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Enterovirus E infects bovine peripheral blood mononuclear cells. Implications for pathogenesis?

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Abstract

Introduction: Enterovirus E (EV-E) is a common viral pathogen endemic in cattle worldwide. Little is known, however, about its potential interactions with bovine immune cells. **Material and Methods:** The EV-E-permissiveness of bovine peripheral blood mononuclear cells (PBMCs) was evaluated. The infectious titres of extracellular virus were measured and the intracellular viral RNA levels were determined by reverse transcription quantitative PCR after cell inoculation. The effects of EV-E on cell viability and proliferative response were investigated with a methyl thiazolyl tetrazolium bromide reduction assay, the percentages of main lymphocyte subsets and oxidative burst activity of blood phagocytes were determined with flow cytometry, and pro-inflammatory cytokine secretion was measured with an ELISA. **Results:** Enterovirus E productively infected bovine PBMCs. The highest infectious dose of EV-E decreased cell viability and T-cell proliferation. All of the tested doses of virus inhibited the proliferation of high responding to lipopolysaccharide B cells and stimulated the secretion of interleukin 1β, interleukin 6 and tumour necrosis factor α pro-inflammatory cytokines. **Conclusion:** Interactions of EV-E with bovine immune cells may indicate potential evasion mechanisms of the virus. There is also a risk that an infection with this virus can predispose the organism to secondary infections, especially bacterial ones.

Keywords: enterovirus E, bovine PBMCs, productive infection, blastogenic response, pro-inflammatory cytokines.

Introduction

Enterovirus E (EV-E) is a member of the large *Enterovirus* genus in the *Picornaviridae* family along with 11 other species of enteroviruses (A, B, C, D, F, G, H, I, J, K and L). Enteroviruses are small, nonenveloped, icosahedral, single-stranded positive-sense RNA viruses that can infect humans and animals. Bovine enteroviruses (BEV) belong to two species: enterovirus E (formerly bovine enterovirus type 1, BEV-1) and enterovirus F (formerly bovine enterovirus type 2, BEV-2) (16, 33).

Enteroviruses are common viral pathogens which usually cause mild and self-limiting diseases, a notable exception being human poliovirus. However, enteroviral infections other than poliovirus can occasionally result in significant morbidity. Host-to-host transmission of enteroviruses occurs *via* the faecal–oral route and is followed by virus replication in the mucosa of the respiratory or gastrointestinal tract. The local lymphatic tissues are also targets, by colonising which the virus can spread to the lymph nodes and bloodstream. Viraemia enables a virus to reach secondary replication sites (34, 35), and while it lasts, leukocytes can act as an additional reservoir of the virus, thereby playing a significant role in the pathogenesis of enterovirus infections, particularly chronic ones (35). The literature data confirm the ability of human enteroviruses to productively infect various types of human immune cells, both T and B lymphocytes, and dendritic cells, monocytes or granulocytes (8, 9, 12, 17, 18, 26, 34, 39). Infection of immune cells with enteroviruses usually results in increased synthesis of numerous cytokines and chemokines by cells (6, 9, 12, 37, 39); from the clinical point of view, the most important of these are the pro-inflammatory cytokines interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumour necrosis factor α (TNF-α),

which sustain the inflammatory process and can contribute to the damage of tissues or even the development of immune-mediated chronic diseases (34). Elevated levels of these cytokines in the blood and cerebrospinal fluid have been observed in patients with a severe and complicated course of enteroviral infection (6, 19).

Bovine enterovirus was first isolated in late 1950s from the faeces of a clinically healthy cow, and was originally classified as enteric cytopathogenic bovine orphan virus (14). Because this virus, widespread in the cattle population worldwide, usually causes subclinical or mild infections, its pathogenicity has not been the subject of intensive research so far. Nevertheless, there are reports in the literature about respiratory, gastrointestinal and fertility disorders caused by BEV, some of which are fatal (3, 36, 42, 43). Bovine enterovirus is also listed as one of the viral pathogens associated with the bovine respiratory disease complex (25). The literature data concerning interactions of BEV with immune cells are only fragmentary. In *in vitro* studies, the ability of bovine enterovirus to infect human cell lines of the monocyte, B cell and T cell lineage has been demonstrated (32, 35). In an *in vivo* study, an experimental infection of mice with BEV resulted in liver lesions, and viral antigens were located around Kupffer cells and neutrophils (21). In turn, following the experimental infection of calves with BEV, the virus was localised in macrophages and lymphocytes of the gastrointestinal lymphatic tissue (3). However, the character of the interactions of this virus with bovine immune cells and the role of such interactions in the pathogenesis of infections remain unknown.

The aim of this study was to determine the ability of enterovirus E to infect bovine peripheral blood leukocytes and learn its influence on the viability and activity of these cells. Its findings have revealed the effect of EV-E on the mitogenic response of leukocytes; synthesis of IL-1β, IL-6 and TNF-α; and ability of phagocytes to carry out oxidative burst.

