ISSN 2347-6893

Evaluating porcine red blood cells in acute African swine fever virus (genotype II) infection in peripheral blood

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ABSTRACT

It has been previously shown that acute ASFV infection cause significant alterations in the population of white blood cells. Thus, it has been assumed that ASFV disrupts hematopoietic homeostasis through unknown mechanism. To test this assumption, we have conducted this study to evaluate the changes of red blood cells (RBCs) in peripheral blood during experimental African swine fever virus infection. Our data show that as acute ASFV infection progressed, the percent of mature erythrocytes (with normal size) gradually decreased. Simultaneously, the number of microcytes (sized 5 µm or smaller in diameter) increased, reaching 20% of all RBCs by 5 day post-infection. From the beginning of infection, juvenile forms of RBCs, such as the largest cells of erythron, rubriblasts, were observed in the peripheral blood of infected pigs. Among the erythroid precursors, up to 60% of all cells were binucleated, which indicates that acute ASFV infection is accompanied with the emergence of pathological forms of RBCs. Our findings show that ASFV affects the erythropoiesis, and justify further investigations to determine how ASFV interferes in erythropoiesis.

Indexing terms/Keywords:-African swine fever virus; binucleation; erythropoiesis; red blood cells.

ISSN 2347-6893

INTRODUCTION

African swine fever virus (ASFV) is a large, cytoplasmic DNA virus that causes African swine fever that is the significant disease of domestic pigs with mortality rates approaching 100%. ASFV predominantly infects cells of the mononuclear-phagocytic system and leads to a marked decrease in white blood cells (Gomez-Villamandos et al. 1997; Takamatsu et al. 1999). However, as juvenile and atypical cells appear in the peripheral blood of infected pigs (Karalyan et al. 2012), it has been suggested that ASFV directly or indirectly influence the hematopoietic homeostasis through activation or impaired hematopoiesis (Gomez-Villamandos et al. 1997; Karalyan et al. 2012). Although it has been also shown that ASFV is associated with red blood cells (RBCs) (Wardley and Wilkinson, 1977; Quintero et al.. 1986), no study has been carried out to investigate subpopulations of circulating RBCs during ASFV infection. Taken together, it is possible that ASFV interferes in erythropoiesis, leading to the emergence of juvenile or pathologic erythrocytes. To test this assumption, the types of RBCs over the course of ASFV infection have been studied in the current work.

Materials and methods

For experimental infection, nine healthy pigs (outbred) of the same age (6-month-old) and weight (30-35 kg) were used in this study. Animal care and euthanasia were done according to the international and local guidelines for animal care and use (Institutional Review Board/Independent Ethics Committee of the Institute of Molecular Biology of NAS, IRB00004079). Infections were carried out by intramuscular injection of ASFV (genotype II) with 10^4 50% hemadsorbing doses (HAD_{50})/ml (Rowlands et al. 2008). For red blood cells analysis and virus titration, blood samples were obtained each day post-infection (DPI) from the ophthalmic venous sinus of either infected (six) or uninfected (three) pigs. Carbon dioxide inhalation (70-80% carbon dioxide for 20-25 minute) was used to euthanize infected pigs after 7 DPI. ASFV titration (in $\log_{10}$ HAD_{50}/ml) was done and expressed as described previously (Enjuanes et al. 1976). For RBC analysis, blood smears were prepared by routine method and fixed in 96% ethanol. Giemsa modified solution (azure B/azure II, eosin and methylene blue) was used to stain blood slides according to the manufacturer's protocol (Sigma-Aldrich). RBC counts were performed on whole blood samples under the light microscope at 1250× (ocular, 12.5×; objective, 100×) magnification. The evaluation of cell sizes was done by routine cytometry using ImageJ software.

Image analysis was employed to recognize and track cells across multiple video frames and simultaneously measure morphologic parameters. In a series of publications, Bacus and colleagues documented the utility of the technique in classifying erythrocytes from patients with various hematologic disorders (Bacus, Ween, 1977, Bacus 1980, Bacus 1984).

Single red cell Soret hemoglobinometry (Bacus, 1982) allows the distinction between heterogeneity of measurement and heterogeneity of population.

