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SIMULATION OF PROTECTIVE IMMUNE MECHANISMS AT AFRICAN SWINE FEVER in vitro

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S u m m a r y

Pigs that survive after infection with African swine fever virus are resistant to reinfection with seroimmunotype-homologous virulent isolates, indicating an immune protection development. Nevertheless, the points regarding the protective importance of various immune mechanisms at African swine fever still remain controversial. Peripheral blood leukocyte cultures prepared from both intact or vaccinated pigs were used as models of implemented virus-specific reactions in vivo. The maximum amount of ASF virus was determined in the cultures with adherent cells from intact or immunized gilt, non-adherent white blood cells and the serum of an intact animal. Replacement of non-adherent cells from intact gilt by those from the immunized one led to a significant decrease in ASF virus titres. It was shown that combination of A-cells from intact or immunized gilt, non-adherent cells from the intact animal and the serum from immunized animal limited the viral propagation more strictly, under the replacement of non-adherent cells from the intact gilt by those from the immunized animal at the most. The results determine the role of cellular and humoral immunity in limiting African swine fever virus replication. The protection mechanisms mediated by cytotoxic T lymphocytes and antibody-mediated cellular anti-effectors are found to have an integrated effect, suggesting their targeting against different epitopes.

Keywords: African swine fever, immune system, antibodies, cytotoxic T-lymphocytes.

Pigs that survived African swine fever (ASF) caused by its virulent or attenuated strains, as a rule, also survive the subsequent infection whose agents are seroimmunotype-homologous virus isolates and strains (1-3). This fact indicates the development of virus-specific immune defense in these animals. However, protective role of humoral and cellular links of immunity against ASF is still being discussed.

Despite the absence of virus neutralizing antibodies to ASF, experimental data show the presence of antibody-dependent component of resistance to the viral infection (4). According to some reports, the transfer of whey or colostrum from ASF survivors to intact animals may detain the expression of clinical symptoms, reduce viremia, and increase survival rate if these animals then catch ASF (1, 5, 6). Antibody-mediated cytolysis of ASF-infected monocytes and macrophages occur during in vitro performed reactions of complement-dependent cytolysis (CDC) and antibody-dependent cellular cytotoxicity (ADCC) (7-9). Antibodies to ASF virus-specific proteins were detected by various methods including ADCC in the blood serum of pigs on the 3rd-7th day after experimental inoculation of ASF. Survivors maintain these antibodies for a long time (9, 10). It was experimentally proved the importance of cytotoxic T lymphocytes (CTL) in formation of virus-specific defense in early period of ASF course (11, 12). At the same time, contributions of ADCC-, CDC-, and CTL-mediated mechanisms to limitation of ASF reproduction are yet insufficiently clear, although this issue had been already studied (13).

The purpose of this research was to determine the value of cellular and humoral immunity components in limitation of the reproduction of African swine fever (ASF) virus using model systems of cultured peripheral blood leukocytes derived from intact pig and the pig immunized with attenuated ASF strain on the 6th day after inoculation.

Technique. The work was carried out using ASF samples from the museum of microorganisms of the All-Russia Research and Development Institute of Veterinary Virology and Microbiology: virulent strains of F-32 IV seroimmunotype and K-73 II seroimmunotype, and their attenuated derivatives (respectively, strains We FC-135 and CC-202) (14).

Production of interleukin-2 (IL-2) in peripheral blood lymphocytes of investigated pigs in response to concanavalin A stimulation was measured from proliferation activity of IL2-dependent cell line of mouse T-lymphocytes (CTLL-2), which was evaluated by incorporated ^{3}H-thymidine. The blood was sampled from the anterior vena cava of young Large White pigs of 40-45 kg weigh – intact and ASF-inoculated, then mixed with heparin (20 units/cm^3) and isolated lymphocytes in density gradient using Ficoll-pack. The lymphocytes (3 millions/cm^3) were cultured in RPMI 1640 medium ("Sigma-Aldrich", USA) supplemented with fetal bovine serum (10%), glutamine (1 mM), 2-mercapto-ethanol (2×10^{-5} M) (culture medium) and 5 ug/cm^3 concanavalin A for 48 hours at 37 °C in a humidified atmosphere (air + 5 % CO2). After that, the cell suspension was centrifuged at 800 g for 30 minutes. Supernatant was clarified at 10 000 g for 30 minutes. Then 10^4 CTLL-2 cells in 0.1 cm^3 culture medium was added with 0.1 cm^3 of the obtained supernatants in which concanavalin A was blocked by 1 % (w/v) methyl-α-D-mannopyranoside. After 24 of culturing, ^{3}H-thymidine was introduced in each mixture (1850 MBq) and after 6 h there was measured incorporation of the radioactive labeled precursor in cells. The assay was performed in 6 replicates, results were statistically processed.