Material and Methods

Virus and cells. Enterovirus E (LCR4 strain, American Type Culture Collection (ATCC) VR-248) was used in this study for *in vitro* infection of bovine peripheral blood leukocytes. Madin-Darby bovine kidney (MDBK, ATCC CCL-22) cells were used for virus propagation, titration and the virus neutralisation assay. MDBK cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 1% nonessential amino acid solution and 1% antibiotic-antimycotic solution (all components from Sigma-Aldrich, Schnelldorf, Germany). The 50% endpoint virus titres (50% cell culture infectious dose, $CCID_{50}$) were determined by an endpoint dilution assay as described before (40) and calculated using the Reed and Muench method (31).

Animals and blood handling. Serum and blood samples were randomly collected from conventionally reared, clinically healthy Holstein-Friesian dairy cows originating from a herd free of bovine viral diarrhoea virus (BVDV) and bovine herpesvirus type 1 (BHV-1). The negative status of the animals used in the study was confirmed by a commercial veterinary laboratory based on ELISA results for both viruses. All samples were collected by an authorised veterinarian, following standard procedures during the routine screening of animals. Both the serum and blood samples used in this study were unused material remaining after other laboratory tests. According to the Local Ethical Committee on Animal Testing at the University of Warmia and Mazury in Olsztyn (Poland), formal ethical approval is not required for this kind of study.

Susceptibility of bovine peripheral blood mononuclear cells to infection by enterovirus E. With confirmation existing in the literature data that human enteroviruses could infect human immune cells, it was decided to verify whether bovine enterovirus E is able to productively infect bovine leukocytes. To this aim, peripheral blood mononuclear cells (PBMCs) isolated from 10 dairy cows were infected with enterovirus E at a multiplicity of infection (MOI) of 1. The intercellular viral RNA levels were determined after 24 h incubation, and the viral titres in the PBMC supernatant after 72 h incubation. In parallel, in order to evaluate a possible protective effect of the acquired immunity, the titres of anti-EV-E antibodies in the sera of the animals the cells of which were infected with the virus were determined.

Virus neutralisation test. The sera were tested for the presence of anti-EV-E antibodies using a microneutralisation test. For this purpose, serum samples prior to testing were inactivated at 56°C for 30 min. Afterwards, sera were serially two-fold diluted starting at 1:5. All dilutions were mixed with the same volume of virus suspension (100 $CCID_{50}$/100 µL) and incubated for 1 h at 37°C, then MDBK cell monolayers were inoculated with the mixtures. All analyses were made in duplicate using 96-well microplates. The plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 3 d. Neutralisation titres (the highest serum dilution that protected cells from a viral cytopathic effect) were evaluated using an IX70 S8F2 inverted phase contrast microscope (Olympus; Tokyo, Japan).

Isolation, culture and infection of peripheral blood mononuclear cells. Peripheral blood mononuclear cells were isolated using Histopaque 1077 density gradient centrifugation at 450 g for 45 min, suspended at a concentration of 1×10^6 cells/mL in RPMI-1640 medium supplemented with 10% horse serum and 1% antibiotic-antimycotic solution (all reagents from Sigma-Aldrich, Schnelldorf, Germany),

and incubated at 37°C in a humidified atmosphere with 5% CO_2.

In order to prove bovine PBMC susceptibility to infection by enterovirus E, cells were put in contact with EV-E at an MOI of 1 and incubated for 1 h at 37°C to allow viral adsorption. Afterwards cells were thoroughly washed three times with phosphate-buffered saline to remove unbound virus, suspended in fresh medium and cultured for 24–72 h. Intracellular viral RNA levels were measured 24 h after infection and the titres of viral progeny in culture supernatants were calculated 72 h after infection.

In other experiments, three different infectious doses of enterovirus E were tested: high (MOI = 10), medium (MOI = 1) and low (MOI = 0.1) and cells were incubated with the virus throughout the whole time of the experiments.

Virus quantification by reverse-transcription quantitative PCR. Isolation of RNA was carried out using a Total RNA Mini Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's recommendations. The purity and amount of RNA isolated was determined using a BioSpectrometer (Eppendorf, Hamburg, Germany). Ribonucleic acid was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Waltham, MA, USA) following the manufacturer's protocol. The sequences of primers used in the study are summarised in Table 1. The conditions of the real-time PCR and the method of determining the virus copy were described in detail in our previous paper (40).

Peripheral blood mononuclear cell viability and blastogenic response to mitogens – MTT reduction assay. The colorimetric 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay is routinely used to assess the number of viable cells the mitochondrial dehydrogenases of which convert water-soluble yellow MTT dye to insoluble purple formazan. Lymphocytes do not proliferate without stimulation, therefore an MTT assay may be used to evaluate both the unstimulated cell viability and their proliferative response to mitogens.

To assess the virus' impact on cell viability, unstimulated PBMCs were infected with three infectious doses of EV-E (MOI = 10, 1 or 0.1) and cultured for 72 h as described above. Then, the viability of the cells was evaluated using an MTT reduction assay as described before (22). The viability of control (uninfected) cells was considered 100% and regarded as a reference value. The blastogenic response of virus-infected or control PBMCs to mitogens was evaluated after 72 h of cell culture. Concanavalin A (ConA) at a concentration of 5 µg/mL was used as a T-cell mitogen, and lipopolysaccharide (LPS) from *E. coli* at a concentration of 5 µg/mL was used as a B-cell mitogen (both mitogens from Sigma-Aldrich). The results obtained were expressed as a stimulation index, which was calculated by dividing the mean values of mitogen-stimulated cells by the values of unstimulated cells as described previously (22).

