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Complex Hemograms of Isolator Raised Specific Pathogen Free (SPF) Chicks

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Abstract: Heterophil/lymphocyte (H/L ratio) statistics and total white blood cell counts (TWBC) determined by light microscopic examination of blood from isolator raised specific pathogen free (SPF) chicks indicated complex hemograms. Brachial vein blood (N =12) obtained at 6 wk was spread into films, air dried, post-fixed in MeOH and Wright's stained. H/L 1 ratios were calculated by dividing all heterophil types, typical (HT) variant (HV) and classic (HC) by the number of small (resting) lymphocytes (Ls) H/L 1 = (HT + HV + HC)/(Ls). A second ratio (H/L 2) obtained by dividing heterophils by all lymphocytes (resting, reactive and atypical, (HT + HV + HC)/(Ls + Lm) was calculated; as was the H/L 1-H/L 2 difference (ΔH/L). TWBC's were determined from the same films. Sorting, non-random distribution of cells and atypia affected all H/L statistics. The mean H/L 1 ratio from standard differential counts (SDC) = 0.17 and H/L 2 = 0.16. Edge based ratios (EB) were ~8 times the SDC values. Atypical cells were in all samples and more common in EB counts. The H/L ratios and ΔH/L calculated from SDC and EB values were significantly different ($p<0.02$). Collectively these observations support earlier conclusions regarding the lack of sensitivity of a single H/L ratio, without a TWBC, or consideration of atypia to estimate welfare. Furthermore, hemograms of SPF chickens reared in isolation may be complex.

Key words: Complex hemograms, atypia, heterophil lymphocyte ratios, welfare

INTRODUCTION

The standard differential blood count (SDC) provides a "blood picture" supplement to clinical observations for determining an animal's health. As an extension, SDC's may measure stress (Burton and Guion, 1968). The idea, an outgrowth of earlier studies (Davidson *et al*., 1983; Gross, 1989; Gross and Siegel, 1983, 1986) finds use in fields ranging from ecology to evolution. The early investigators described how stress affected the ratio of heterophils to lymphocytes (H/L). Stress generally increased heterophils and decreased lymphocytes; handling or cold increased eosinophils, but basophils did not change (Wolford and Ringer, 1962; McFarlane *et al*., 1989; McFarlane and Curtis, 1989). However, in a large study of blood pictures of commercial laying hens, the standard H/L method was unreliable as a stress measure. Low (non-stress) H/L' s often accompanied high total white blood counts (TWBC) an apparent contradiction (Cotter, 2015a). Moreover atypical cells were commonly found (Cotter, 2015b, c). It is unknown if the unusual hematology is unique to commercial hens and their caging systems. Therefore, a study of blood films of certified specific pathogen free (SPF) chickens raised in isolators is the subject here. Study-wide hematological measures, H/L 1, H/L 2, their difference (ΔH/L) total white blood counts (TWBC) and standard differential counts (SDC) are the data. Wright stained peripheral blood films of 6 wk SPF chickens (N =12) housed in isolators are the data source. Each slide was counted at least twice and due to non-random cell distributions, ("sorting") repeat SDC's and edge based counts (EB) were obtained, giving 31 SDC, 15 EB and 27 TWBC estimates. Complex hemograms occurring in SPF/isolation settings are remarkable. The response to low grade bacteremia seen in these SPF chicks apparently adds complexity to the blood pictures (Cotter and Heller, 2015). The present observations support and extend earlier conclusions (Cotter, 2015a-c) on the need for integrating ratios and other statistics, with cytoarchitecture for good use of hematology.

MATERIALS AND METHODS

Chicks: Hatching eggs were purchased from an SPF flock certified free of 33 avian pathogens, Sunrise Farms, Catskill, NY 12414, USA. They were incubated for 18 days in a clean and disinfected incubator (37.5 C) and 85% humidity. On day 18 eggs were candled and transferred to clean (16% glutaraldehyde in 4 quaternary ammonium compounds, (Glutamon by Biovac, Or-Akiva, Israel) 0.56 m^3 Horsefall-type negative-pressure isolators equipped with 0.84/m^2 wire flooring designed to allow droppings to fall through. The chicks were given food, free of anticoccidials and water *ad libitum*. The chickens were examined daily and were determined to be clinically healthy and sero-negative to all known chicken diseases.

