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Veterinary Immunology and Immunopathology 110 (2006) 363–367

Veterinary
immunology
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immunopathology

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A monoclonal antibody to equine interleukin-4

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Received 20 November 2005; accepted 6 January 2006

Abstract

Interleukin-4 (IL-4) is secreted by T helper type 2 cells, mast cells, basophils and eosinophils. Detection of IL-4 can contribute the evaluation of cellular immune responses during infectious diseases, immunological disorders or vaccination. We used recombinant equine IL-4 to generate a monoclonal antibody (mAb) to equine IL-4. The mAb detected recombinant IL-4 in mammalian cells transfected with different plasmids containing IL-4 cDNA. After mitogen stimulation of equine peripheral blood mononuclear cells, an intracellular protein was recognized by the new mAb in 1–2% of lymphocytes using flow cytometric analysis. In the presence of the secretion blocker Brefeldin A, the protein accumulated and was detected in 4–8% of lymphocytes stimulated with phorbol 12-myristate 13-acetate and ionomycin. Double staining with the new mAb and T-cell or B-cell markers identified a subpopulation of CD4⁺ T-cells expressing the protein recognized by the mAb. In addition, the protein was detectable in cell culture supernatants of mitogen stimulated cells by ELISA when using the new mAb for coating of the plates and a polyclonal antiserum to equine IL-4 for detection. In conclusion, the new mAb detects equine IL-4 and can be used for intracellular staining and ELISA to measure this important cytokine.

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Keywords: Interleukin-4; Horse; Equine; Monoclonal antibody

IL-4 is derived from Th2 cells (Mosmann and Coffman, 1989) and can also be produced by mast cells, basophils and eosinophils (Gessner et al., 2005). It has multiple immunoregulatory effects on B-cells, T-cells, monocytes, dendritic cells, and non-immune cells including endothelial cells and fibroblasts (Chomarat and Banchereau, 1998; Galli et al., 2005). In humans and mice, IL-4 has been found to

be the major regulatory cytokine inducing immunoglobulin class switching to IgE (Siebenkotten et al., 1992; Stavnezer and Amemiya, 2004). In horses, the detection of IL-4 and its functions during immune responses has been inhibited by the lack of specific reagents to this important Th2 cytokine.

We have generated a monoclonal antibody (mAb) to equine IL-4 using rIL-4 for immunization. The equine rIL-4 was obtained from supernatant of a stable transfected mammalian cell line expressing rIL-4/IgG1 fusion protein using Protein G purification and enterokinase digestion procedures established earlier

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Table 1
Detection of recombinant equine cytokines and cytokine/IgG1 fusion proteins by new anti-equine IL-4 mAb

	rIL-4/IgG1	rIL-2/IgG1	rIFN- γ /IgG1	rIL-4/myc	rIL-2/myc	IgG1
ELISA ^a	+	–	–	+	–	–
Flow cytometry ^b	+	–	–	+	–	N/A

N/A = not tested.

^a Supernatants of transfected Chinese Hamster Ovary cells were used to detect the recombinant cytokines.

^b Intracellular staining and flow cytometric analysis of transfected cells.

(Wagner et al., 2005). The immunization of mice and the cell fusion were performed as previously described (Wagner et al., 2003). The supernatants of hybridomas were tested for detection of equine rIL-4/IgG1 by ELISA and the corresponding cells were cloned twice. Positive supernatants were further analyzed for recognition of different recombinant equine cytokine/IgG1 fusion proteins (Wagner et al., 2005) or myc-tagged cytokines (Dohmann et al., 2000) in transient transfectants or their supernatants by intracellular staining and flow cytometric analysis or by ELISA, respectively (Table 1). Using both techniques the mAb specifically detected rIL-4/IgG1 fusion protein and myc-tagged rIL-4, but not rIFN- γ /IgG1, rIL-2/IgG1 fusion proteins, myc-tagged rIL-2 or IgG1 alone. The mAb was isotypized as murine IgG1.

