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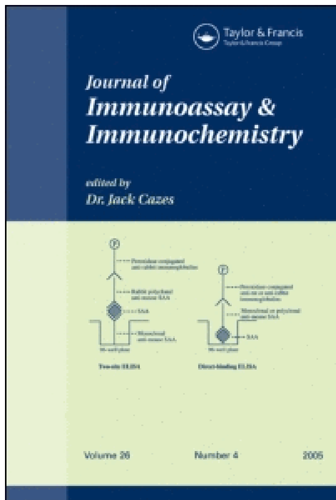

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
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Development and Application of a Pig IL-8 ELISA Detection System

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Online publication date: 05 December 2003

To cite this Article Splichal, I. , Muneta, Y. , Mori, Y. and Takahashi, E.(2003) 'Development and Application of a Pig IL-8 ELISA Detection System', Journal of Immunoassay and Immunochemistry, 24: 2, 219 — 232
To link to this Article: DOI: 10.1081/IAS-120020086
URL: http://dx.doi.org/10.1081/IAS-120020086

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JOURNAL OF IMMUNOASSAY & IMMUNOCHEMISTRY
Vol. 24, No. 2, pp. 219–232, 2003

Development and Application of a Pig IL-8 ELISA Detection System

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ABSTRACT

Interleukin 8 (IL-8) is a chemotactic and activating chemokine, especially for neutrophils, which plays an important role in inflammatory process. A pig IL-8 specific enzyme-linked immunosorbent assay (ELISA) was developed to measure IL-8 concentrations in cell culture supernatants and biological fluids. A streptavidin-biotin

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DOI: 10.1081/IAS-120020086 1532-1819 (Print); 1532-4230 (Online)
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amplified sandwich method uses mouse capture mAb IZ8.03 and detection biotinylated mouse mAb IZ8.04 against recombinant pig IL-8. The assay specifically and reproducibly recognizes both recombinant and natural pig IL-8. A working range of the assay is 16–1000 pg/mL and takes a mere 3.5 h of incubation time. This pig IL-8 ELISA is a suitable alternative way of measurement of IL-8 concentrations to time consuming and laborious IL-8 bioassays.

Key Words: ELISA; Interleukin 8; Chemokine; Monoclonal antibody; Pig.

INTRODUCTION

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Locally produced cytokines are presumed to mediate the sequence of events leading to the infiltration at inflammatory sites where they maintain inflammation and favor organ dysfunction, which may lead to lethality. Interleukin 8 (IL-8), a CXC chemokine produced by various types of cells including monocytes/macrophages, eosinophils, T-cells, NK cells, fibroblasts, endothelial cells, keratinocytes, hepatocytes, astrocytes, chondrocytes, and neutrophils upon stimulation with proinflammatory stimuli, exerts a variety of functions particularly on leukocytes.[1,2] The IL-8 attracts polymorphonuclear neutrophils (PMN), basophils, and T-cells toward tissue[3] and anti-IL-8 treatment prevents neutrophil-dependent tissue damage, as well as neutrophil infiltration in these conditions.[4] The IL-8 levels in different body fluids very often correlate with the severity of the diseases, as found in bronchoalveolar lavage of patients with acute respiratory distress syndrome,[5] in plasma of patients with multiple organ failure,[6,7] and in amniotic fluid during intraamniotic infections.[8,9]

The IL-8 molecule was originally reported as 10 kDa protein but later precise determinations have demonstrated that natural IL-8 occurs as a 6–8 kDa doublet.[10] The mature protein is nonglycosylated, contains 72–77 amino acids and its sequence analysis shows that it occurs in multiple forms that differ in truncation at the NH_2 terminus which depends on cell source, probably due to the presence of different specific proteases.[2] In the pig, IL-8 was identified as the alveolar macrophage-derived neutrophil chemotactic factor I,[11] and its biological effect described.[12]

We developed the pig IL-8 ELISA sandwich detecting system to make possible the measurement of pig IL-8 levels in different experimental in vivo and in vitro states. This ELISA sandwich system is based on mouse capture mAb IZ8.03 and biotinylated detection mAb IZ8.04 against recombinant pig IL-8. It is fast, reliable, and suitable method

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for the detection of pig IL-8 in cell culture supernatants and different body fluids, the lower limit of sensitivity is 16 pg/mL.