Blood and stain procedures: Whole blood drawn from the wing veins was immediately spread across the length of standard microscope slides and dried in air. Slides were

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post fixed in absolute MeOH for 10 -15 min. Films were stained by Wright's method following the manufacturer's recommendations (Sigma Chemicals, St. Louis, Mo., Procedure WSGD-128). Gram stains were with commercial reagents (Product# 291-476, Fisher Scientific Co. Kalamazoo, MI, USA).

Differential counts: Population-wide statistics are based on standard differential counts (SDC) using sets of a minimum of 200 leukocytes. SDC's used counts from central microscopic fields magnified at 40x (high-dry). Leukocytes were categorized as small or medium lymphocytes (Ls, Lm) monocytes (Mn), heterophils (typical, variant, classic types; HT, HV, HC), basophils (Ba), or eosinophils (Eo). Morphological criteria described in avian hematology texts (Campbell and Ellis, 2007; Lucas and Jamroz, 1961; Reagan *et al*., 2008; Weiss and Wardrop, 2010) and in Cotter (2015c), are the category basis. Atypical cells were included in the differential counts, their locations noted and later photographed. Blast cells were counted, but thrombocytes were not included in differential counts. Total white blood cell counts (TWBC) were estimated from the same slides and fields as SDC's by a method described in Campbell and Ellis (2007).
Additional sets of 200 cell counts were made when an atypical or a"sentinel" cell or some other unusual hematological phenomenon (leukergy, Otremski *et al*., 1993), reactive cluster formations (RC) or sorting (Rebar and Raskin, 2006), was identified during the initial pass (Table 1). Counts of cells located at the ends of the smears are identified as "edge based" (EB) in Table 1.

Photomicrography: Olympus CX-41 light microscope (Olympus America, Center Valley, PA 18034-0610) equipped with a Plan N, 1.25 N.A. 100x oil objective. Images captured with an Infinity-2 1.4 megapixel CCD USB 2.0 Camera, were processed with Infinity Analyze software (Release 5.0.2) (Lumenera, Inc. Ottawa, Ontario, CA K2E 8A7).

Statistics: Significance of SDC/ED mean differences were determined by a t-test with Minitab statistical software Release 17, Minitab Inc, State College, PA, 16801, USA.

RESULTS

Study-wide hematology: Atypical cells of all leukocyte categories and irregular (anisocytosis) erythrocytes were detected by SDC and EB counts in all samples. Sorting, the non-random distribution of leukocytes (accumulation at ends) was evident in all samples. Hematology statistics were severely affected by sorting as seen by differences in SDC/EB leukocyte percentages (Table 1). Standard differential counts (SDC) are from middle region microscopic fields. Edge basis counts (EB) were determined from fields located at the ends of the slide. Their difference, calculated by subtraction, gives an estimate of specific cellular sorting tendencies. The Table 1 data indicate small "resting" lymphocytes (Ls) are more likely to be found in middle microscopic fields during SDC's; monocytes (Mn) heterophils (HT, HC) and basophils (Ba) are more often located at the edges. The sorting differences carry-over to the study-wide statistics of Table 2. H/L ratios computed from EB data are approximately 8 times those of SDC data. Each ratio statistic was highly significant. TWBC's cannot be determined reliably from EB counts.