According to the literature data, cows can be divided into low- and high-responder animals in terms of their blastogenic response to LPS (1, 5); therefore, it was decided to divide the experimental animals into two categories in this respect. In each experimental setup, the PBMCs obtained from 10 animals were analysed, and the control (uninfected) cells constituted the reference point in each case.

Immunophenotyping of bovine peripheral blood mononuclear cells. Immunophenotyping of peripheral blood lymphocytes was conducted in order to determine whether the EV-E effect was focused particularly on either of the main populations of these cells. This experiment was carried out using cells from the high- responder animals (n = 5), and supernatants from these cells were used for measurement of the levels of pro-inflammatory cytokines.

Table 1. Primer sequences used for the detection of intracellular enterovirus E RNA

Primer	Primer sequence (5′–3′)	Amplicon size	GenBank accession No.
EV-E802 forward	AAAGGGGGCTGTCGAAACCA	802	DQ092769.1
EV-E 802 reverse	GCTAGTGGGCTCAGACTCCG		
EV-E 183 forward	TACGCCTTTCGTGGCTTGGA	183	
EV-E 183 reverse	TTGCTTTTCCTGGCTTGCCG		

Table 2. Monoclonal antibodies used in the study

Marker	Expressed by	Fluorochrome	Clone	Isotype
CD4	subset of T cells	FITC	CC8	IgG2a
CD8	subset of T cells	Alexa Fluor 647	CC63	IgG2a
WC1	gamma/delta (γδ) T cells	FITC	CC15	IgG2a
CD21	B cells	RPE	CC51	IgG2b

Both unstimulated and mitogen-stimulated PBMCs were cultured for 72 h in the presence of the same three doses of EV-E (MOI = 10, 1 or 0.1). Afterwards, cells were harvested, washed and stained with fluorochrome-conjugated monoclonal antibodies specific to bovine CD4, CD8, WC1 or CD21 markers (all from Bio-Rad Laboratories Inc., Hercules, CA, USA), as described before (24). The properties of antibodies used in the study are summarised in Table 2. Fluorescence minus one (FMO) controls were used to determine the cut-off point between background fluorescence and positive populations. Flow cytometry analysis was performed using a FACSCelesta cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The data were acquired by FACSDiva version 10.0 software (BD Biosciences) and analysed with FlowJo software (Tree Star Inc., Stanford, CA, USA).

Analysis of cytokine production by ELISA. Culturing of PBMCs took place in 48-well plates for 72 h after inoculation with the virus. Control cells remained uninfected. Lipopolysaccharide from *Escherichia coli* (5 µg/mL) was used to stimulate production of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. Cytokine production by unstimulated cells was also evaluated. After incubation, the plates were centrifuged at 300 × g for 10 min, and the supernatants were collected and tested in duplicate. Cytokine levels in supernatants were determined using commercial ELISA kits (Cloud-Clone Corp., Katy, TX, USA), according to the manufacturer's protocol.

Oxidative burst activity of peripheral blood phagocytes. The whole blood samples were incubated for 3 h at 37°C with the three doses of EV-E (MOI = 10, 1 or 0.1) to evaluate virus impact on the oxidative burst activity of blood phagocytes. Control cells were not infected. After incubation, a commercial Bursttest (ORPEGEN Pharma, Heidelberg, Germany) was performed according to the manufacturer's instructions, as previously described (23). Bursttest measures the percentages of active monocytes and granulocytes separately and the oxidative burst intensity within cells as mean fluorescence intensity (after stimulation with opsonised *E. coli* bacteria. The marker of cell activity is cell-permeable dihydrorhodamine 123 dye, which is oxidised by reactive oxygen species produced by phagocytes to fluorescent rhodamine 123.

The fluorescence of the samples was measured by flow cytometry using the FACSCelesta cytometer. The data were acquired by FACSDiva version 10.0 software and analysed with FlowJo software.

Statistical analysis. All the results were expressed as the mean values ± standard deviations (SD). After validation of normality with the Shapiro–Wilk test and homogeneity of variances with Levene's test, data were submitted to one-way analysis of variance and Tukey's *post-hoc* test was used to determine differences between control and EV-E-infected cells. Statistical evaluation of the results was performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA).

Results

Susceptibility of bovine peripheral blood mononuclear cells to infection by enterovirus E. In nine out of ten animals the PBMCs of which were infected with the virus, the presence of anti-EV-E antibodies was confirmed, and the titre values ranged from 1:20 to 1:160 (Table 3). Regardless of the presence of antibodies and value of the titre, enterovirus E was able to cause productive infection of bovine PBMCs. The viral titres (log $CCID_{50}$/1 mL) in the supernatant ranged from 1.75 to 3.875, and the number of copies of the viral RNA (per µL of RNA) varied from a few to over 50 thousand (Table 3).