A crucial step in the generation of mAbs using a recombinant protein is to provide sufficient evidence for specific detection of the corresponding native protein by the reagent. We designed different experiments using mitogen stimulated PBMC to show that the new mAb detects native equine IL-4 using intracellular staining and ELISA. For both methods, the mAb was purified from serum-free supernatants using large scale cell cultures and Protein G affinity purification as previously described (Wagner et al., 2003). For intracellular staining, the mAb was conjugated with Alexa Fluor647 (Molecular Probes, Eugene, OR) according to the manufacturer's protocol.

PBMC were isolated from heparinized blood of four clinical healthy, adult (5–19 years of age) Thoroughbred horses by density gradient centrifugation (Ficoll-PaqueTM Plus, Amersham Bioscience, Piscataway, NJ). A total of 3×10^6 PBMC were incubated for 4 h in medium (DMEM containing 10% (v/v) FCS, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol, 50 μ g/ml gentamycin) or in medium supplemented with PMA (25 ng/ml) and ionomycin (1 μ M; both Sigma, St.

Louis, MO). To block the secretion of intracellular protein during stimulation, Brefeldin A (10 μ g/ml; Sigma, St. Louis, MO) was added to all cultures. Afterwards, the cells were washed in PBS and fixed in 2% formaldehyde for 20 min at room temperature. Intracellular staining was performed with the Alexa647 conjugated new mAb in saponin buffer (PBS, supplemented with 0.5% (w/v) BSA, 0.5% (w/v) saponin and 0.02% (w/v) NaN₃). Double staining was performed with anti-equine CD4 (VMRD, Pullman, WA), anti-equine CD8 (CVS8, kindly provided by Dr. Paul Lunn) to label T-cells, or FITC conjugated goat anti-horse IgG(H + L) for detection of B-cells (Jackson ImmunoResearch, West Grove, PA). The anti-CD4 and anti-CD8 antibodies were conjugated with Alexa488 (Molecular Probes, Eugene, OR). After antibody incubation, the cells were washed twice in saponin buffer and measured by flow cytometry. Between 4 and 8% positive lymphocytes were detected by the new mAb in cells incubated in the presence of PMA and ionomycin. Cells incubated in medium alone and stained with the mAb, or PMA stimulated cells stained with an isotype control antibody did not show this positive population (Fig. 1A–D). Double staining with T and B-cell markers showed that a subpopulation of CD4 cells expressed the protein detected by the new mAb (Fig. 1E–G). Compared to CD8⁺ T-cells and Ig⁺ B-cells in the same PBMC sample, the expression of CD4 on T-cells was down-regulated after 4 h of PMA stimulation. This time-dependent effect on the expression of CD4 on equine T-cells from peripheral blood has been described previously. CD4 down-regulation was greatest after 4 h of incubation at 37 °C, and was followed by re-expression of CD4 after 24 h in culture (Zhang et al., 1994). Consistent staining results with the new mAb were observed in PMA stimulated lymphocytes of four randomly selected horses (Fig. 1H).