Cytology: Sorting, accumulations of groups of cells, common in films of effusion fluids (Rebar and Raskin, 2006) was evident in all 12 samples; in addition to normal cells, Accumulations contained varying numbers and kinds of atypia. Fields with atypical cells often contained bacteria. Erythrocytes, present in all fields, have an average long axis of 10.1 (+/-0.48) µm and exhibited slight to moderate anisocytosis (Fig. 2).
The accompanying figures convey the overall complexity of hemograms of isolator raised SPF chicks. The selections came from 6 of 12 samples whose H/L 1 ratios ranged from 0.12-0.32, H/L 2 from 0.11-0.27 and TWBC's from 20-50K.
Figure 2 is of an EB field enriched with normal cells (HC, Ls, Mn) and atypia. A large developmental monocyte (dMn) reactive lymphocytes (Lm) reactive thrombocytes (Th) and dysplastic basophils (Ba). The cytoplasm of one basophil appearing to contain phagocytosed bacteria is located by an arrow. The shapes of a majority of erythrocytes are irregular (anisocytosis).
Cells with features of atypical basophils or heterophils were among all samples; an example is in Fig. 3a. This large diameter cell (10 µm) has an area of 75 μm^2 and a nuclear/cytoplasmic (N/C) ratio of 0.75. A slightly larger cell with an eccentric nucleus and cytoplasmic features similar to 3A was in the same sample (Fig. 3d).
A four-cell cluster containing 2 medium lymphocytes (Lm) and 2 cells with distinctly basophilic hues (Ba) are in Fig. 3b. Clustering, a feature of blood films from infected sources is highly remarkable and originally described by Fleck (1949). Leukergy is considered to indicate bone infection by Otremski *et al*. (1993).
Examples of atypical heterophils common throughout this study are in Fig. 3c. The left cell has a 2 lobed nucleus, the nucleus of the other cell has 3 lobes and would represent a more mature stage. The condition of the cytoplasm and granules of each suggests mild toxicity.
Rare and unusual cells of the circulation are in Fig. 4. The left most cell of panel A is an eosinophil, the cell on the right is a classic heterophil (HC) unusual because its deep red cytoplasmic granules are spindle shaped. Eosinophils were scarce throughout the study, only 3 were among a total of 5014 leukocytes (0.06%). One of the eosinophils a developmental stage, metamyelocyte, is in Fig. 3b.
Plasmacytes, by themselves remarkable features of leukograms, were found in 10/12 (83%) samples. A classic plasmacyte displaying a characteristic extranuclear Hof

Table 1: Standard differential counts (SDC) and edge basis counts (EB) and the difference (SDC-EB) as a % of total leukocytes of N = 12 isolator raised SPF chickens at 6 wk. Probability (p) by T-test; ns = non-significant

Count	No	HT[1]	HV	HC	Ls	Lm	NK	Bst	Mn	Ba	Eo
SDC	31	5.9	0.4	6.2	75.8	6.0	0.3	0.2	2.4	2.8	0.0
EB	15	17.8	0.0	12.6	29.2	5.9	0.1	0.8	29.6	3.9	0.0
	SDC-EB	(11.9)	0.3	(6.4)	46.6	0.1	0.3	(0.7)	(27.2)	(1.1)	0.0
T-test	P	0.00	0.01	ns	0.00	ns	0.04	0.04	0.00	ns	ns

[1]Table abbreviations: Heterophil (typical HT, variant HV, classic, HC) small lymphocyte Ls, medium lymphocyte Lm, natural killer lymphocyte NK, blast Bst, monocyte Mn, basophil Ba, eosinophil Eo

Table 2: SDC and EB heterophil/lymphocyte statistics H/L 1, H/L 2 and ΔH/L's. Total SDC cellularity (TWBC in thousands/μL, K) of N = 12 isolator raised SPF chickens at 6 wk. TWBC' are not determined from EB data. P = probability by t-test

Count	No	H/L 1	H/L 2	ΔH/L	TWBC (K)
SDC	31	0.17	0.16	0.02	35
EB	15	1.46	1.13	0.33	-
T-test	P	0.001	0.000	0.023	
	SDC-EB	(1.29)	(0.98)	(0.31)	