Image analysis can distinguish the changes in spherocytes by the changes in integrated optical density (IOD) in evaluated erythrocytes.

All preparations were treated with the combined Feulgen-Naphthol Yellow staining (FNYS) procedure (Gaub et al. 1975). This method permits simultaneous microspectrophotometric analyses of DNA and protein in single cells and the protein value is closely correlated to the amount of dry mass of the cell.

The classification of RBCs was performed as described previously (Douglas, Weiss 2010). Cells were classified according to their size and shape. At least 10^4 cells (from all samples) were observed for RBC analysis, and results are expressed as mean ± standard error.

Results and Discussion

In this study, the clinical signs of experimental infection were not different from those in cited research (Karalyan et al. 2012). The first two days PI proceeded virtually without symptoms. ASFV infection reached the premortal stage at 6 DPI when bloody diarrhea and lethargy were seen, and therefore infected animals were sacrificed at 7 DPI. Although infected animals were asymptomatic up to 3 DPI, viremia were observed from 2 DPI and increased up to 5.0 $\log_{10}$ HAD_{50}/ml by 5 DPI. The highest titer of ASFV was determined in all pigs up to 7 DPI (data not shown).

The observation of peripheral blood of uninfected pigs showed that the population of RBCs mainly consisted of mature erythrocytes and an insignificant number of reticulocytes. The average diameter of mature erythrocytes (normocytes) was 7.3 µm whereas the average diameter of a small number of erythrocytes considered as microcytes and macrocytes was respectively 5 µm (or less) and 9 µm (or greater). Interestingly, the number of macrocytes in the peripheral blood of infected pigs increased at 1 DPI and then totally disappeared from 2 DPI (Table 1). As acute ASFV infection progressed, the number of microcytes gradually increased, reaching 20% of the total RBCs at 5 DPI (Table 1). From 5 DPI onward, their number decreased slightly, averaging 17% of the total population of erythrocytes at 7 DPI.

From the early stages of infection, nucleated RBCs that were various forms of early (basophilic) and late (metarubricytes and polychromatophilic rubricytes) erythroid precursors appeared in the blood of pigs. Changes observed in the population of nucleated RBCs are summarized in table 2. The number of nucleated RBCs did not exceed 0.5% of all RBCs. Early erythroid precursors in peripheral blood arose from the beginning of infection (Table 2). At 1 DPI, early erythroid precursors were represented by prorubricytes whose sizes varied from 11 µm to 13 µm (Fig. 1, G, H, I, and Table 3). Throughout infection, prorubricytes exhibited a reduction in size, and had a diameter reduced by 16% at 7 DPI compared to the diameter of prorubricytes at 1 DPI (Table 3). Moreover, basophilic rubricytes (Fig. 1, F) were observed at 3 DPI and remained detectable up to 5 DPI (Table 2). The largest cells of erythron, rubriblasts, were detected from 3 DPI and absent by 7 DPI. Their size was mainly less than 20 µm and they contained a large nucleus with one or two nucleoli (Fig. 1, J).

Although metarubricytes (Fig. 1, A, B) and a small number of polychromatophilic (Fig. 1, E) rubricytes were clearly observed at 1 DPI, the bulk of late erythroid precursors in peripheral blood were mainly comprised of polychromatophilic rubricytes, as well as fewer metarubricytes (Table 2). Surprisingly, no polychromatophilic rubricytes were detected in the final phase of infection. The average size of late erythroid precursors was slightly smaller than the size of these cells in the bone marrow of healthy pigs (unpublished data). While metarubricytes varied insignificantly in size, polychromatophilic rubricytes showed high variability in average diameter (Table 3). Importantly, among the late erythroid precursors, up to 60% of all cells had additional nuclei (Fig. 1, C, D), particularly in the population of metarubricytes where this phenomenon was observed for up to 80% of cells (data not shown).

