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Short communication

Hemophagocytic lymphohistiocytosis in acute African swine fever clinic

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ABSTRACT

Introduction: Hemophagocytic lymphohistiocytosis (HLH) usually has been defined as the combination of a proliferation of cytologically benign, actively phagocytic macrophages in bone marrow, spleen, lymph nodes, etc. in association with fever, cytopenia, splenomegaly, and hypertriglyceridemia. HLH is often triggered by viral infection. The aim of this study was to ascertain the features of HLH involvement in African swine fever virus (ASFV) (genotype II) pathogenesis.

Methods: The serum levels of macrophage colony-stimulating factor (MCSF) and granulocyte-macrophage colony-stimulating factor (GMCSF), as well as the histological constitution (for hemophagocytic macrophages detection) of various organs of pigs infected with ASFV genotype II were investigated. The diagnosis of HLH was made according to universally accepted human criteria.

Results: The association of fever, cytopenias, splenomegaly, and hemophagocytosis was present in 87.5% of the infected pigs (absence of hyperthermia in one of eight pigs). Marked hypertriglyceridemia was observed at 3–4 days post infection. Previously it was shown that ASFV induced a significant decrease in the level of fibrinogen from day 5 till the end of experiment. Progression of the HLH coincided with a temporary increase in the serum levels of MCSF levels (early stage of disease) and GMCSF levels (2–3 pays post infection).

Conclusions: Hemophagocytic syndrome should be suspected in ASFV (genotypeII) infected pigs.

1. Introduction

No animal virus disease has undergone a more dramatic change in epidemiology and emergency during the past years than African swine fever (ASF). ASF is a highly contagious infection of pigs caused by the African swine fever virus (sole member of the genus Asfivirus in the family *Asfarviridae*). Clinically ASF in domestic pigs varies from peracute, acute, subacute, and chronic to inapparent infections. Acute ASF is characterized by hemorrhaging, disseminated intravascular coagulation (DIC), severe lymphopenia and high lethality (Freeman and Ramanan, 2011; Villeda et al., 1993).

Despite intensive research efforts, most of pathogenetical aspects of ASF are still far from being understood. Generally the pathogenesis of ASF shows similarities with viral haemorrhagic fevers of mammalians (primary replication in cells of the monocyte/macrophage system; cytokine-mediated lesions, abnormal activation of endothelial cells and the coagulation system, often activation of cells of the monocyte/macrophage lineage) (Blome et al., 2013). Diseases like human viral hemorrhagic fevers leads to the systemic inflammatory response syndrome (SIRS), which often includes abnormal activation of macrophages.

The term Hemophagocytic lymphohistiocytosis (HLH) refers to a condition caused by excessive activation and expansion of macrophagic histiocytes that exhibit hemophagocytic activity (Favara, 1992). HLH has a prominent link with a variety of viral, bacterial, fungal, and parasitic infections (Fisman, 2000). HLH also known as hemophagocytic syndrome is an uncommon systemic inflammatory clinical syndrome associated with numerous infectious conditions. Virus-associated HLH is a disorder characterized by a nonmalignant macrophagal proliferation with marked hemophagocytosis in the background of a systemic viral infection, usually sepsis (Fisman, 2000). Inadequate stimulation of macrophages in bone marrow and resultant phagocytosis of different blood cells with the production of high amounts of proinflammatory cytokines are the pathologic hallmarks of HLH (Karras and Hermine, 2002). Briefly HLH is characterized by defective cytotoxic cell function coupled with macrophage hyperactivity, leading hypercytokinemia and immune dysregulation, with further tissue damage. It is important to remark that hemophagocytosis in secondary cases sometimes may not be visible until late in disease progression (George, 2014).

This article considers some important recent observations that

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contribute to our understanding of the pathogenesis and pathophysiology of ASF. Information on mechanism of pathogenesis may suggest strategies for development of an antiviral therapy and predict potential mechanisms of disease resistance to therapy.

2. Materials and methods

2.1. Animal experiment and viral stock

In our study, ten pigs (Landrace breed) of the same age (3-month-old) and weight (30–32 kg) were used for infection and control. All animals were clinically healthy at the beginning of experiment. They had access to a commercial feed twice a day and to clear water at all times.

Infections were carried out using ASF virus (ASFV) (genotype II) distributed in the Republic of Armenia and the Republic of Georgia (Rowlands et al., 2008). Eight pigs were infected by intramuscular injection and two pigs were used as the uninfected control with intramuscular injection of physiological solution. The titre of ASFV for each intramuscular injection was 10^4 hemadsorbing doses (HAD50)/ml. Virus titration was done as described previously and expressed as log10 HAD50/ml (Enjuanes et al., 1976). Preinoculation blood samples were collected from all pigs for control values. Considerable care was taken in the collection of the blood samples from the ophthalmic venous sinus of either infected or uninfected pigs as described previously (Stier and Leucht, 1980). At the time of sacrifice, all visceral organs were autopsied for evidence of gross pathologies and tissues were dissected for study.