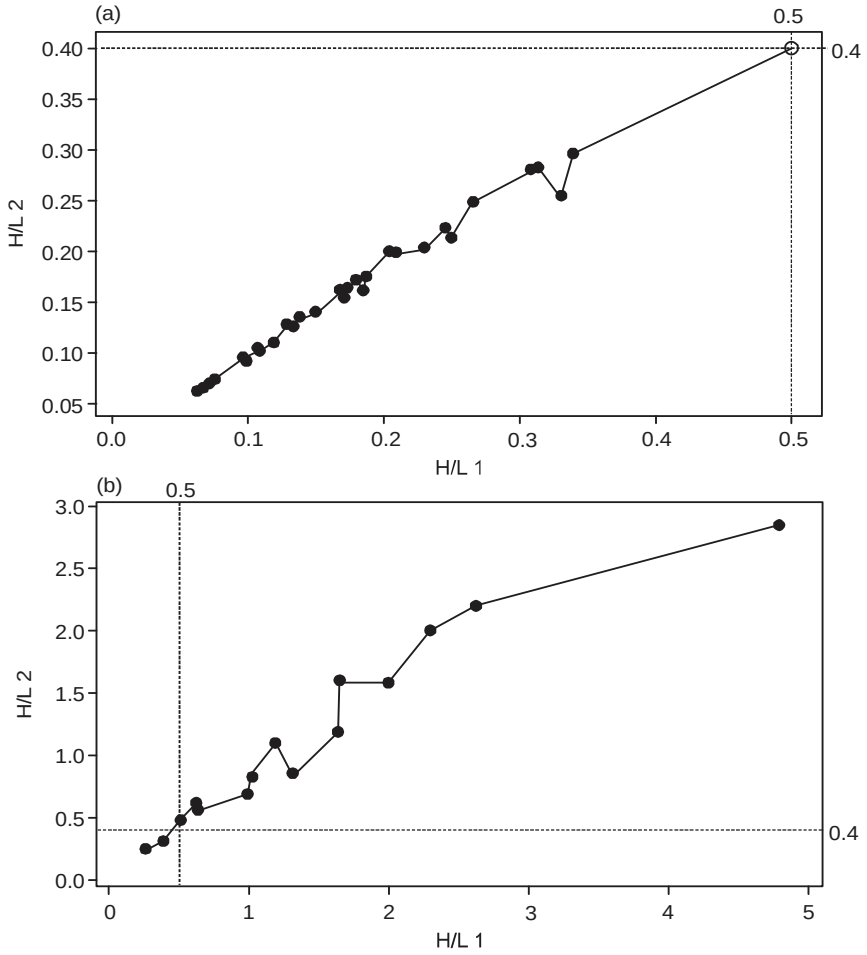

Fig. 1: H/L 2 vs. H/L 1 scatter plot determined from 31 SDC's of 12 isolator raised 6 wk SPF chicks by examination of Wright's stained venous blood. Reference lines are SDC cut-off levels for non-stress/stress. The open circle data point is artificial was needed to allow placement of reference lines in the graphic H/L 2 vs. H/L 1 scatter plot and reference lines determined from 15 edge based (EB) differential counts (Right)

is in Fig. 3c. An atypical plasmacyte with Mott cell features (Russell bodies) is in Fig. 4d.

Free, cell surface associated and phagocytosed bacteria were found among circulating blood cells in 5/12 (42%) of