EXPERIMENTAL

Recombinant Pig IL-8 (rpIL-8)

Recombinant pig IL-8 (rpIL-8) was produced in the form of the 6x histidine affinity tagged protein by using the QIAexpressionist^{TM} *Escherichia coli* system (Qiagen, Washington, USA). A first strand cDNA was synthesized by the RNA PCR kit (Takara Shuzou, Osaka, Japan) from the total RNA which was extracted from pig alveolar macrophages stimulated with LPS for 16 h. The coding region for the mature pIL-8 containing ELR sequence was amplified by PCR with primers that were designed from the mRNA sequence listed on the Database (M86923, GenBank). Then, *Nco* I and *Bgl* II restriction enzyme sites were added to the 5′ and 3′ ends of the PCR product, respectively, and ligated into the *Nco* I–*Bgl* II site of the *p*QE-60 vector (Qiagen) using the Ready-To-Go T4 DNA Ligase ligation kit (Pharmacia, Uppsala, Sweden). The resulting plasmid, *p*QEpIL-8, was used for expression of the 6x His-tagged rpIL-8 in *E. coli*. Recombinant plasmid was introduced into *E. coli* JM109 by transformation. The cells induced by IPTG were harvested, resuspended in Tris–HCl buffer (pH 7.5) and sonicated for 15–20 min on ice. The cell suspension was clarified by centrifugation at 15,000 rpm for 20 min, and supernatant was loaded onto Ni^{2+} HiTrap Chelating adsorbent column (Pharmacia). After washing the column, the rpIL-8 was eluted with Tris–HCl buffer containing 500 mM imidazole. The fractions containing rpIL-8 were dialyzed against distilled water.

Monoclonal Antibodies to rpIL-8

BALB/c mice were immunized by 10 µg of recombinant pig IL-8 (rpIL-8) in complete Freund's adjuvant (Wako Pure Chemical Industries, Osaka, Japan) i.p. and s.c. They were two times repeatedly immunized by 5 µg of rpIL-8 in incomplete Freund's adjuvant (Wako Pure Chemical Industries) at three-week intervals and i.p. boosted with 20 µg of rpIL-8 in PBS three days before fusion. Splenocytes were fused with P3U1 mouse myeloma cells using polyethylene glycol. Hybridoma supernatants were tested by ELISA and selected on reactivity to rpIL-8 relative to the reactivity to the recombinant *Mycoplasma hyopneumoniae*

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protein[13] produced by the same expression system. The mAbs against rpIL-8 producing hybridomas were two times subcloned by the limiting dilution method. Isotypes of mAb were estimated in cell culture supernatants using a monoclonal antibody isotyping ELISA kit (Zymed Laboratories Inc., San Francisco, CA).

The mAb were precipitated from cell culture supernatants by 45% $(NH_4)_2SO_4$, dialyzed in PBS and purified on 1 mL Hi-Trap protein A column (Pharmacia) according to manufacturer's instructions. After elution from the column, part of the mAb was dialyzed against PBS or 0.1 M $NaHCO_3$ buffer pH 8.4 in cases of coupling with biotin (Sigma, St. Louis, MO) performed according to manufacturer instructions. All antibodies were stored in PBS with 50% glycerol at −20°C.