To simulate in vitro the mechanisms of immune defense to ASF, the blood was sampled from the anterior vena cava of a young pig on day 0 and 6 days after its immunization with ASF strain FC-35 (107.5 Had_{50}). Half volume of the sampled venous blood was mixed with heparin (20 units/cm^3) and thermostated for 1.5 hours at 37 °C. The white blood was collected with pipette and precipitated at 800 g for 20 min. The precipitate was resuspended in 0.1 % lactalbumin hydrolyzate in Earle's saline with 5% fetal bovine serum (culture medium); the suspension (5 million cells/cm^3) was dispensed into Carrel d-flasks of 9 cm^3 and cultured at 37 °C. The second half volume of the sampled venous blood was a source for obtaining serum by a conventional method.

On the 8th day after the start of the experiment, non-adherent cells in the culture medium of each d-flask with cultured blood leucocytes were separately poured into sterile centrifuge tubes, A-cells were perfused with the culture medium (5 cm^3). Non-adherent cells from each cup were precipitated at 800 g for 20 min. Resulting precipitates were resuspended in 9 cm^3 of culture medium and transferred into d-flasks with A-cells according to the specially developed experimental pattern after preliminary elimination of the

culture medium. Then into each d-flask introduced 1.0 cm^3 (10 %) of serum obtained from the sampled blood of intact (day 0) and immunized (day 6) pig according to the developed pattern. Then into all d-flasks introduced ASF strain F-32 (100 Had_{50} in 0.5 cm^3 volume) and incubated 4 days at 37 °C. Accumulation of ASF virus was determined by titration in the culture of pig leukocytes in terms of hemadsorption expressed in HAd_{50}/cm^3.

Statistical data analysis was performed using a standard computer program BIOSIS-1.

Results. Cultures of peripheral blood leukocytes of pigs can be considered as models in vitro that show realization of virus-specific immunological reactions as it occurs in vivo. Methodological basis of this work was developed using cell cultures of pig blood leucocytes with different variations of three components: target cells (A-cells, macrophages) adherent on the glass, effector cells (lymphocytes) not attached to the glass surface, and cells of the blood serum. It's important to note that all investigations were conducted in autologous systems with uniform antigen-presenting capacity of cells (9).

Finding solution to the target task necessitated proper strains representing one seroimmunotype group: one immunogen, the other – virulent strain. In this case, the immunogenic strain mustn't express immunosuppressive properties. That's why the first phase of research included evaluation of the functional state of peripheral blood lymphocytes of pigs inoculated with ASF strains of different virulence and seroimmunotype. To do this, there was investigated the ability of T-lymphocytes to produce IL-2 that, along with virus-specific antigens, induces activation and clonal expansion of antigen-specific resting cytotoxic T-lymphocytes.

1. Production of IL-2 by concavalin A-stimulated lymphocytes isolated from the peripheral blood of Large White pigs — intact, immunized with ASF strains FK-135 and KK-202, and infected with ASF strains F-32 and K-73.

Condition of pigs	Dilution of supernatants	Production of IL-2, impulses per minute			
Number of individual		1st	2nd	3rd	4th
Intact	1:2	4127±217	3944±108	4321±95	4111±73
	1:4	2345±301	2227±94	2531±119	2004±54
	1:8	1672±74	1471±104	1750±96	1421±139
Immunized,	1:2	4435±282	3884±171		
strain FK-135	1:4	2212±86	2035±74	–	–
	1:8	1325±77	1118±49		
Infected,	1:2	3874±118	3439±109	1917±91	1812±145
strain F-32	1:4	2031±43	1931±72	235±120	1098±81
	1:8	1675±92	920±67	629±66	531±42
Number of individual		5th	6th	7th	8th
Intact	1:2	3452±194	3160±97	2930±162	3001±108
	1:4	1878±104	1662±64	1645±79	2012±98
	1:8	1103±59	863±57	865±41	977±73
Immunized,	1:2	2308±98	1876±209		
strain KK-202	1:4	1146±38	864±46	–	–
	1:8	712±27	409±29		
Infected,	1:2	1966±157	2142±174	1322±96	1541±62
strain K-73	1:4	886±84	975±33	598±34	843±51
	1:8	448±16	462±28	280±16	377±39

N o t e . Dashes — measurements were not conducted.