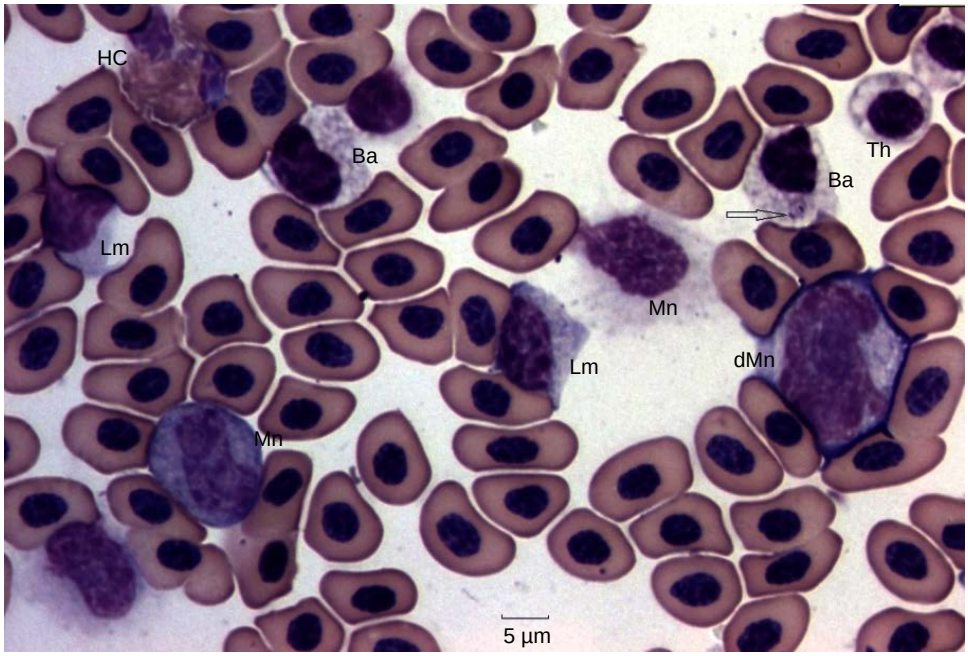

Fig. 2: An edge based (EB) assortment of atypical cells is presented on a background of erythrocyte anisocytosis. (Ba) are basophils with weakly stained cytoplasmic granules. The arrow at one basophil locates 2 internal structures resembling bacteria. A classic heterophil (HC) is at the upper left. Two medium size (reactive) lymphocytes (Lm) are at the left and center. Several versions of monocytes are located throughout. A large developmental monocyte (area ~300 µm^2, diameter ~21 µm, perimeter ~76 µm) is polygonal with an irregular nuclear membrane and deeply stained edges (dMn) is at the right. Portions of the dMn cell membrane have been squeezed by neighboring erythrocytes into spike like projections seen at 12, 2, 4 and 6 o'clock. More mature monocytes are also present (Mn). Two thrombocytes are at the top right (Th)

samples. An example of free bacteria is located at the arrow and by circles in Fig. 5a. After decolorizing with 70% EtOH and Gram staining, the atypical basophil of Fig. 5a appeared to contain both surface associated and internalized bacteria not seen with Wright's stain (Fig. 5b).

DISCUSSION

Hen welfare has received more attention recently due to phasing out of the use of conventionally styled cages. Replacement by other styles raises issues of how this might affect performance, welfare and ultimately egg supply. Welfare issues may consider the physical environment, or be based on animal data. A physiologic approach using blood information, are descriptions of formed elements (cells) humoral factors or hormones (Yan *et al*., 2014). The simple H/L ratio is a method widely relied upon, but few authors describe the kinds of cells used for its computation. Moreover, description of cellularity (TWBC) rarely accompanies H/L estimates. This makes the interpretation of a simple ratio as problematic and the lack of consistency among studies makes comparative work difficult. These problems were clearly demonstrated in commercial hens (Cotter, 2015a) whose hemograms were complex. Abnormal cellularity (leukocytosis/ leukemoid reactions), high H/L ratios and atypia were common (Cotter, 2015a, c). Whether observations on commercial animals apply to other circumstances was not answered by data from that study. It could be argued that the complex hemograms are solely the result of multiple stressors of the commercial environment (McFarlane *et al*., 1989). The current observations on isolator housed SPF chickens address some of these issues. The data show complex hemograms occur even in a non-commercial environment. Although the degree of bacteremia in isolator/SPF chicks did not approach levels seen in a commercial setting, it was enough to affect the hemogram. Bacteremia results in sorting likely due to physical changes in plasma composition, a consequence of inflammation. This explanation is supported by the detection of sorting and "Rouleaux" erythrocyte formations and anisocytosis in all SPF samples. The magnitude of the SDC/EB difference indicates how certain cells are so disposed. Furthermore, H/L ratio statistics and total cellularity, important components of an accurate blood picture, were also affected.

Because of its apparent simplicity, use of the H/L method is likely to continue. However, by itself, the H/L lacks sufficient power to resolve a complex blood picture. The

addition of the H/L 2 and the difference (ΔH/L) increases resolving power. Use of H/L 1, H/L 2 scatter plots helps convey study-wide hematology. In a non-stress situation a majority of data points should be found in quadrant located below and to the left of the 0.4 H/L 1-0.5 H/L 2 intersection, the non-stress/stress cut-off. When ΔH/L exceeds 0.1, non-resting lymphocytes, plasmacytes, Mott cells, etc., are common and must be a part of the blood picture. The improvement obtained by additional H/L statistics is inadequate without cellularity. A TWBC should be integrated with H/L statics and include leukocytosis/ leukemoid reaction cut-off values. However, neither H/L2 nor ΔH/L includes monocytes, or non-heterophil granulocytes as basophils.