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Capture ELISA Method

The 96-Well ELISA MaxiSorp microplate (Nunc, Roskilde, Denmark) was filled by 100 μL of 0.05 M Na_2CO_3 buffer pH 9.6 containing 4 μg/mL of IZ8.03 mAb and incubated overnight at 4°C. After four-fold washing by a washing solution—WS (0.15 M NaCl, 0.05% Tween-20), the wells were filled with rpIL-8 or samples diluted in a diluting solution 1—DS1 (0.01 M Tris–HCl, pH 7.2, 0.15 M NaCl, 0.5% skim milk, 0.05% Tween-20) and incubated for 2 h at room temperature (RT). The wells washed five times with WS were filled with 100 μL of 0.6 μg/mL biotinylated IZ8.04 mAb in diluting solution 2—DS2 (0.01 M Tris–HCl, pH 7.2, 0.15 M NaCl, 0.1% skim milk, 0.1% Tween-20) and incubated for 1 h at RT. After five-fold washing by WS, 1/5000 diluted streptavidin/peroxidase ELISA grade conjugate (Biosource International Inc., Camarillo, CA) in DS2 was added and incubated for 30 min at RT. After a five-fold washing a color reaction was developed for 30 min in a dark room using 100 μL of TMB substrate per well.[14] The reaction was stopped by addition of 100 μL of 2 M H_2SO_4 and absorbance was read at 450 and 620 nm in RC Multiskan ELISA reader (Thermo Labsystems, Helsinki, Finland). The absorbance difference was evaluated and a standard curve counted by Genesis Lite software (Thermo Labsystems).

Cross Reactivity

The cross reactivity was tested against pig recombinant IL-1β, IL-10, and IFN-γ (Biosource International Inc., Camarillo, CA), IL-18[15] and

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TNF-α (Endogen, Woburn, MA). All cytokines in comparative studies were at concentration of 1000 pg/mL.

Influence of Body Fluids on IL-8 Estimation

(a) Seven blood serum samples from adult pigs, (b) six citrated blood plasma samples from adult pigs, and (c) seven amniotic fluid samples representing different periods of gestation were used to determine a possible interference of body fluids with pIL-8 estimation. All samples were taken from clinically healthy pigs.

Natural Pig IL-8 Containing Cell Culture Supernatant and Body Fluids

Pig peripheral blood mononuclear cells (PBMC) of three 7-week-old piglets were separated from citrated blood on Ficoll Histopaque 1.077 (Sigma) density gradient for 20 min at 1500 *g* and RT. The cells were washed three times in Dulbecco PBS (DPBS), resuspended in RPMI 1640 with 25 mM HEPES supplemented by 5% FCS, 100 μg/mL of penicillin, 100 U/mL of streptomycin and 2 mM pyruvate (all PAA, Linz, Austria). A 2×10^6/mL cells were nonstimulated, stimulated with 5 μg/mL of Con-A (Pharmacia) or stimulated with 10 μg/mL of LPS from *E. coli* O55 (Sigma), and incubated in 5% CO_2 atmosphere at 37°C. Supernatants were harvested after 2, 5, and 20 h of cultivation.

Samples of citrated blood plasma and amniotic fluids of pig fetuses infected experimentally in utero with *E. coli* O86 (2×10^2–10^6 CFU in 3 mL of Hank's phosphate buffered saline—/HPBS/ injected in an amniotic cavity) were used 10 h after infection as body fluids containing natural IL-8. The same body fluids from sham-infected fetuses (HPBS only) were used as controls.

Experiments with animals were approved by the Ethical Committee of the Institute according to the rules of the Animal Protection Act.

RESULTS

More than 2000 hybridoma culture supernatants from three independent fusions were tested. Hybridomas producing anti-rpIL-8 mAb, but

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not against recombinant *M. hyopneumonieae* protein were selected. Four stable hybridomas (IZ8.01–IZ8.04) producing anti rpIL-8 mAb were obtained. All four mAb were determined as IgG_1 isotype, mAb IZ8.01, IZ8.03, and IZ8.04 having κ light chain and IZ8.02 λ light chain, as determined by an ELISA mAb isotyping kit.