Viability and proliferative response of PBMCs infected with EV-E. The high infectious dose (MOI = 10) considerably decreased the viability of cells and their proliferative response to concanavalin A; however, the two lower doses of the virus did not have any effect on these parameters. None of the doses of the virus had a significant effect on the blastogenic response to LPS among the low-responder animals, while all the doses considerably decreased the proliferative activity of LPS-stimulated cells among the high-responder animals (Table 4).

Immunophenotyping of peripheral blood mononuclear cells infected with enterovirus E. None of the doses of the virus had a substantial impact on the distribution of the main populations of unstimulated lymphocytes, and the two lower doses did not affect the percentages of ConA-stimulated cells either (Table 5). The highest infectious dose of the virus considerably increased the percentage of double positive (DP) CD4+ CD8+ T cells following the stimulation with ConA (Table 5, Fig. 1). As regards the LPS-stimulated cells, all doses of the virus caused a considerable decrease in the percentage of CD21+ B cells and an increase in the CD4+ and CD8+ T cells (Table 5).

The flow cytometry cytograms obtained from control (uninfected) cells stimulated with mitogens displayed the occurrence of a large cloud of lymphoblasts, cells bigger and with higher density than resting lymphocytes. The doses of the virus that inhibited the proliferation of lymphocytes caused a strong reduction in the number of lymphoblasts (Fig. 1).

Pro-inflammatory cytokine responses of bovine peripheral blood mononuclear cells infected with enterovirus E. Enterovirus E increased the production of pro-inflammatory cytokines by unstimulated PBMCs isolated from high-responder animals. The lower doses of the virus, which did not decrease the viability of cells, considerably stimulated the production of all determined cytokines, and the highest dose of the virus stimulated only the production of IL-1β (Table 6).

Regarding the LPS-stimulated cells, the two lower doses of the virus (MOI = 1 or 0.1) did not affect the production of cytokines, while the highest dose of the virus decreased the production of IL-1β (Table 6).

Table 3. Serum anti-EV-E antibody titres, intracellular viral RNA levels and extracellular virus titres from bovine PBMCs

Parameter	Individual									
	1	2	3	4	5	6	7	8	9	10
anti-EV-E antibody titer in serum	ND	1:40	1:20	1:80	1:20	1:40	1:80	1:40	1:20	1:160
extracellular virus titer (log $CCID_{50}$/1 mL)	3.625	1.75	3.25	3.125	3.875	3.125	2.125	3.625	2.75	3.125
intracellular viral RNA (copy number/µL of RNA)	13.39	37.48	1.866	4913	7.133	14.82	3.852	54740	20.1	9.610

Table 4. Enterovirus E effect on the viability and blastogenic response of bovine peripheral blood mononuclear cells to mitogens shown by the MTT reduction assay, n=10

Parameter	C	EV-E (MOI)		
		10	1	0.1
viability (%)	100 ±0	58.928** ±19.969	80.927 ±20.681	104.685 ±31.217
proliferation ConA (SI)	4.332 ±0.957	2.198*** ±0.731	4.712 ±1.274	3.960 ±0.779
proliferation LPS (SI) high responders	2.085 ±0.46	1.479*** ±0.152	1.212*** ±0.298	1.095*** ±0.255
proliferation LPS (SI) low responders	0.924 ±0.112	1.084 ±0.120	0.925 ±0.160	0.880 ±0.140

All data expressed as means values ± standard deviation. EV-E – enterovirus E; C – control (uninfected) cells; MOI – multiplicity of infection; ConA – concanavalin A; LPS – lipopolysaccharide from *E. coli*; SI – stimulation index. ** – statistically significant difference between control and EV-E-infected cells at $P < 0.01$; *** – statistically significant difference between control and EV-E infected cells at $P < 0.001$

Table 5. Immunophenotyping of bovine peripheral blood lymphocytes cultured for 72 h in the presence of enterovirus E, n = 5

Population	Unstimulated				LPS-stimulated				ConA-stimulated			
	C	EV-E (MOI)			C	EV-E (MOI)			C	EV-E (MOI)		
		10	1	0.1		10	1	0.1		10	1	0.1
CD4+CD8−	44.80 ±8.73	44.86 ±6.30	42.98 ±6.93	42.46 ±7.24	19.38 ±3.15	34.55** ±6.53	31.02* ±5.99	38.30*** ±5.05	45.98 ±10.56	47.48 ±9.67	45.30 ±7.44	42.24 ±7.33
CD4-CD8+	22.20 ±0.92	20.28 ±1.53	21.58 ±2.53	22.10 ±1.92	12.72 ±4.66	23.30* ±5.86	22.22* ±6.17	24.05* ±5.80	13.36 ±4.18	19.54 ±9.54	13.21 ±5.96	14.45 ±5.74
CD4+CD8+	0.97 ±0.38	1.92 ±0.88	1.17 ±0.40	1.04 ±0.18	2.89 ±0.61	6.09 ±2.16	5.83 ±2.61	6.90 ±3.27	2.39 ±0.68	7.34** ±4.05	2.93 ±0.82	2.31 ±0.50
CD21+	15.38 ±8.23	21.96 ±6.49	20.30 ±8.61	19.59 ±8.30	47.86 ±9.94	16.39*** ±8.63	19.71*** ±9.12	11.53*** ±5.86	18.55 ±6.67	9.49 ±4.88	18.46 ±5.31	17.66 ±9.02
WC1+	4.31 ±3.01	1.80 ±1.40	5.29 ±3.66	5.81 ±4.57	4.28 ±1.94	2.28 ±1.48	3.45 ±1.78	3.32 ±0.99	16.98 ±10.99	18.86 ±12.10	14.53 ±9.16	13.55 ±9.18

