

Phenotypic and cytologic studies of lymphoid cells and monocytes in primary culture of porcine bone marrow during infection of African swine fever virus

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Abstract We have modeled in vitro infection of African swine fever virus (ASFV) in primary unstimulated cells of the porcine bone marrow and have studied the phenotypical changes in the population of porcine lymphoid cells by cytophotometry. Monocytes and large-sized lymphocytes completely vanished in 72 h of infection which is result of high sensitivity of those cells to ASFV. We describe DNA synthesis in monocytes at 24 h post infection. Cytophotometry of the uninfected cells revealed the few number of atypical lymphocytes and lymphoblasts after 72 h of cultivation; whereas in viral infected cultures, atypical cells appeared in large quantity (about 14%) with 24 h. Most of atypical lymphocytes and lymphoblasts had altered nucleus, and only a small number of atypical cells had additional nucleus. The cytophotometry of main and additional nuclei showed that DNA content didn't exceed diploid standard which indicates that the additional nuclei were consequence of fragmentation of nuclei in lymphocytes.

Keywords African swine fever virus · Atypical lymphocyte · Cytophotometry · Lymphoblast · Monocyte · Nucleus

African swine fever (ASF) is a highly lethal and significant disease of domestic pigs, for which there is no vaccine or disease control strategy other than animal slaughter. The acute forms are characterized by high fever, reddening of the skin, pronounced hemorrhages in lymph nodes and internal organs, and occasionally enlargement of the spleen. The disease does not affect people or animals outside of the pig family. ASF results from infection by the African swine fever virus (ASFV). ASFV is currently the sole member of the new genus *Asfivirus* in the family *Asfarviridae* and it is the only virus with a DNA genome that is transmitted by arthropods. In common with other viral haemorrhagic fevers, the main target cells for replication of ASFV is the mononuclear-phagocytic system, including highly differentiated fixed-tissue macrophages and specific lineages of reticular cells in the spleen, lymph node, lung, kidney, and liver (Colgrove et al., 1969; Konno et al., 1971; Mebus 1987; Dixon et al. 2005). These tissues show extensive damage with highly virulent strains of ASFV, and the ability of ASFV to replicate and induce cytopathology in these tissues in vivo appears to be a critical factor in ASFV virulence. The role of the proinflammatory cytokines in pathology of lymphoid tissue have been demonstrated (Whittall and Parkhouse 1997; Gomez et al. 1999), however the biology of pathological changes in lymphoid tissue caused by ASFV remains poorly studied.

The aim of our study was to define changes in the population of lymphoid cells during infection by ASFV. For this purpose, we first of all have modeled in vitro infection of ASFV in primary unstimulated cells of the bone marrow of swine, and have studied the morphological changes in

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the population of porcine lymphoid cells (Plowright et al. 1968; Gomez-Villamandos et al. 1997). We correlated these morphological changes over the course of infection with changes in cellular DNA content by scanning image cytophotometry, thus describing the atypical lymphocytes in ASFV-infected population.