It has been previously shown that some animal and human viruses interfere in erythropoiesis, leading to its deregulation. For instance, Friend spleen focus-forming virus (SFFV) encodes a unique glycoprotein mimicking erythropoietin (Epo) which regulates RBCs production. SFFV glycoprotein interacts with specific cell surface receptor, resulting in activation of erythroid signal transduction pathways. This, in turn, leads to Epo-independent erythroid hyperplasia, a condition in which the number of immature RBCs sharply increases, and they release from bone marrow (Ruscetti 1999). We have previously reported that ASFV (genotype II) infection is accompanied with profound changes in white blood cells, indicating that hematopoietic homeostasis is disrupted in ASFV-infected pigs (Karalyan et al. 2012). Herein, our data demonstrate that acute ASFV infection also leads to serious alterations in the population of circulating RBCs. The current study points to a disturbance of erythropoiesis, which is manifested by a decrease in the number of normocytes, and the appearance of nucleated RBC, such as rubriblasts, in the peripheral blood of infected animals. In addition, binucleated erythroid precursors have been observed, indicating that acute ASFV infection is accompanied with the emergence of pathological forms of RBCs in the host blood. These results reinforce the statement that haematopoiesis undergoes significant changes during infection. Further experiments will be required to determine whether ASFV affects the erythropoiesis via hormones/cytokines that are thought to be involved in erythropoiesis (Lodish et al. 2010) and regulated by ASFV (Gil et al. 2003), as well as to determine the nature of binucleation. This and further studies may increase our understanding of the pathology of ASFV.