Pig IL-8 ELISA

Twelve possible combinations of capture and biotinylated (bi) detection antibodies in different dilutions were tested for their ability to create a sandwich pIL-8 ELISA detection system. Six combinations with sensitivity limit approximately 1 ng/mL of rpIL-8 were found. The most suitable pIL-8 ELISA sandwich combination was IZ8.03 as the capture and IZ8.04 as the biotinylated detection antibody. This combination compromises between high sensitivity for both recombinant and natural pig IL-8 and a low interference of pig serum, plasma or amniotic fluid. Several buffer compositions and blocking reagents such as PBS, TBS (0.15 M NaCl, 0.05 M Tris, pH 7.2) containing 0.15 or 0.3 M NaCl, 0.5–5% BSA, 0.1–2% skim milk (SM), 1–5% FCS, all with or without addition of Tween-20, were compared. TBS containing 0.5% SM and 0.05% Tween-20 for standard (DS1) and TBS containing 0.1% SM and 0.1% Tween-20 (DS2) for biotinylated capture mAb IZ8.04bi and streptavidin/peroxidase conjugate were chosen as the most suitable diluting solutions. A commonly used separate blocking step before an addition of samples was omitted since it was found to exert no influence on the quality of the detection system.

All values of the standard curve (Fig. 1) were performed in quadriplicate in six independent experiments. The limit of the pig IL-8 detection system was established to be lower than 10 pg/mL using calculation of the subtraction of OD450-OD620 mean value > OD450-OD620 for 0 pg/mL plus three standard deviations at 0 pg/mL. The suitable range for common use was estimated at 16–1000 pg/mL.

Cross Reactivity

No significantly increased values of OD450-OD620 in comparison with the IL-8 zero value of ELISA test were found for 1000 pg/mL for rpIL-1β, rpIL-10, rpIL-18, rpIFN-γ, and rpTNF-α of the above-mentioned origins (Table 1).

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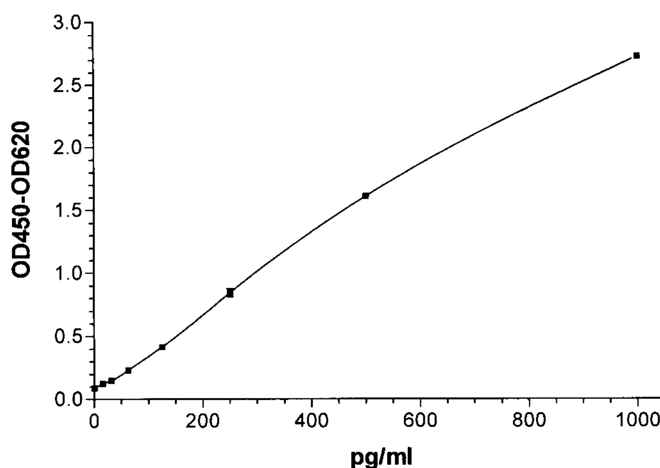

Figure 1. Standard curve of pig IL-8 ELISA (a working range of the assay is 16–1000 pg/mL).

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Table 1. Cross reactivity of the assay to selected pig cytokines at 1000 pg/mL level.

Recombinant pig cytokine	Concentration (pg/mL)	OD450-OD620 Mean ± SD	Source of cytokine
IL-8	1000	2.736 ± 0.108	Prepared in laboratory
IL-1β	1000	0.056 ± 0.004	BioSource International
IL-10	1000	0.052 ± 0.010	BioSource International
IL-18	1000	0.054 ± 0.006	(15)
IFN-γ	1000	0.060 ± 0.004	BioSource International
TNF-α	1000	0.066 ± 0.011	Endogen
Diluting solution	0	0.057 ± 0.006	Prepared in laboratory

Influence of Body Fluids on IL-8 Estimation

A possible interference of body fluids with IL-8 estimation was tested by the recovery of rpIL-8 in the presence of blood serum, blood plasma or amniotic fluid (Table 2). A higher content (50%) of serum or plasma reduced the values of estimated rpIL-8 by approximately 10 or 20%, respectively. Lower tested contents of serum or plasma did not

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Table 2. Influence of body fluids on IL-8 detection.

Body fluid %	Serum ($n=7$)			Plasma ($n=6$)			Amniotic fluid ($n=7$)		
	Mean (pg/mL)	SD (pg/mL)	Recovery %	Mean (pg/mL)	SD (pg/mL)	Recovery %	Mean (pg/mL)	SD (pg/mL)	Recovery %
50.0	180.1	32.4	89.4	159.8	17.8	79.5	201.5	10.3	98.1
25.0	200.4	9.9	99.5	201.1	16.6	100.0	199.8	4.2	97.3
12.5	193.3	15.9	96.0	194.4	15.6	96.7	210.2	11.7	102.4
6.3	198.2	8.7	98.4	197.3	12.3	98.1	206.6	8.4	100.6
0.0	201.4	—	100.0	201.1	—	100.0	205.3	—	100.0

significantly influence the estimation of IL-8. Amniotic fluid did not influence the detection of IL-8 at all.