All data expressed as means values ± standard deviation. EVE-E – enterovirus E; C – control (uninfected) cells; MOI – multiplicity of infection; ConA – concanavalin A; LPS – lipopolysaccharide from *E. coli*. * – statistically significant difference between control and EV-E-infected cells at $P < 0.05$; ** – statistically significant difference between control and EV-E-infected cells at $P < 0.01$; *** – statistically significant difference between control and EV-E-infected cells at $P < 0.001$

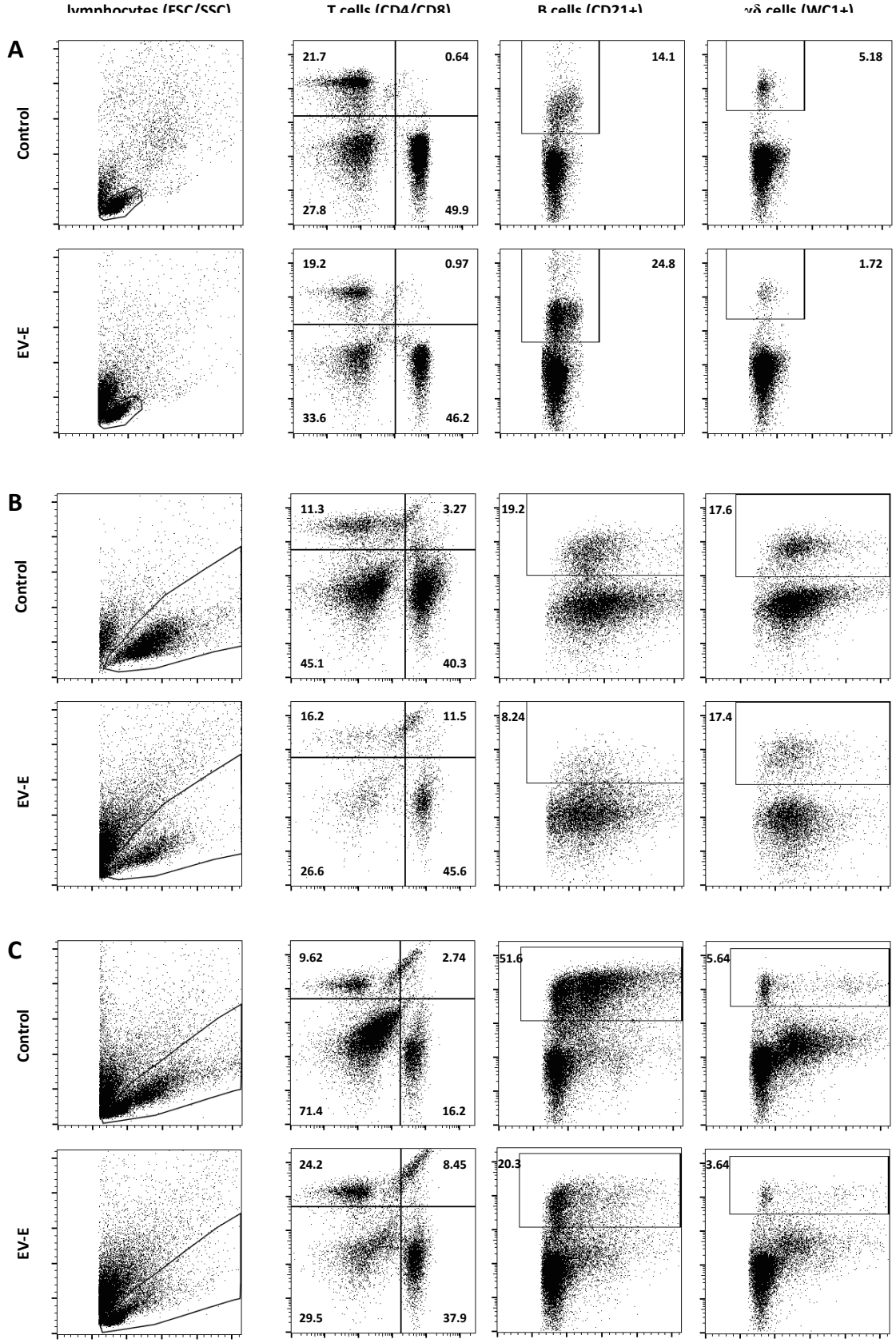

Fig. 1. Representative dot plot cytograms showing distribution of the main lymphocyte subsets of bovine peripheral blood mononuclear cells after 72 h incubation with a high infectious dose of enterovirus E (EV-E): panel (A) unstimulated cells; (B) concanavalin A-stimulated cells; (C) lipopolysaccharide-stimulated cells. In each panel: upper row – control (uninfected) cells, bottom row – EV-E-infected cells; columns from left to right: lymphocyte gating based on their forward and side scatter (FSC/SSC) properties, T-cell gating according to the expression of CD4 and CD8 markers, B-cell gating according to the expression of CD21 marker and gamma-delta-cell gating according to the expression of WC1 marker

