Cytokine Production by Porcine Mononuclear Leukocytes Stimulated by Mitogens

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Abstract

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The aim of the study was to contribute to the understanding of the postnatal development of the immune system in domestic pigs by studying *in vitro* cytokine production by mononuclear leukocytes stimulated with pokeweed mitogen (PWM) and concanavalin A (ConA). The production of interleukin 1β (IL-1β), interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α) was evaluated. The mitogenic effect was studied in cultures of two different cell concentrations (1×10^5/ml and 2×10^5/ml) and assessed by the ELISpot method. The stimulatory effect was detected in IL-1β-secreting cells after exposure to PWM (stimulation index, 6.15 and 5.42, for 1×10^5/ml and 2×10^5/ml, respectively) and to ConA (stimulation index, 2.12 and 2.63, for 1×10^5/ml and 2×10^5/ml, respectively). IFN-γ-secreting cells at the concentration of 1×10^5 cells/ml responded to PWM to a low degree (stimulation index, 1.9). ConA alone or combined with PWM had no stimulatory effect on either IFN-γ- or TNF-α-secreting cells, as compared with the controls. It can be concluded that the porcine immune system at 60 days postnatally is mature enough to be able to selectively control the response of cytokine producing cells to mitogenic stimulation.

Cytokine, ELISpot, mitogen, piglet

Cytokines are soluble proteins produced and released by individual cells for the purpose of transmitting distinct messages of activation, inhibition, chemoattraction, apoptosis, etc. They form a heterogeneous group involving hundreds of mediators of low molecular weight (5-70 kDa) which influence physiological functions, particularly the immune responses of the organism (Murtaugh 1994). The most frequently studied cytokines include IL-1β, IFN-γ and TNF-α, which are produced by a whole range of immune cells.

Cytokines play an important role in the specific immunity. In pigs, that are similarly to human infants, born with an immature immune system, a good coordination of immune responses and reactions to stress, in which cytokines are involved, is necessary.

For *in vitro* studies of mechanisms involved in immune responses, the ability of mitogens to stimulate the production of cytokines is of great importance. Mitogens, also called polyclonal lymphocyte activators, are able to induce mitotic division in cells. Their reaction is non-specific, which means that they can influence various lymphocyte subpopulations, and not only those involved in the expression of complementary receptors. The most frequently used mitogens are concanavalin A (ConA), pokeweed mitogen (PWM) and phytohemagglutinin (PHA) (Wimer 1996). The primary target of their action is the plasma membrane, where they bind to the saccharide domains of membrane glycoproteins. ConA binds to α-D-manosyle, PWM and PHA to N-acetyl-D-glucosamine. The binding of mitogen molecules makes membrane receptors reticulate and, by means of adenylate cyclase (followed by the synthesis of cyclic adenosine monophosphate) or guanylate cyclase (followed by the synthesis of cyclic guanosine monophosphate), the signal is transferred from the membrane to the nucleus of lymphocytes (Wimer and Mann 2002).

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The aim of the study was *in vitro* cytokine production by mononuclear leukocytes stimulated by ConA and PWM with the aim to contribute to the understanding of the postnatal development of the immune system in domestic pigs (*Sus scrofa*).

Materials and Methods

Cells

Mononuclear leukocytes were used for the experiment. They were isolated from blood samples taken from conventionally bred piglets (n = 7) aged 60 days.

Blood samples containing 20 U of heparin per ml of blood (Léčiva) were diluted 1:1 in X-vivo 10 tissue medium (BioWittacker). Mononuclear leukocytes were separated on discontinuous gradient of Histopaque 1077 (Sigma-Aldrich) (Tlaskalová et al. 1985).

Mitogens

The following mitogens (Sigma-Aldrich, Germany) were used: concanavalin A (ConA) isolated from the seeds of *Canavalia ensiformis* and pokeweed mitogen (PWM) obtained from pokeweed rootstocks (*Phytolaca americana*).

Cultivation and stimulation of mononuclear leukocytes

Separated mononuclear leukocytes were transferred to X-vivo 10 tissue medium, washed and diluted in the same medium to give 10^6 cells per ml, the starting concentration for production of cytokines.