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Detection of IL-8 in Cell Culture Supernatants

IL-8 was found in pig PBMC cell culture (2×10^6/mL) derived from three 7-week-old piglets stimulated by 5 μg/mL Con-A after 20 h of stimulation and in cell culture supernatants of PBMC stimulated by 10 μg/mL LPS in all observed periods (2, 5, and 20 h) in a time dependent manner. By contrast, no IL-8 was detected in non-stimulated controls (Fig. 2).

Detection of IL-8 in Body Fluids

IL-8 was detected in samples of blood plasma and amniotic fluid of *E. coli* O86 in utero infected fetuses ($n=4$) but not in cases of their sham-infected counterparts ($n=4$) (Fig. 3).

DISCUSSION AND CONCLUSION

Bacteria produce many molecules having profound effects on the capacity of leukocytes and tissue cells to trigger the production of inflammatory mediators. The diagnosis of inflammatory processes is an important goal in medicine and experimental work. IL-8, a member of the CXC chemokine family, plays a key role in host defense mechanism through its effects on neutrophil attraction and activation, and has been implicated in a variety of inflammatory diseases such as nephropathy,[16] gastritis,[17]

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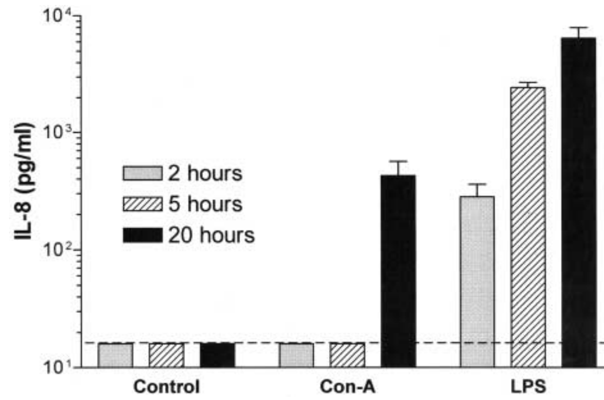

Figure 2. Detection of pig IL-8 in the cell culture of PBMC (2×10^6/mL) of 7-week-old piglets ($n = 3$) stimulated in vitro by Con-A (5 μg/mL) or LPS 10 μg/mL) vs. nonstimulated controls. Absence of detectable IL-8 is depicted by values below the limit of sensitivity of the assay (dashed line).

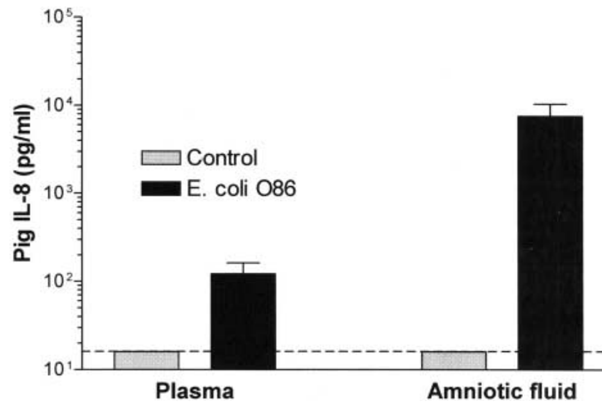

Figure 3. Detection of IL-8 in citrated blood plasma and amniotic fluid of *E. coli* O86 infected fetuses ($n = 4$) and noninfected control fetuses ($n = 4$).

chronic ileal lesions,[18] and in sepsis.[19] IL-8 is able to stimulate many PMN activities, including oxidative burst, exocytosis of specific granules and release of proteases, and to enhance the expression of integrin on the surface of PMN.[20] The release of IL-8 is triggered by inflammatory signals in a stimulus-specific manner by a wide variety of cell types and is regulated primarily at the level of gene transcription.