Two pigs had been immunized with the strain FK-135 ($10^{7.5}$ HAd_{50}), the other two – with the strain KK-202 ($10^{7.5}$ HAd_{50}). After 2 weeks, both pigs earlier immunized with the strain FK-135 and two intact pigs were infected with the strain F-32 ($10^{3.0}$ HAd_{50}), while the two pigs earlier immunized with the strain KK-202 and two intact pigs were infected with the strain K-73 ($10^{3.0}$ HAd_{50}). On the 6th day after each inoculation, there was determined the production of IL-2 by peripheral blood lymphocytes stimulated by concanavalin A.

The obtained results (Table 1) show that immunization of pigs with the strain FC-135 and their subsequent infestation with the strain F-32 didn't reduce functional activity of T-lymphocytes. Infection of intact pigs with ASF strain F-32 resulted in suppression of T-lymphocytes, which was expressed as the decline in production of IL-2 more than 2.0 times. Inoculation of pigs with the attenuated strain KK-202 as well as with the virulent strain K-73 caused a significant decrease in production of IL-2 (up to 1.5-2.0 times). Experimental simulation of immune defense mechanisms in vitro was performed using ASF strains of IV seroimmunotype group F-32 and FK-135, because the latter doesn't cause immunosuppression in pig lymphocytes.

In the model of immune response to ASF virus in vitro, on the 6th day after immunization it was found (Table 2) that maximum accumulation of the virus (7.50-7.67 lg HAd_{50}/cm^3) occurred in culture systems ¹ 1 and ¹ 5 that contained adherent cells from intact or immunized pig, as well as non-adherent cells of the white blood, and the serum from intact animal.

2. Accumulation of ASF virus (strain F-32) in autologous cultures of peripheral blood leucocytes of intact (IN) and immunized (IM) Large White pig in a simulated model experiment performed according to the developed experimental pattern

№ systems	A-cells		Serum		Lymphocytes		Virus titer, lg HAd_{500}/cm^3
	IN	IM	IN	IM	IN	IM	
1	+	–	+	–	+	–	7,67±0,33
2	+	–	+	–	–	+	7,00±0,21
3	+	–	–	+	+	–	6,33±0,43
4	+	–	–	+	–	+	5,67±0,21
5	–	+	+	–	+	–	7,50±0,07
6	–	+	+	–	–	+	6,67±0,26
7	–	+	–	+	+	–	5,67±0,43
8	–	+	–	+	–	+	5,00±0,21

N o t e . "+" and "–" — respectively, presence and absence of component.

In the culture system where non-adherent cells from intact pig were replaced by non-adherent cells from immunized one (¹ 2 and № 6), there was observed a significant reduce in accumulation of the virus (by 0.67-0.83 lg HAd_{50}/cm^3). In this variant, the

possible limiting mechanism could be CTLs, because the serum was derived from intact individual and the mechanisms of virus-specific antibody-dependent defense were not enabled.

In the culture system containing A-cells of intact or immunized pig, non-adherent cells of intact animal, and the serum of immunized pig ([1] 3 and [1] 7), accumulation of ASF virus reduced by 1,34-1,83 lg HAd_{50}/cm^3. In this case, accumulation of the virus was limited by antibody-dependant mechanisms of antivirus defense.

And, finally, in the system containing A-cells of intact or immunized pig, the serum and non-adherent white blood cells derived from immunized animal ([1] 4 and [1] 8), there was reduced accumulation of ASF virus compared with control ([1]1 and [1] 5) by 2,00-2,50 lg HAd_{50}/cm^3. In this variant, both links of the immune system were enabled – cells (CTLs) and antibody-mediated link (ADCC, CDC).

Thus, on the 6th day after immunization of pigs with AFS strain FK-135 in model systems it was observed that the mechanisms of antibody-mediated anticellular immunity exceed CTL-dependant mechanisms by ability to limit reproduction of African swine fever virus. Findings of this work show that CTL- and antibody-mediated mechanisms of anticellular immune defense cooperate as integrated system, which allows assuming their action against different epitopes.

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