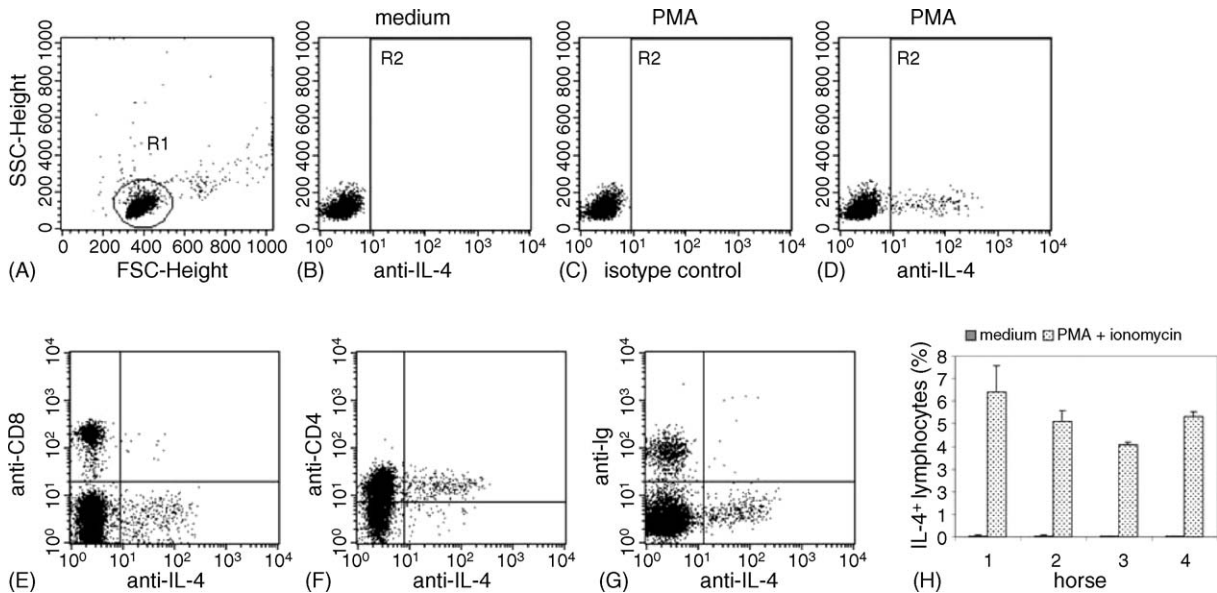


Fig. 1. Flow cytometric analysis of equine PBMC using the new monoclonal antibody to IL-4 for intracellular staining. Equine PBMC were stimulated with PMA and ionomycin in the presence of the secretion inhibitor Brefeldin A. The cells were fixed after 4 h of stimulation and stained. (A) Lymphocytes in gate R1 were used for the analysis; (B) non-stimulated PBMC (medium + Brefeldin A) stained with anti-IL-4; (C) PMA-stimulated PBMC stained with an isotype control or (D) the anti-IL-4 mAb; (E) double staining using anti-CD8; (F) anti-CD4 or (G) anti-Ig antibodies for membrane staining and the anti-IL-4 mAb for intracellular staining; (H) IL-4 positive lymphocytes in gate R2 were determined in PMA-stimulated and non-stimulated PBMC of four horses, with triplicates for each (shown as means and standard deviations).

In a second experiment, 3×10^6 PBMC obtained from four horses were stimulated for 4 days with LPS (12.5 $\mu\text{g/ml}$), PHA (5 $\mu\text{g/ml}$), PWM (2.5 $\mu\text{g/ml}$), or ConA (1 $\mu\text{g/ml}$) (all from Sigma, St. Louis, MO) or cultured in medium alone. No secretion inhibitor was used. The cells were fixed and stained with the new mAb as described above and analyzed by flow cytometry. In PBMC stimulated with PHA or PWM, 1–2% positive cells were detected by the new mAb. Less than 0.05% of positive cells were found in the medium control or LPS stimulated PBMC (Fig. 2). A time-kinetics was performed in an additional experiment using 2 and 6 h, and 1–4 days of PHA, PWM, or ConA stimulation. Staining of these cells with the mAb indicated that the highest percentages of positive cells were detectable after 4 days of stimulation (data not shown). The results using different T-cell mitogens and the secretion inhibitor Brefeldin A or not suggest that the new mAb detects a secreted protein expressed by equine CD4⁺ T-cells.

The supernatants of mitogen-stimulated PBMC obtained from the experiment shown in Fig. 2 and the time-kinetics study were tested by ELISA for the

secreted protein. All ELISA buffers, washing steps, the substrate solution and the measurement of the assay were the same as described before in detail (Wagner et al., 2003). To detect equine IL-4 the following steps were used: The affinity purified new mAb (5 $\mu\text{g/ml}$) was coated to ELISA plates overnight at 4 °C. After washing the plates, the cell culture supernatants were applied in two-fold dilutions (undiluted to 1:16). For quantification, purified rIL-4 was obtained by enterokinase digestion of rIL-4/IgG1 and was added in two-fold dilutions ranging from 2 $\mu\text{g/ml}$ to 125 ng/ml. Samples and standard were incubated for 2 h at room temperature. For detection a polyclonal anti-equine IL-4 antiserum was used (kindly provided by Pierce Biotechnology, Endogen Brand (Rockford, IL)), which was produced in rabbits immunized with equine rIL-4 expressed in yeast (*Pichia pastoris*). The incubation with the anti-equine IL-4 antiserum was followed by a secondary peroxidase conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) and substrate solution. Using this ELISA and consistent with the results obtained by intracellular staining, a