Table 6. Cytokine levels in supernatants from bovine peripheral blood mononuclear cells cultured for 72 h in the presence of enterovirus E, n = 5

Cytokine (pg/mL)	Unstimulated				LPS-stimulated			
	C	EV-E (MOI)			C	EV-E (MOI)		
		10	1	0.1		10	1	0.1
IL-1β	3.56 ±2.38	62.09*** ±13.39	96.55*** ±17.96	24.67* ±5.97	21.81 ±6.46	5.35** ±3.81	24.36 ±4.95	17.53 ±7.21
IL-6	89.32 ±53.37	141.89 ±84.48	784.94*** ±284.26	864.22*** ±82.46	1292.09 ±279.69	1209.51 ±179.79	1151.45 ±167.52	1152.22 ±197.26
TNF-α	129.09 ±84.32	207.14 ±90.41	1057.82*** ±441.88	1236.32*** ±96.40	1780.26 ±456.88	2037.78 ±550.99	1684.56 ±363.51	1682.79 ±356.02

All data expressed as means values ± standard deviation. EV-E – enterovirus E; C – control (uninfected) cells; MOI – multiplicity of infection; LPS – lipopolysaccharide from *E. coli*; * – statistically significant difference between control and EV-E-infected cells at $P < 0.05$; ** – statistically significant difference between control and EV-E-infected cells at $P < 0.01$; *** – statistically significant difference between control and EV-E-infected cells at $P < 0.001$

Table 7. Oxidative burst activity of bovine peripheral blood phagocytes after 3 h incubation with enterovirus E, n = 5

Cell type	Parameter	C	EV-E (MOI)		
			10	1	0.1
granulocytes	%	91.62 ± 4.3	92.18 ± 3.98	92.94 ± 3.86	92.76 ± 4.23
	MFI	1797.6 ± 269.67	1774.6 ± 291.22	1831.4 ± 280.50	1793.8 ± 286.99
monocytes	%	43.32 ± 7.70	32.62 ± 3.74	35.90 ± 6.94	37.46 ± 6.65
	MFI	299.2 ± 87.04	208.4 ± 71.53	240.2 ± 74.89	247.2 ± 93.38

All data expressed as means values ± standard deviation. EV-E – enterovirus E; C – control (uninfected) cells; MOI – multiplicity of infection; MFI – mean fluorescence intensity

Oxidative burst activity of peripheral blood phagocytes infected with EV-E. Because enterovirus E had a considerable effect on the synthesis of typical monocyte pro-inflammatory cytokines, we decided to verify whether it also influenced the oxidative burst activity of peripheral blood phagocytes. To this end, full peripheral blood of animals was put in contact with three infectious doses of EV-E for 3 h. Afterwards, oxidative burst activity of granulocytes and monocytes stimulated with *E. coli* bacteria was measured using flow cytometry.

Regardless of the dose, the virus did not affect significantly either the percentages of cells undergoing oxidative burst or their mean fluorescence intensity (Table 7).

Discussion

Bovine enterovirus is highly prevalent in cattle populations worldwide. Isolated from both healthy and diseased individuals, the virus induces production of antibodies, which are detected in most of the tested animals (2). In the first stage of our study, we confirmed the presence of antibodies (titres from 1:20 to 1:160) against BEV-E in nine out of ten cows the blood of which was used as our research material. This finding is fully consistent with results reported by other authors (2, 11). For example, in their study conducted in Turkey, Birdane and Gür (2) determined the presence of specific antibodies against EV-E in 153 out of 155 clinically healthy dams (98.7%), and the titres ranged from 1:5 to 1:160. In another study conducted in Brazil, neutralising antibodies against BEV were detected in 411 out of 414 tested cattle of both sexes (99.2%), and the titres varied from 1:5 to >1:640 (11).

Our experimental results suggested that previous contact of animals with bovine enterovirus, as was confirmed by the presence of antibodies in their blood serum, did not affect the permissiveness of their PBMCs to EV-E. The infection of cells was productive; we confirmed not only the presence of intracellular viral RNA but also the presence of viral progeny in the supernatant from the cell cultures. The virus achieved relatively low $CCID_{50}$ (1.75–3.875 log/mL) titres; in comparison, the titre of the same strain of virus in the MDBK cell line in our earlier studies typically oscillated around 7 logs (40). The only literature data available concerning the replication of enteroviruses in immune cells concern human pathogens. The productive infection of human cells has been confirmed in the case of enterovirus 71 (EV71), coxsackievirus B, enterovirus D and echoviruses (8, 9, 12, 17, 18, 26, 34, 39). Enterovirus 71 in supernatant from human PBMCs and monocyte-derived macrophages was measured at titres of 10^4–10^5 PFU/mL and 4.5 log $TCID_{50}$/mL