Animal experiments were approved by the Institutional Review Board/Independent Ethics Committee of the Institute of Molecular Biology of NAS RA (reference number IRB00004079). Carbon dioxide inhalation (75–80% carbon dioxide for 20 min) was used to euthanatize infected animals (in accordance with the AVMA Guidelines for the Euthanasia of Animals) after 7 days post infection (dpi).

2.2. Blood smears and Giemsa staining

Fresh blood was used in preparing the blood smears by a routine method. For macrophage visualization, slides were fixed in pure methanol and stained by Giemsa modified solution (azure B/azure II, eosin and methylene blue) according to the manufacturer's protocol (Sigma-Aldrich, Chemie Gmbh Munich, Germany distributor to Yerevan, Armenia). Macrophages in blood were detected by scanning of a blood film with the low-power objective (Smith, 1964).

2.3. Bone marrow smears and preparations

For bone marrow, the needle was advanced in the femoral bone marrow cavity with a twisting motion and rotated to obtain a solid piece of bone marrow, after which the imprints were made.

2.4. Lymph nodes smears and preparations

Fresh lymphoid nodes were sent to the laboratory with minimal delay. Before fixation each lymph node was carefully cut with a sharp scalpel blade through the hilum, and lymph node imprints were made by gently touching the freshly cut surface of the node with a clean glass slide. Sideways movement of the slide was avoided to minimize artefactual distortion of cells (Stevens et al., 1987). For histological studies the same regions of the lungs were obtained, fixed in 10% buffered formalin solution (pH 7.2), and embedded in paraffin.

2.5. Lung smears and preparations

The lungs were removed from the animal, impression smears were made from each lobe. For histological studies the same regions of the lungs were obtained, fixed in 10% buffered formalin solution (pH 7.2), and embedded in paraffin. The lung smears and preparations were free of bacterial and fungal infection at the light-microscope level.

2.6. Spleen smears and preparations

The pieces of organs were fixed in 10% neutral phosphate-buffered formalin for 24 h. After fixation the tissues were dehydrated (using graded alcohols and xylene) and embedded in paraffin. Before fixation each spleen was carefully cut with a sharp scalpel blade imprints were made by gently touching the freshly cut surface of the node with a clean glass slide.

2.7. Macrophage hemophagocytosis investigation

At least three smears and 1000 nucleated cells were observed for hemophagocytosis for every case. Hemophagocytosis was considered significant when at least two histiocytes showing hemophagocytosis were observed per slide, similar to the selection criterion of Favara (Singh et al., 2005).

The quantitation of macrophage accumulation in tissues was conducted by direct evaluation. For each tissue sample, 12 serial sections were evaluated quantitatively with a micrometer eyepiece grid. Each square represented an area of 3780 (70×54) μm^2 on the original section. Average cell counts were made for bone marrow, lymph nodes and lungs in each animal.

2.8. Blood collection and ELISA

From 1 to 7 dpi, blood samples were collected from the ophthalmic venous sinus as described previously (Stier and Leucht, 1980). Pre-inoculation (0 dpi) blood samples were taken to obtain control values. For the detection of serum levels of macrophage colony stimulating factor (MCSF) and granulocyte macrophage colony-stimulating factor (GMCSF) commercial ELISA kits (Elabscience Biotechnology Co., Ltd, Bethesda, MD 20824) were used. The levels of cytokines (pg/ml) were measured using a colorimetric reader (Stat Fax 303 Plus, Awareness Technology Inc) and calculated according to the cytokine standard curve supplied in the kits. All samples were tested in duplicate according to manufacturers' instructions.

2.9. Porcine serum triglycerides

Blood for triglycerides was collected in serum tubes. Porcine serum triglycerides were determined by standard enzymatic colorimetric analysis using an analyzer for clinical chemistry (COBAS INTEGRA 400, Hoffmann-La Roche Ltd Representation in Armenia).

2.10. Statistical analysis

Statistical tests were performed using SPSS version 17.0 software package (SPSS Inc., Chicago, Ilinois). The normality of the data was analyzed using the Kolmogorov–Smironov test. The results of platelet number and aggregated platelets were evaluated by Student's *t*-test. Mann–Whitney *U* test was used to compare the results of coagulation tests between control and infected group, as well as to compare changes in platelet sizes. Pierson *r*-test was used to define a correlation between the virus titer and the number of platelets. All results are expressed as mean value ($n = 8$ and $n = 2$ for infection and control, respectively) ± standard deviation. Differences between control and infection were considered significant at the $P < 0.05$ level.

3. Results

ASFV inoculated animals began to present unspecific signs from 2 to 3 dpi, consisting in pyrexia, loss of appetite, depression and diarrhea