Other forms of atypia, large plasmacytoid lymphocytes, dysplastic heterophils and developmental cells ordinarily

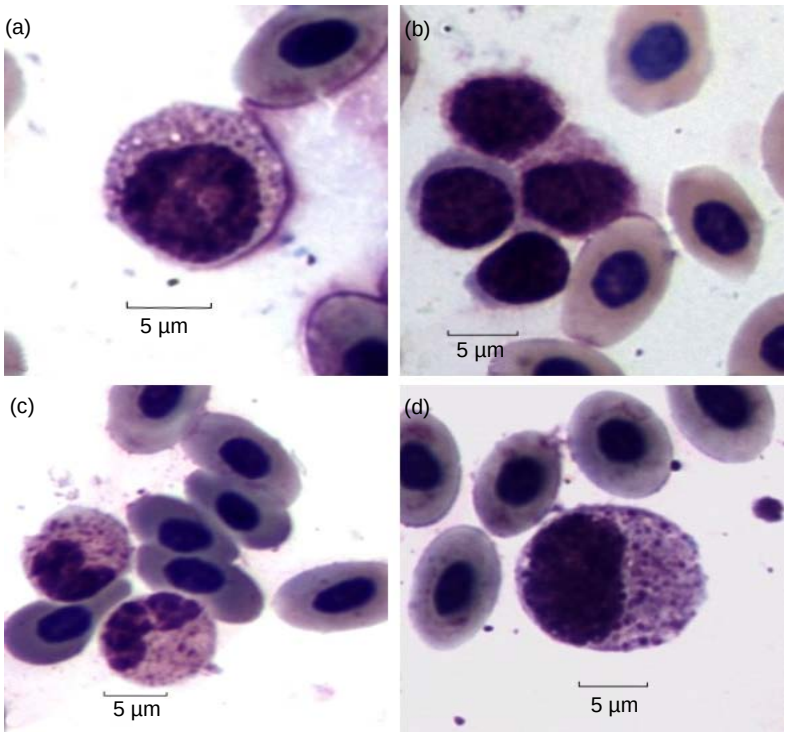

Fig. 3: (a) An atypical leukocyte with basophilic granules and cytoplasmic vacuoles has a diameter of 10 µm and an N/C ratio ~0.75. The slightly eccentric rounded nucleus has coarse patchy chromatin; no nucleoli are evident, (b) A four cell cluster has 2 large lymphocytes at the left edge and 2 atypical (dysplastic) basophils at the right edge, (c) Two heterophils display atypical cytoplasmic granulation, (d) A granulocyte developmental stage (metamyelocyte) shares cytoplasmic features with the cell of panel a and b is ~12.5 µm in diameter; its nucleus is coarse and patchy. All cells were from the same sample