(9, 39), respectively, whereas echoviruses in dendritic cells were not seen to exceed titres of 3 logs (18). The factors influencing the levels of viral titres are undoubtedly viral tropism and the percentage of virus-permissive cells. The available data suggest that different enteroviruses demonstrate tropism to different types of immune cells. Enterovirus 71 and coxsackievirus B infected T and B lymphocytes as well as monocytes/macrophages (8, 9, 12, 17, 26), enterovirus D was able to replicate not only in lymphocytes and monocytes but also in granulocytes (34), while echoviruses infected dendritic cells (18). Typically, the percentage of immune cells infected with enteroviruses was low. In a study by Wongsa *et al.* (39), monocytes were more sensitive than lymphocytes to EV71 infection, both carrying rather low percentages of infected cells at around 10% and 3%, respectively. Also, the productive infection of B lymphocytes by coxsackievirus involved only 1 to 10% of these cells (26).

Regrettably, we were unable in our study to determine which populations of bovine PBMCs were sensitive to EV-E, or what percentage of cells was infected with the virus. The rather low titres of the virus may indicate the limited susceptibility of the cows' peripheral blood cells to EV-E infection. We also determined that most of the analysed cells contained a small number of copies of the viral RNA. This finding can be attributed to the late time of determination (24 h after infection). Plekhova *et al.* (30) demonstrated that the release of newly synthesised virions of enteroviruses from macrophages takes place as early as 4 h after infection, and the number of cells containing the virus decreases substantially with time. In the study by these researchers, the number of cells detected to contain the virus decreased by 60% after 24 h, and then after 72 h post infection the virus was undetectable in the cells.

In the subsequent stage of our research, we verified that the infection of bovine cells with the highly infectious dose of enterovirus E considerably decreased the cells' viability. However, we did not observe statistically significant differences in the lymphocyte distribution relative to the uninfected cells, which may indicate similar sensitivity of different populations of these cells to EV-E infection. The literature data on the impact of enteroviruses on the viability of immune cells studied *in vitro* are divergent and what they indicate depends on the tested virus or even its serotype. Coxsackievirus B3 did not decrease the viability of human monocytes considerably, although it is capable of productively infecting them (12). In turn, Smura *et al.* (34) found that the EV-94 serotype of enterovirus D significantly decreased the viability of monocytes, granulocytes and B and T cells, while the EV-68 serotype did not affect the viability of cells despite generating viral progeny. It was also confirmed that enterovirus 71 in a high dose stimulated the apoptosis of T cells, which may represent a mechanism of evasion (7). On the other hand, human dendritic cells infected with echovirus showed signs of both necrosis and apoptosis (18). The elucidation of the mechanism through which high infectious doses of EV-E affect the viability of bovine PBMCs requires further research.

Infection of bovine PBMCs with enterovirus E had an effect on their blastogenic response to mitogens. A considerable decrease in the proliferation of T lymphocytes infected with the highest dose of the virus correlated with and was probably due to the decrease in the viability of these cells. Our cytometric analysis confirmed a decrease in the number of ConA-stimulated cells. However, the only significant change in the percentages of main lymphocyte populations caused by the highest virus dose was a substantial rise in the percentage of the relatively small population of double positive CD4+ CD8+ T cells. In humans, DP T cells are mature effector memory cells engaged in acquired immune response to viral antigens (27). This scenario cannot be excluded in cattle, especially because the cells analysed in this research originated from animals which had had previous contact with EV-E. This previous contact was implicated by the presence of antibodies in their serum, as detected in the first stage of the study. On the other hand, Onah *et al.* (28), who observed induction of DP T cells in sheep infected with *Trypanosoma evansi* after vaccination, attributed this finding to the generalised immunosuppression caused by trypanosomes in hosts since this phenomenon was followed by a substantial decline in all lymphocyte subset sizes. According to these authors, the appearance of DP T cells suggested the induction of the programmed cell death of lymphocytes. In our study, apart from increasing the percentage of DP T cells, the high dose of EV-E also caused a considerable decline in the viability of bovine PBMCs, and therefore lymphocyte apoptosis is also likely in this case. Nevertheless, the clarification of the mechanism of action of a high EV-E dose calls for further, more detailed investigations.

Another interesting finding made in the course of our research was a contrary response of LPS-stimulated cells to the virus which depended on their initial responsiveness. Based on the proliferative response of PBMCs to LPS, cows can be divided into high and low responders. The cells of low responders either do not divide after LPS stimulation or their response is negligible. It has been confirmed that such cows are more susceptible to infections after calving (developing metritis, mastitis or interdigital dermatitis) than high responders (1, 5). Based on our research results, we divided our material into two analogous categories. The low-responding cells did not proliferate after 72 h incubation with LPS, and none of the infectious doses of the virus had a significant effect on their mitogenic response. On the other hand, all the doses of the virus significantly decreased the LPS-induced proliferation of high-responding cells. Immunophenotyping confirmed