The cells were stimulated by either 10 μg/ml ConA or 2 μg/ml PWM or a combination of 10 μg/ml ConA and 2 μg/ml PWM. Cells cultivated in the medium only were used as controls. After incubation (3 days at 37 °C and 5% CO_2), the mononuclear leukocytes were centrifuged at 150 *g* and distributed in 96-well plates.

Enzyme-Linked ImmunoSpot assay (ELISpot)

For each cytokine, a polyvinylidene difluoride (PVDF)-backed microplate pre-coated with a monoclonal antibody specific for this cytokine, was used. Stimulated cells at two concentrations (1×10^5 and 2×10^5 cells/ml) were pipetted into the wells and the microplates were incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 3 days. Subsequently, after washing away unbound substances, the following agents were added to each well: cytokine-specific biotinylated polyclonal antibody, streptavidin conjugated to alkaline phosphatase and substrate solution (BCIP/NBT) for visual detection of the reaction. A blue-black precipitate at the site of cytokine localization appeared as a spot, each representing a cytokine-secreting cell. The spots were counted with an automated ELISpot reader system (AID, Germany). The values obtained in wells with control, non-stimulated cells were used as standards.

For numerical evaluation of the stimulatory effect of each mitogen, a stimulation index was calculated by dividing the stimulated cell count by the control (non-stimulated) cell count. The results are shown in Table 1.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test and the results were expressed as mean ± SE values. Significant differences were defined at $p \leq 0.01$.

All experiments were approved by the Ethics Committee of the Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences in Brno.

Results and Discussion

ConA and PWM are potent mitogens most frequently used in studies of leukocyte proliferation and differentiation (Symons et al. 1977; Becker and Misfeldt 1993; Dvořák et al. 1993). Because their effects are non-specific, it had not been possible to evaluate the response of individual porcine cells that produce cytokines until recently, when the ELISpot kit for porcine mononuclear leukocytes became available. In piglets, the ability of and the potential for cytokine production may serve as criteria by which the development of their immune system can be assessed; this is known to be at a low level of maturity in the early postnatal period (Wilson 1974). IL-1β, IFN-γ a TNF-α, studied in this paper, are cytokines secreted immediately in response to immune system activation, without need for any mediators.

In IL-1β-secreting cells, the highest cell proliferation, as compared with the control ($p \leq 0.01$), was found after stimulation with PWM. ConA had a lower stimulatory effect and the combination of mitogens (ConA + PWM) did not stimulate cell proliferation at all, because the cell counts were lower than the control value (Fig. 1).

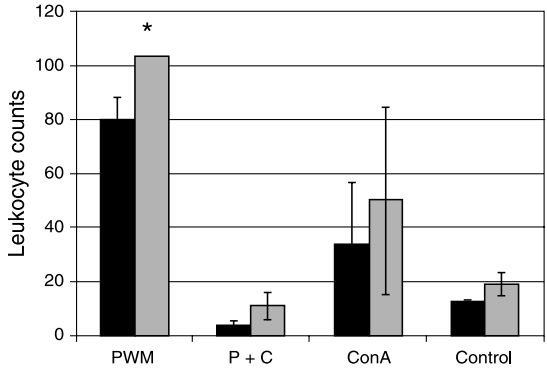

Fig. 1. Effects of mitogens on the cells producing interleukin 1β. PWM, pokeweed mitogen; P + C, PWM + ConA; ConA, concanavalin A; dark area ■, concentration of 1×10^5 cells/ml; shaded area ■, concentration of 2×10^5 cells/ml.

In IFN-γ–secreting cells, the response to mitogens differed in relation to cell concentration, with the stimulatory effect being shown only by PWM in the 1×10^5 cells/ml culture (Fig. 2).

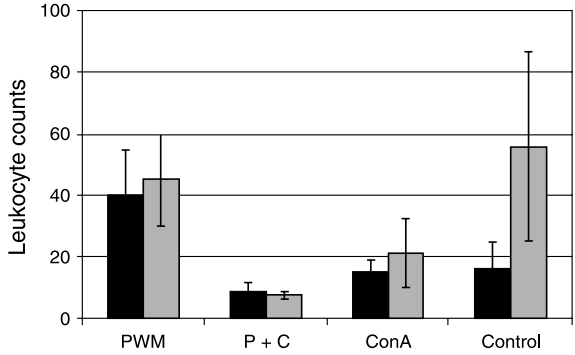

Fig. 2. Effects of mitogens on the cells producing interferon γ. PWM, pokeweed mitogen; P + C, PWM + ConA; ConA, concanavalin A; dark area ■, concentration of 1×10^5 cells/ml; shaded area ■, concentration of 2×10^5 cells/ml.