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Measurements of IL-8 in biological samples by bioassays, based on chemotaxis,[21] release of intracellular enzymes,[22] production of reactive oxygen metabolites,[23] are time-consuming, relatively complicated, and often semi-quantitative.[24] They can often lack the specificity due to the presence of chemoattractants such as C5a, leukotriene B4 and platelet activating factor in many samples.[25]

The measurement of IL-8 in biological fluids by ELISA can also interfere with the presence of elusive substances or the presence of circulating autoantibodies to IL-8.[26,27] Standardization of ELISA techniques for cytokine measurement in body fluids is therefore inevitable.[28] The major goal of our work was to develop a sensitive and specific system to detect IL-8 in pig body fluids obtained in various infection events.

Some highly sensitive combinations of anti-IL-8 mAbs were strongly influenced by the presence of blood serum or plasma. The developed ELISA method using the capture IZ8.03 mAb and biotinylated detection IZ8.04 mAb is sensitive and specific for the measurement of the pig IL-8 in body fluids and cell culture supernatants. The reliability of the method was confirmed by measurements of rpIL-8 in the presence of pig serum, citrated plasma, and amniotic fluid. Amniotic fluid in 50% and lower concentrations did not interfere with the estimation of rpIL-8 at tested concentrations, i.e., at approximately 200 pg/mL. The presence of 50% blood serum or plasma resulted in a reduced recovery of rpIL-8 but this interference was possible to efficiently reduce by diluting of serum/plasma sample by DS1.

In vivo, amniotic IL-8 is a possible marker of intraamniotic infection.[8] The positive correlation between maternal and cord serum IL-8 levels was found to be useful for rapid prenatal screening of chorioamnionitis at term.[29] We detected IL-8 appearance in pig amniotic fluids and fetal plasma 10 h after experimental intraamniotic infection with *E. coli* O86, in contrast to the negative findings in sham-operated counterparts.

In vitro, IL-8 was at first detected 20 h after stimulation of pig PBMC with Con-A. The lack of IL-8 in earlier observed periods is in accordance with the findings of Yancy et al.[30] who detected IL-8 protein not earlier than 12 h after stimulation with Con-A. On the other hand, IL-8 was detected as early as 2 h after stimulation with bacterial lipopolysaccharide. This quick response showed a great importance of this chemokine in bacterial infection.

The pig is an important domestic animal, its importance as biological model has increased during the last decade with the possibility of using it as a graft donor for a xenotransplantations in humans.

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We have developed a sensitive and specific pig IL-8 ELISA to facilitate the detection of infection states in the pig.

ABBREVIATIONS

BSA	Bovine serum albumin
Con-A	Concanavalin A
DS1	Diluting solution No. 1
DS2	Diluting solution No. 2
FCS	Fetal calf serum
ELISA	Enzyme linked immunosorbent assay
i.p.	Intraperitoneally
IPTG	Isopropylthiogalactopyranoside
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
NKcells	Natural killer cells
NPS	Normal pig serum
PBMC	Peripheral blood mononuclear cells
PBS	Phospate buffered saline
PMN	Polymorphonuclear neutrophils
P3U1	Mouse myeloma cell line
rpIFN-γ	Recombinant pig interferon gamma
rpIL-8	Recombinant pig interleukin-8
rpIL-18	Recombinant pig interleukin-18
rpTNF-α	Recombinant pig tumor necrosis factor alpha
RT	Room temperature
s.c.	Subcutaneously
SM	Skim milk
TBS	Tris buffered saline
TMB	Tetramethylbenzidine
WS	Washing solution

ACKNOWLEDGMENTS

Igor Splichal's stay at the University of Tokyo, and at the National Institute of Animal Health, Tsukuba, was supported by a Postdoctoral Fellowship of the Japan Society for the Promotion of Science No. P 96336. Samples of pig fetus-derived body fluids were provided from research grant 524/99/0518 supported by the Grant Agency of the Czech Republic.

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Received November 30, 2002
Accepted January 6, 2003
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