a decline in the number of lymphoblasts after the stimulation of infected cells with LPS and a considerable decrease in the percentage of B cells, correlated with a compensatory rise in the percentages of the main T-cell subsets. There are no literature data that could elucidate this phenomenon. However, some publications suggest that the infection of other types of immune cells with enteroviruses can lead to a decline in their responsiveness to bacterial LPS. Henke *et al.* (13) reported from their experiment that the infection of human monocytes with coxsackievirus B3 resulted in the activation of cells, but also made them unresponsive to further activating stimuli, thereby considerably inhibiting the production of cytokines and prostaglandin E_2 in response to LPS. In the case of human monocyte-derived dendritic cells, enterovirus B not only failed to activate cells but also caused their loss of responsiveness to LPS, thus reducing their maturation and decreasing the production of TNF-α and IL-12p70 in comparison with uninfected cells stimulated with LPS (18). The poor responsiveness of B lymphocytes infected with EV-E to LPS confirmed in our study is a worrying finding. The literature data prove that B lymphocytes play an important role in enteroviral infections. Peripheral B-cell deficiency in humans predisposed patients to adverse outcomes of enteroviral infections (10); antibodies playing a critical role in the control of human infections with coxsackieviruses and EV71 (20, 26), an insufficiency of the B cells to stimulate antibody secretio hampers the immune system's response to these pathogens. B lymphocytes are a site of early replication of coxsackieviruses, and participate in their dissemination, which can be of key importance in an infection's course and whether it becomes chronic (15, 17, 26). In turn, infection of mice with the human enterovirus 71 resulted in a reduced number of B cells in the animals' peripheral blood and spleen (20, 41). The amount of EV71 in tissues was particularly high in the case of B-cell–deficient mice, and the survival rate of these animals was considerably lower than that of wild-type mice (20). Likewise, in piglets infected orally with the porcine enterovirus T80, antibodies played a more important role in counteracting the infection, IgA antibodies being particularly prominent, while the specific cellular response was weak, local in character and not connected with the considerable antiviral activity (4).

In the present study, apart from its effect on the viability and blastogenic response of bovine PBMCs, enterovirus E intensified the production of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) by unstimulated cells isolated from high-responder animals. These cytokines are particularly important in the pathogenesis of enteroviral infections in humans, and sometimes it is even suggested that they may be more important than the virulence of the virus (19). Although they initiate the inflammatory and immune responses, which is beneficial for overcoming the infection, their excessive or persistent release can lead to immunopathological processes (12, 37). Patients with severe courses of EV71 infection were observed to produce considerably more IL-1β, IL-6 and TNF-α (6, 19). In *in vitro* studies, EV71 also stimulated the production of TNF-α in human PBMCs (8), and IL-1β, IL-6 and TNF-α in human monocyte-derived macrophages (9). According to the authors of that research paper, the stimulation of TNF-α may be important during the early phases of enteroviral infection, and monocytes/macrophages, the main producers of this cytokines, can be both the target and the effector cells in an EV71 infection (9). Coxsackievirus B3 also stimulated the production of IL-1β, IL-6 and TNF-α in human monocytes, the production of the last of these being the most sensitive indicator of the activation of bovine cells, as it also was in the case of EV71 (12).

It is well known that primary viral infections predispose animals to secondary bacterial infections. Among the bovine viruses having had their immunosuppressive effects well described are nearly all those which participate in development of the bovine respiratory disease complex in common with bovine enterovirus, *i.e.* bovine herpesvirus type 1, bovine parainfluenza virus type 3, bovine respiratory syncytial virus and bovine viral diarrhoea virus (29). Bovine enterovirus has always been perceived as a minor viral pathogen of cattle because of its low virulence. However, it is now known that BEV is not as mild as was claimed. As research techniques have advanced over many years, the opinions of researchers have changed about "commensal viruses" which have been detected in healthy subjects and until recently were considered completely harmless. It has been demonstrated that their presence in an organism can be associated with the development of immunosuppression, immunological anergy or tolerance. Their constant presence in the organism can also influence the course of infections caused by bacterial pathogens (trans-kingdom interactions) (38). In light of the results obtained in this study, the potential effect of the bovine enterovirus on the immune system may prove to be far more significant from the clinical viewpoint than its virulence. Nonetheless, confirmation of this hypothesis needs further, more extensive studies.

Our research shows that bovine PBMCs are permissive to enterovirus E and the infection is productive. Despite the relatively small amount of the viral RNA in cells and the low titres of the viral progeny in the supernatant, EV-E affected the viability and functions of bovine immune cells. High infectious doses of the virus decreased the viability of cells and inhibited the proliferation of T lymphocytes. However, the virus was found to have the strongest impact on B cells high responding to LPS, as their proliferation was significantly inhibited by the virus, regardless of the size of its infectious dose. All doses of the virus

also stimulated the production of early pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α). In our opinion, the clinical importance of EV-E, a virus widespread in cattle populations, may be underestimated. There is a risk that this virus can increase the susceptibility of infected cattle to secondary infections, particularly bacterial ones, which to a large extent are otherwise controlled through the antibody response. The interactions between the virus and the immune cells observed in our experiment may also implicate a potential evasion mechanism of the virus. However, continued and more broadly based studies are needed to verify these hypotheses.

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