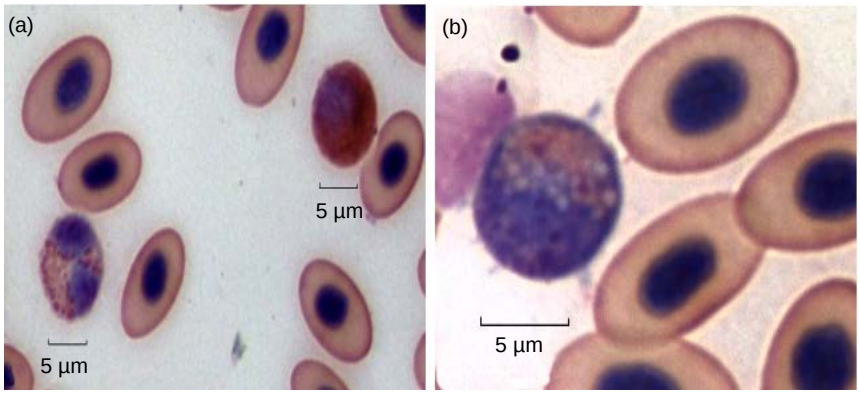

Fig. 4: Continue

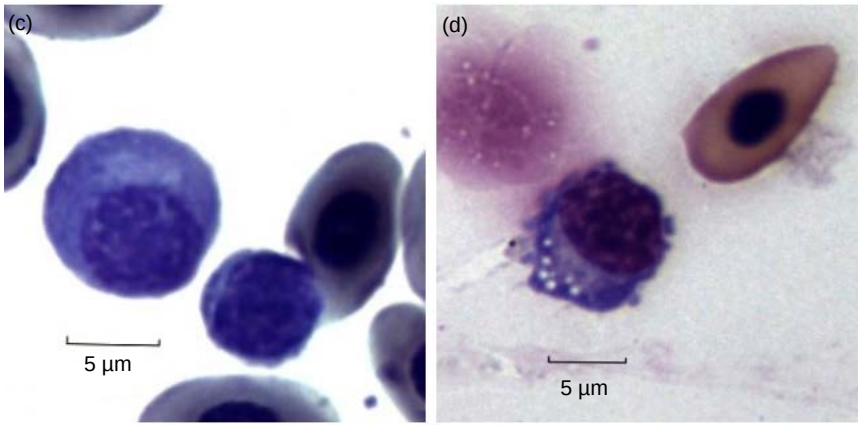

Fig. 4: Rare and unusual circulating cells, (a) Lower left is an eosinophil with hollow loose packed cytoplasmic granules and a two-lobe nucleus; right is a "classic heterophil" with tightly packed deep red spindle cytoplasmic granules, (b) An eosinophil metamyelocyte with an eccentric nucleus contains patchy chromatin, (c) A pair of lymphocytes; the larger left member has a faintly stained extra nuclear Hof, an indication of a circulating plasmacyte, (d) An atypical plasmacyte with Russell bodies a Mott cell cytoplasmic feature

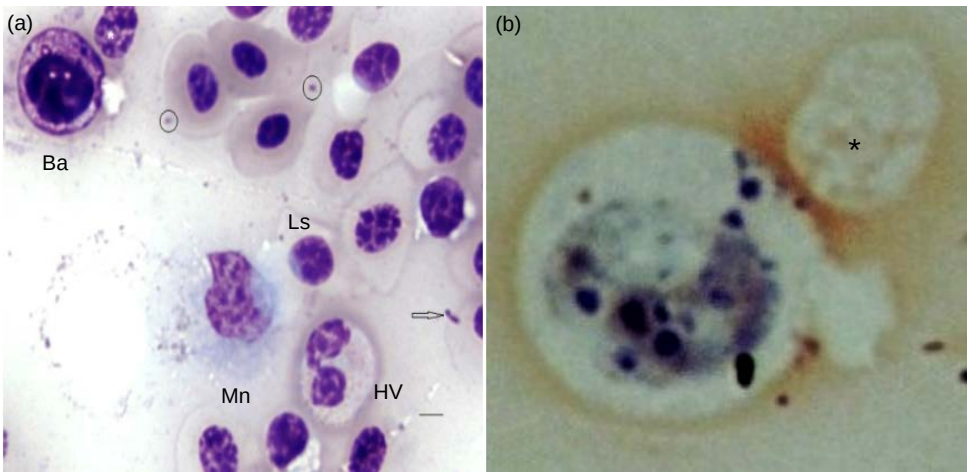

Fig. 5: (a) An edge based field (EB) in which a basophil (Ba, top left) a monocyte (Mn) a small lymphocyte (Ls, center) and a variant heterophil (HV, right) are found. Several small encapsulated bacteria are circled, a bacillus is located at the arrow (Bar 5 µm), (b) The basophil of panel a after decolorization and staining with Gram reagents reveals multiple internal and surface associated bacteria-like structures not seen in the Wright stained version. The asterisk is at a common location to cells of Fig. 5a and b

restricted to bone marrow were circulating in these SPF chicks. Atypical behavior (leukergy), a sign of infection, was also seen. Coccinocytes, possible developmental stages of natural killer (NK) cells, accompanied bacteremia in commercial hens and SPF chicks (Cotter *et al*., 2013; Cotter and Heller, 2015; Gobel *et al*., 1994). Collectively atypical forms as these are "sentinels," indications of hemogram complexity. When they occur in an SDC, a more detailed hematological examination is needed.

Conclusion: In conclusion, the current observations indicate SPF status or isolation housing does not guarantee a non-stress environment nor do they exclude the possibility of complex hemograms. The issues raised by the present observations parallel many of those made on commercial pullets and hens not in isolation (Cotter, 2105a-c). They draw attention to the detail necessary for application hematological information to questions of welfare, disease status and related issues.

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