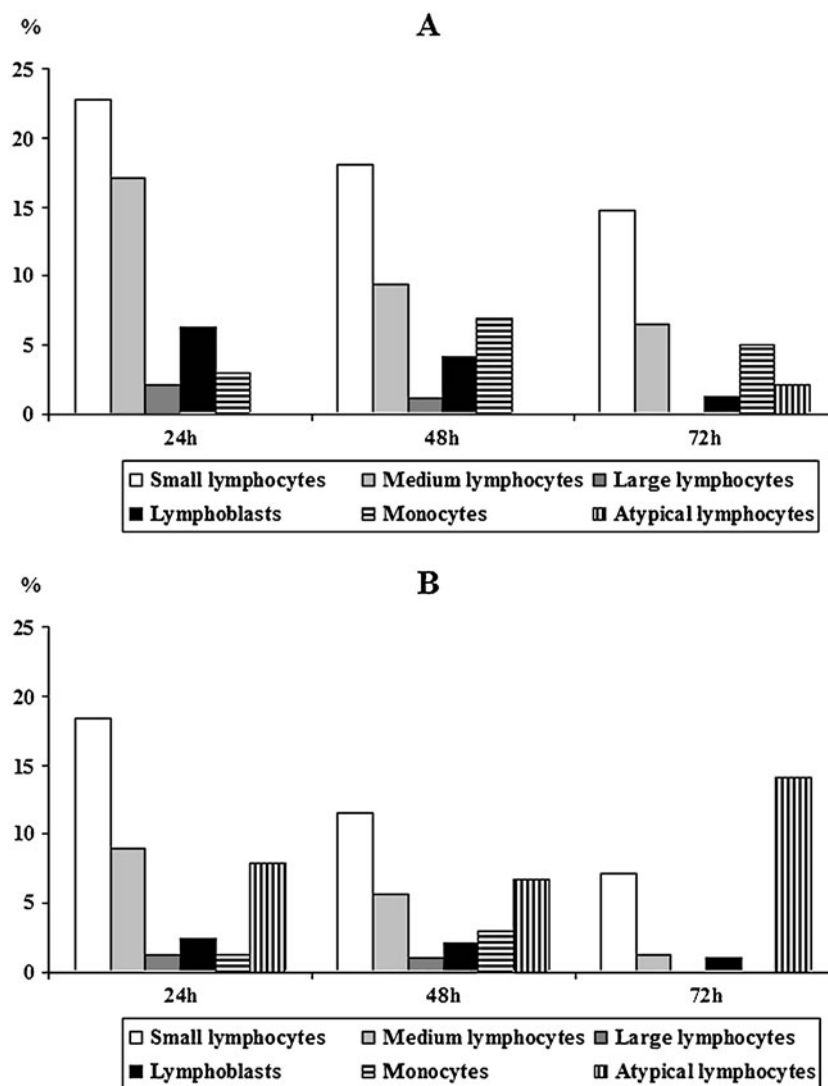
In our study, we used suspension of primary cells (initial cell number was 10^6 cells/ml.) from bone marrow that was prepared from the thigh of five healthy piglets (Sansone 1978) and was cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics in CO₂ atmosphere. Euthanasia of 3-mo-old piglets was done according to Guide for the Care and Use of Laboratory Animals, AVMA Guidelines on Euthanasia, and local guideline for animal care and use.

We used ASFV (genotype II) distributed in Republic of Georgia and Republic of Armenia (Rowlands et al. 2008). African swine fever virus was added to culture vials

simultaneous with cultivation of cells. The titer of ASFV in each experimental culture was 10^4 hemadsorption units (HADU)/ml.

For cytochemical and morphological studies, the first group of preparations was stained by hematoxylin–eosin vital dye according to the method of Romanovsky-Giemsa. Schiff's reagent was used for staining the second group of preparations by the method of Feulgen (Deich 1966). DNA content (in conventional units) was determined at 575 nm wavelength (λ), and cytomorphometry of nuclei of all studied forms of lymphoid cells, atypical lymphocytes, and monocytes were carried out at 24, 48, and 72 h of cultivation. One hundred nuclei were used for each measurement. Measurements were performed by the scanning image analyzer (magnification 12.5×100). Before the scanning process, each nucleus was contoured. We defined small-, medium-, and large-sized lymphocytes based on the shape of their nuclei during cytophotometry (Feldman et al. 2000). The

Figure 1. Percentage ratio of cells in control (A) and ASFV-infected (B) population.



nuclear sizes of small, medium, and large-sized lymphocytes were significantly different.