ISSN 2347-6893

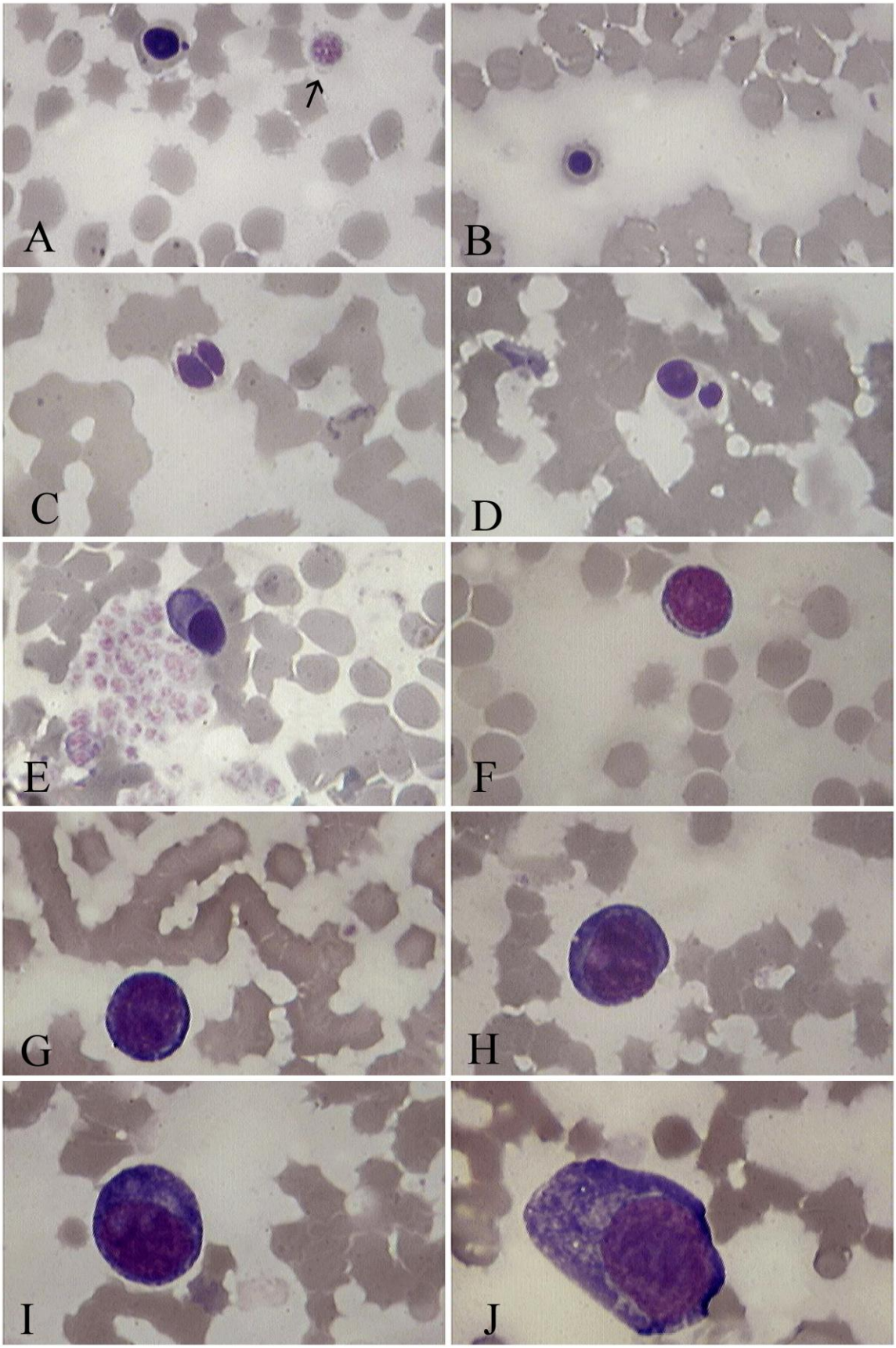

Fig 1. Peripheral RBCs of ASFV-infected pigs.

ISSN 2347-6893

A – Metarubricytes (arrow on platelet); B. Metarubricyte; C, D - Metarubricytes with additional nuclei; E - Polychromatophile rubricyte; F - basophilic rubricyte; G, H, I - prorubricytes; J - Rubriblast.

Cells were examined under the light microscope at 1250× magnification. Scale bare is 10 µm.

Tab 1: The percent and protein content in microcytes, normocytes and macrocytes in the peripheral blood of ASFV-infected pigs.

DPI	Erythrocytes					
	microcyte		normocyte		macrocyte	
	%	Mean Corpuscular Hemoglobin (pg)		Mean Corpuscular Hemoglobin (pg)		Mean Corpuscular Hemoglobin (pg)
0	1.5±0.4	11.2±1.9	98.5±7.3	18.4±2.1**	-	-
1	-	-	97.3±7.0	20.4±2.2	2.7±0.6	27.0±3.3*
2	8.2±1.1	13.0±1.2	91.8±7.8	20.9±2.7	-	-
3	16.5±3.6	10.7±1.8	83.5±8.1	16.8±2.5**	-	-
4	19.2±2.7	12.5±2.1	80.8±9.9	18.6±1.8**	-	-
5	19.9±3.2	11.5±2.0	80.1±9.0	18.0±2.2**	-	-
6	17.9±3.7	10.1±1.9	82.1±9.1	17.1±2.1**	-	-
7	17.0±3.5	8.8±1.5	83.0±9.7	14.5±2.0**	-	-

*significant, compared with microcytes (p<0.01-0.001)

** significant, compared with macrocytes (p<0.05-0.01)

Tab 2: Changes detected in the population of nucleated RBCs during acute ASFV infection.

DPI	Nucleated cells				
	Metarubricytes	Polychromatophile rubricytes	basophilic rubricyte	prorubricyte	Rubriblasts
0	-	-	-	-	-
1	+	+	-	+	-
2	+	+	-	+	-
3	+	+++	+	+	+
4	+	++	+	+	+
5	+	+	+	+	+
6	+	-	-	+	+
7	+	-	-	+	-

-, none; +, slight; ++, moderate; +++, abundant

Tab 3: Average size (diameter, μm^2) of RBCs during acute ASFV infection

Cells	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	Control
Erythrocyte	8.1±1.5	5.8±0.7	5.8±1.0	5.5±0.9	5.2±0.8	5.5±0.8	5.8±1.0	7.3±1.3
Rubriblasts	-	-	16.5±1.9	16.8±2.1	19.1±2.1	19.9±2.2	-	-
basophilic rubricytes	-	-	7.3±0.9	8.4±0.9	9.1±1.0	-	-	-
prorubricytes	12.2±1.1	11.9±1.2	11.2±1.0	10.9±0.9	10.6±0.9	10.5±0.9	10.3±0.9	-
Metarubricytes	9.5±1.0	9.5±1.0	10.0±1.0	9.6±0.9	10.0±0.9	9.6±0.9	9.6±0.9	-
Polychromatophile rubricytes	10.1±0.8	9.1±0.9	7.7±0.8	9.3±1.0	9.1±1.0	-	-	-

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ISSN 2347-6893

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