblast; 2, Reactive lymphocyte; 3, Classic plasmacyte. Panel B. Binuclear PC with primitive cytoplasm, primitive Ls, reactive Lm. Panel C. Binuclear plasmacyte with an irregular edge and micronuclei. Panel D. A mitotic PC at the anaphase/telophase transition with chromosomal bridges. Additional descriptions of the cells are in the text.

atypical types. A CAB and a pyrenocyte (nucleated erythroplastid [14] are located at the top-left edge (*).

Discussion

The present observations are intended to demonstrate the role of the fungus (*Hemomycetes avium*) as a cytotoxic agent. Ha has been detected microscopically in the blood of a wide variety of poultry [2] often in the company of free or cell-associated bacteria, themselves potential sources of toxins. When Ha is present in BM it is invariably accompanied by cells expressing cytogenetic and cytologic anomalies. These atypical forms involve all series. Since the Hematopoietic Stem Cell (HSC) is the common progenitor of all series, likely, it is sometimes the target. Since HSCs divide and pass this capacity to their immediate descendants; it is not surprising both mitosis and chromosomal anomalies should be seen in all series of BM and at multiple developmental stages.

When the Plasmacyte Series (PC) is involved, multinuclear cells result. More often primitive PC, those with basophilic cytoplasm, becomes bi and tri-nuclear. Such cells are seen in MM, a neoplastic

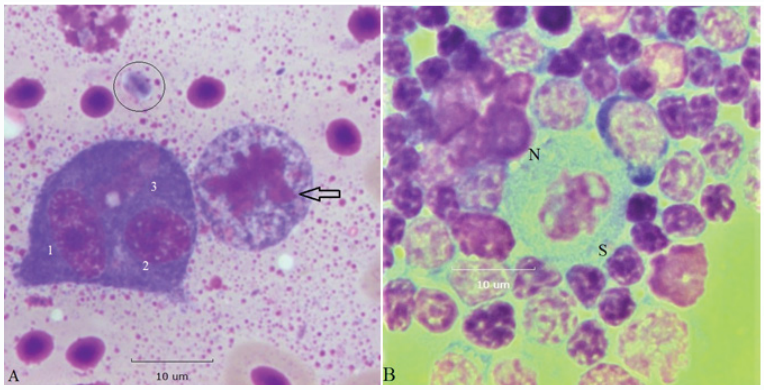

Figure 3: Panel A. Left, a giant trinuclear PC containing a crystalline Dutcher body. Right, a giant mesomyelocyte at metaphase, displays thickened chromosome arms, likely as a result of polyteny (arrow). A Ha shard is encircled. Panel B. A mononuclear giant plasmacytoid cell at anaphase with sticky chromosomes. Additional descriptions of cells are in the text.

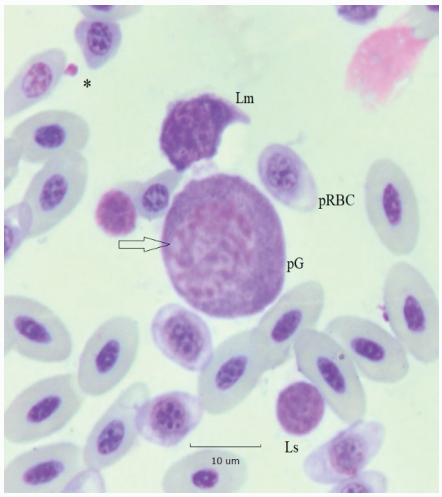

Figure 5: A polyploid (4C) progranulocyte (pG) at prometaphase has a retained nuclear membrane (arrow). Additional descriptions of neighboring cells are in the text.

condition, so this supports the notion some toxins can act early. PC nuclei may be of equal size; presumably, the result of successful mitosis; suggests a toxin interferes with cytokinesis. In other cases, daughter nuclei are unequal, suggesting a toxin acts directly on karyokinesis, perhaps at assembly (metaphase) or during segregation (anaphase). The consequence of which is an unequal distribution of daughter chromosomes. Binuclear RBC, described in turkeys, Bloom [15] result from the action of a recessive gene (*bn*). Perhaps some *duck gene* equivalent to *bn* is operative on PC and its expression is controlled by exposure to the toxin.

Giant cells are a special case, with little information available on their significance. They can result from polyploidy, multiple chromosomal sets residing in the same cell. Giant cells may also arise through endoduplication of their DNA, Robinson et al. [16]. Do such cells offer more targets for toxin action? Are multiple chromosomal sets in a single cell too unwieldy for successful mitosis? Can amitosis occur in a giant cell? Do supernumerary chromosomal sets offer a survival value, as has been suggested in some cancers [17,18]? How should giant cells originating in BM be interpreted when seen in the circulation [19]? In what discussion space should polytene cells be placed?

Erythroid cells are the most common element in BM, [1]. Because of this anatomical property, it may not be surprising to find diverse atypia in this group. Cells displaying chromosomal bridges, vagrant chromosomes, and binuclear erythrocytes are common in BM samples also having Ha.

Mycotoxins are known to have the capacity to damage DNA, and it was shown that the DON toxin caused DNA fragmentation in chicken spleen cells, [20]. However, the exact mechanisms of genotoxicity are not yet completely clear [21]. Found that dietary inclusion of T-2 toxin also increased lipid peroxidation in the livers of quails. The present observations reinforce these studies by providing visual evidence of damaged DNA and processes dependent on intact nucleic acids.

Conclusions

In conclusion, the present observations support the role of Ha as a toxin source and likely contributing to the development of lameness. Since these observations are made from microscopic examinations, identification of individual toxic substances is not possible. Hopefully, these observations are of sufficient value to overcome this limitation and stimulate such an effort.

Acknowledgments

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