As mentioned above, we correlated morphological alterations with changes in cellular DNA content. We examined DNA content by scanning image cytophotometry and expressed it on a “c” scale, in which 1c is the haploid amount of nuclear DNA seen in normal (non-pathological) diploid populations in G_0/G_1 . DNA cytophotometric measurements identify nuclei as aneuploid if they deviate more than 10% from 2c, 4c, 8c, or 16c; i.e., if they are outside $2c \pm 0.2$, $4c \pm 0.4$, $8c \pm 0.8$, or $16c \pm 1.6$. The total number of all cells in euploid regions of the DNA histogram rescaled by the mean corrective factor (1.8c–2.2c, 3.6c–4.4c, 7.2c–8.8c, and 14.4c–17.6c) was also calculated. The ploidy status of cells was determined by the amount of DNA expressed as the DNA index. We have used unstimulated lymphocytes of swine as a diploid standard for DNA measurement. Cytophotometry of standards showed that the variability of DNA content did not exceed 10%.

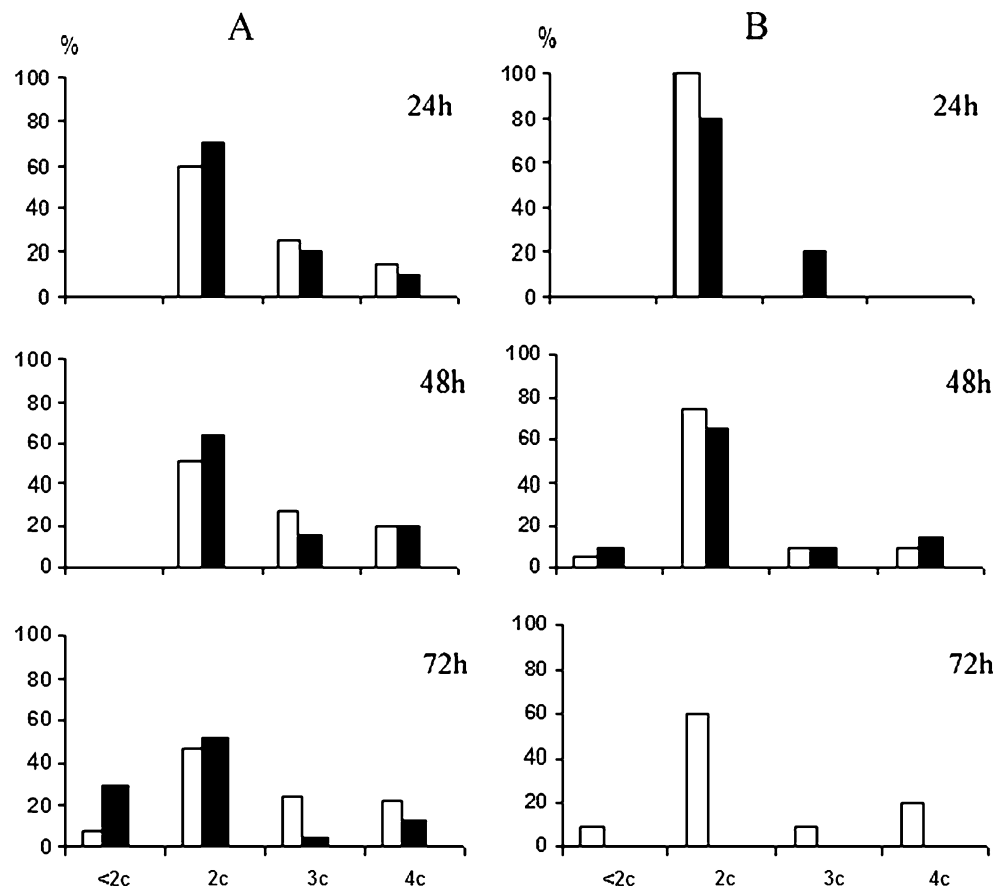
Changes in Composition of Cellular Population

Our data showed that after 24 h of cultivation, about 48.8% of the primary unstimulated cells from the bone marrow of

intact piglets were destroyed. The remaining cells were represented mainly by small- and medium-sized lymphocytes (about 40% of the initial total cells), whereas the number of large-sized lymphocytes and monocytes didn't exceed 5% of the initial seeding (Fig. 1). At 24 h, the number of lymphoblasts was about 6% of the total cell number. During the next 2 d of cultivation (24–72 h) large-sized lymphocytes completely disappeared, and the number of small- and medium-sized lymphocytes decreased more than twofold. The number of lymphoblasts didn't exceed 1%. In striking contrast to these dramatic decreases, during this same interval the percent of monocytes in the population increased more than twofold (Fig. 1).