In TNF-α–secreting cells, no stimulatory effect of mitogens was detected. In comparison with the controls, cell proliferation was markedly lower after exposure to both PWM and ConA, and significantly low after exposure to their combined effect ($p \leq 0.01$) (Fig. 3).

In agreement with other authors, PWM proved to be the strongest mitogenic stimulator (Naidoo and Derbyshire 1992; Schwager and Schulze 1998; Chen et al. 2004). Compared with that, ConA had a much lower effect, and exposure of cells to a combined effect of both mitogens showed the lowest response. In terms of cell proliferation, the response to PWM can be regarded as stimulatory only in IL-1β-producing cells (stimulation index, 6.15 and 5.42, for 1×10^5/ml and 2×10^5/ml, respectively) and in IFN-γ-producing cells at the concentration of 1×10^5/ml (stimulation index, 1.9). In the other stimulated cultures the cell counts were lower than those in the controls (Table 1). The differences in the response to mitogens among the cytokine-producing cells became even more pronounced when they were expressed by the stimulation indices (Krátká et al. 2002).

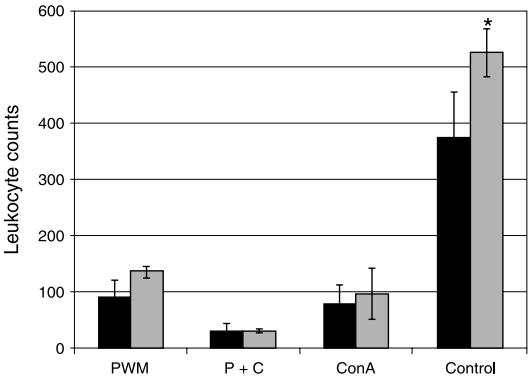

Fig. 3. Effects of mitogens on the cells producing tumor necrosis factor α. PWM, pokeweed mitogen; P + C, PWM + ConA; ConA, concanavalin A; dark area ■, concentration of 1×10^5 cells/ml; shaded area ■, concentration of 2×10^5 cells/ml.

Table 1. The mitogen effect expressed by means of the stimulation index

Cytokine-producing cells	Cell concentration	Stimulation index for PWM	Stimulation index for P + C	Stimulation index for ConA
IL-1β cells	1×10^5 /ml	6.15	0.31	2.12
IL-1β cells	2×10^5 /ml	5.42	0.58	2.63
IFN-γ cells	1×10^5 /ml	1.9	0.05	0.71
IFN-γ cells	2×10^5 /ml	0.8	0.14	0.38
TNF-α cells	1×10^5 /ml	0.24	0.08	0.21
TNF-α cells	2×10^5 /ml	0.26	0.6	0.18

PWM, pokeweed mitogen; P + C, PWM + ConA; ConA, concanavalin A; IL-1β, interleukin 1β; IFN-γ, interferon γ; TNF-α, tumor necrosis factor α.

It is known that PWM has two specific domains available for binding to membrane receptors (Fujii et al. 2004), and therefore to have a higher potential for its action. This may explain the fact that the mitogenic effect of PWM on porcine mononuclear leukocytes was higher than that of ConA. In terms of cell proliferation, the response to ConA can be regarded as stimulatory only in IL-1β-producing cells (stimulation index, 2.12 and 2.63, for 1×10^5/ml and 2×10^5/ml, respectively). The combined effect of PWM and ConA can be regarded as inhibitory, since the cell counts in all stimulated cultures were much lower than those in the controls, regardless of whether the cells produced IL-1β, IFN-γ or TNF-α. It can be accounted for by a competition of the two mitogens for the binding sites available.

Such marked differences in the reactions of cytokine-producing cells, as observed in this study, were not expected in 60-day-old piglets. Our results thus imply that the porcine immune system at that age is mature enough to produce an adequate response to mitogenic stimuli and that its non-responsiveness to mitogens under certain conditions can be understood as a defense mechanism.

With the ELISpot method it was not possible to distinguish among the responses of individual leukocyte subpopulations to mitogenic stimulation. It is suggested that the flow cytometric analysis of cluster-of-differentiation markers, which are specific for each subpopulation, may be the tool for further research into these cellular mechanisms.