When ASFV was added to cultures of primary cells, replication of viruses took place in lymphoid cells, and the viral titer increased up to 10^6 HADU/ml (after 48 h). After 24 h of infection we observed a significant decrease of the number of lymphoblasts and monocytes as well as small, medium, and large-sized lymphocytes, compared with uninfected controls ($p < 0.05$). By 72 h the percent of dead cells reached 70.5% in infected populations and 76.5% in uninfected populations. Destroyed cells mostly died by necrosis and only 4–6% of destroyed cells died by apoptosis. ASFV caused a decrease in the number of small and

Figure 2. Distribution of lymphoblasts (A) and monocytes (B) by ploidy during cultivation and infection. *White histogram bar* is control and *black histogram bar* is effect of ASFV.



medium-sized lymphocytes, whereas the number of lymphoblasts decreased but was not statistically significant. By 72 h large-sized lymphocytes and monocytes completely vanished, small-sized lymphocytes fell to 7%, and medium-sized lymphocytes with lymphoblasts were just 2% of the total cell number (Fig. 1). Thus we can assume that the most viable cells during cultivation and infection were small-sized lymphocytes whose number decreased an average of 45%, while medium-sized lymphocytes decreased on average 75%. Large-sized lymphocytes completely vanished in cultured population. These phenomena were enhanced during infection. Monocytes and large-sized lymphocytes completely vanished in 72 h of infection and this can be explained by high sensitivity of those cells to ASFV (Genovese et al. 1990). The permanent reduction of all types of lymphoid cells both in control and particularly in ASFV-infected population can be explained by the fact that ASFV replication in monocytes and lymphoid cells is occasionally associated with necrosis (Hervás et al. 1996; Tulman and Rock 2001). However, the correlation between lymphocyte size and sensitivity to ASFV warrants further investigation.

Ploidy of Lymphoblasts and Monocytes

Our data showed that the percent of diploid cells slightly reduced during cultivation process whereas the percent of tetraploid lymphoblasts increased. In ASFV-infected population, the number of diploid lymphoblasts reduced up to 25%, DNA synthesizing lymphoblasts (3c cells) decreased threefold while the percent of tetraploid lymphoblasts changed in minor ranges in third day of cultivation (Fig. 2).

After 24 h of cultivation, monocytes were diploid in control. The tetraploid and DNA synthesizing monocytes appeared at 48 h (Fig. 2). In contrast to control DNA synthesizing monocytes appeared at 24 h of infection, and became 10% of all monocytes as well as hypodiploid monocytes at 48 h. After 72 h of infection all monocytes completely vanished from population (Fig. 2). At the early stage of infection (24 h), DNA content in monocytes increased, indicating that DNA synthesis occurred in these cells. DNA synthesis under the influence of ASFV may be caused by tumor necrosis factor- α (TNF- α) that is one of the characteristic proteins synthesized in ASFV-infected cells at the early stages of infection, and is the important pathogenic agent of ASFV during both in vivo and in vitro infections (Gomez-Villamandos et al. 1995; Gomez et al. 1999; Gil et al. 2008). TNF- α can induce DNA synthesis in various cell types, and particularly in mononuclear-phagocytic system cells (Branch et al. 1989; Chen and Mueller 1990; Okamoto et al. 2009).