Produkce cytokinů mononukleárními leukocyty stimulovanými mitogeny u prasete domácího

Cílem práce bylo přispět k pochopení postnatálního vývoje imunitního systému prasete domácího. Studována byla *in vitro* produkce cytokinů mononukleárními leukocyty stimulovanými pokeweed mitogenem (PWM) a konkanavalinem A (ConA). Hodnocena byla produkce interleukinu 1β (IL-1β), interferonu γ (IFN-γ) a faktoru nekrotizujícího tumory α (TNF-α). Účinek mitogenů byl stanovován u dvou různých koncentrací buněk (1×10^5/ml a 2×10^5/ml) metodou ELISpot. Stimulační účinek byl zjištěn u buněk secernujících IL-1β po expozici PWM (stimulační index 6.15 respektive 5.42 pro 1×10^5/ml respektive 2×10^5/ml,) a ConA (stimulační index 2.12 respektive 2.63 pro 1×10^5/ml respektive 2×10^5/ml). Méně výrazný stimulační efekt PWM byl prokázán u buněk secernujících IFN-γ při koncentraci 1×10^5/ml (stimulační index 1.9). ConA samotný, ani v kombinaci s PWM, neměl ve srovnání s kontrolami u buněk secernujících IFN-γ i TNF-α žádný stimulační účinek. Tuto skutečnost lze vysvětlit tím, že imunitní systém 60 dní starých selat je již vyzrálý natolik, aby byl schopen selektivně kontrolovat odpověď cytokiny secernujících buněk na mitogenní stimulaci.

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References

BECKER BA, MISFELDT ML 1993: Evaluation of the mitogen-induced proliferation and cell surface differentiation antigens of lymphocytes from pigs 1 to 30 days of age. J Anim Sci **71**: 2073-2078

DVOŘÁK P, HRUBAN V, STANĚK R, HAMPL A 1993: Characterization of a new porcine differentiation antigen involved in the proliferative response to mitogens. Vet Immunol Immunopathol **35**: 365-373

FUJII T, HAYASHIDA M, HAMASU M, ISHIGURO M, HATA Y 2004: Structures of two lectins from the roots of pokeweed (*Phytolacca americana*). Acta Crystallogr D Biol Crystallogr **60**: 665-673

CHEN XD, LEI T, XIA T, GAN L, YANG ZQ 2004: Increased expression of resistin and tumour necrosis factor-alpha in pig adipose tissue as well as effect of feeding treatment on resistin and cAMP pathway. Diabetes Obes Metab **6**: 271-279

KRÁTKÁ Z, BÁRTOVÁ J, KRYŠTŮFKOVÁ O, BENETKOVÁ K, MRKLAS L, FUČÍKOVÁ T 2002: Effect of intravenous immunoglobulins on in vitro immunoglobulin formation in patients with antibody immunodeficiency. APMIS **110**: 205-213

MURTAUGH M 1994: Porcine cytokines. Vet Immunol Immunopathol **43**: 37-44

NAIDOO D, DERBYSHIRE JB 1992: Interferon induction in porcine leukocytes with transmissible gastroenteritis virus. Vet Microbiol **30**: 317-327

SCHWAGER, J, SCHULZE, J 1998: Modulation of interleukin production by ascorbic acid. Vet Immunol Immunopathol **64**: 45-57

SYMONS DB, LAY CA, MACDONALD AN 1977: Stimulation of pig lymphocytes with anti-immunoglobulin serum and mitogens. Int Arch Allergy Appl Immunol **54**: 67-77

TLASKALOVÁ H-HOGENOVÁ, BÁRTOVÁ J, MRKLAS L, HANIKÝŘOVÁ G 1985: Stimulation of human blood lymphocyte by different polyclonal B-cell activators of bacterial and plant origin: production of IgM, IgG and IgA estimated by ELISA METHOD. Folia Microbiol **30**: 258-266

WILSON MR 1974: Immunologic development of the neonatal pig. J Anim Sci **38**: 1018-1021

WIMER BM 1996: Putative effects of mitogenic lectin therapy corroborated by alloactivation data. Cancer Biother Radiopharm **11**: 57-75

WIMER BM, MANN PL 2002: Mitogen information summaries. Cancer Biother Radiopharm **17**: 569-597