Atypical Lymphocytes

In our study, we have identified atypical lymphocytes in primary cell culture of porcine bone marrow during ASFV infection. Cytophotometry of the uninfected cells revealed atypical lymphocytes only in third day of cultivation (2%), whereas in viral-infected cultures atypical lymphocytes

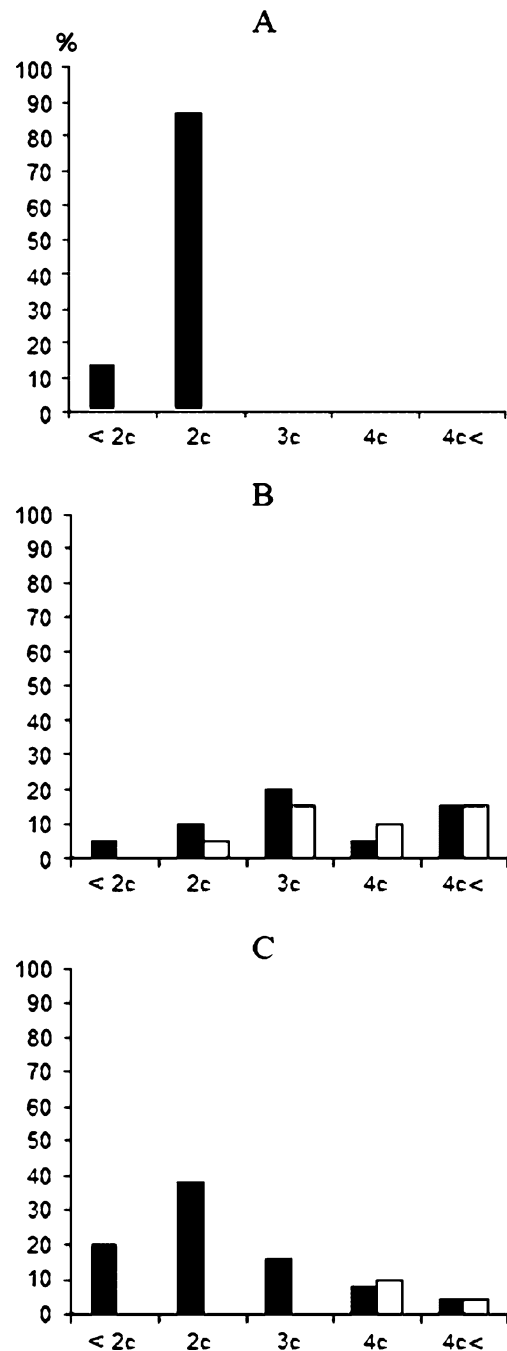


Figure 3. Distribution of atypical lymphocytes and atypical lymphoblasts by ploidy at 24 h (A), 48 h (B), and 72 h (C) of infection. *White histogram bar* is atypical lymphoblasts and *black histogram bar* is atypical lymphocytes.

appeared with 24 h (Fig. 1). Most of atypical lymphocytes with altered nuclei were diploid at 24 h of infection whereas their percent decreased twofold at 72 h (Fig. 3). While the first atypical lymphocytes were identified at 24 h post infection, the atypical lymphoblasts appeared at 48 h post infection and their total number decreased at 72 h (Fig. 3). In contrast to atypical lymphocytes, the atypical lymphoblasts were presented by tetraploid and hyper-tetraploid cells (Fig. 3).

Only the low number of atypical lymphocytes had an additional nucleus, and cytophotometry of both nuclei showed that DNA content didn't exceed diploid standard (exception is atypical lymphocytes at 48 h post infection) which indicates that the additional nuclei were consequence of fragmentation of nuclei in lymphocytes.

During some viral infections atypical lymphocytes are formed and can be used as important diagnostic characteristic. The emergence of atypical cells during infections is probably caused by stress factors and response of lymphoid cells to multiple, often differently directed stimuli (Inman and Cooper 1965; Simon 2003). ASFV-induced synthesis of the large number of regulatory cytokines (including antagonists) is probably involved in the emergence of atypical lymphocytes (Gil et al. 2008). The high percent of atypical lymphocytes and lymphoblasts in ASFV-infected populations can be of benefit to those who want to develop early diagnostic tests, in vitro assays, and study drug targets for ASFV.

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