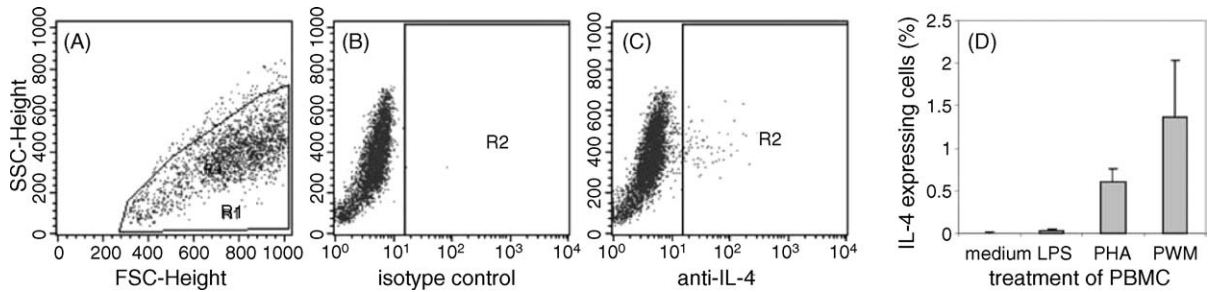


Fig. 2. Intracellular staining of mitogen stimulated equine PBMC using the new anti-equine IL-4 mAb. PBMC of four horses were stimulated for 4 days with LPS, PHA or PWM, or were cultured in medium alone. The cells were fixed, stained and analyzed by flow cytometric analysis. (A)–(C) Show cells after stimulation with PWM. (A) Lymphocytes and lymphoblast shown in gate R1 were used for analysis; (B) the cells were stained using an isotype control or (C) the anti-equine IL-4 antibody. (D) The percentages of anti-IL-4 positive cells in the gate R2 are shown as means and standard deviations for all horses and stimuli.

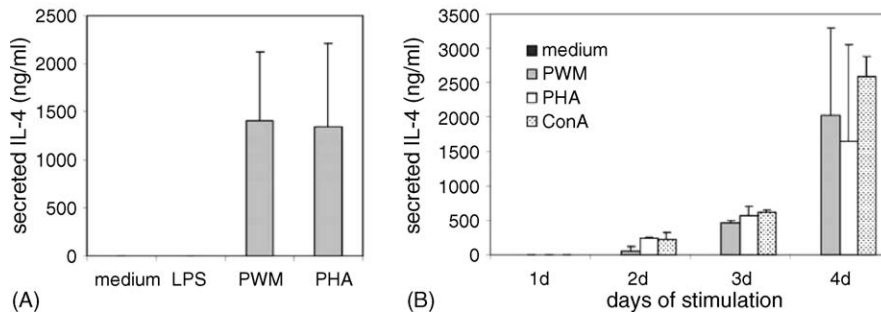


Fig. 3. IL-4 was detected in cell culture supernatants of mitogen stimulated PBMC by ELISA. The new anti-IL-4 mAb was used for coating of the plates and a polyclonal anti-equine IL-4 antiserum for detection. Purified equine rIL-4 served as standard for quantification. The bars show means and standard deviations of the protein detected in cell culture supernatants of PBMC from different horses. (A) PBMC were obtained from four horses and were stimulated for 4 days with different mitogens or cultured in medium alone. (B) PBMC of two horses were stimulated with mitogens and the protein was detected in the supernatants over several days.

protein was detectable in supernatants of PHA, PWM and ConA stimulated cells, but not in supernatants from LPS stimulations or medium alone (Fig. 3). The protein was found in supernatants after 2–4 days of stimulation (Fig. 3B). On day 4, the concentration of the protein ranged between 1 and 3 $\mu\text{g}/\text{ml}$. This ELISA used two independently generated antibodies which were obtained using equine IL-4 from two different expression systems, namely mammalian cells and yeast. Thus, in all likelihood the protein detected in this ELISA was equine IL-4.

In summary, our results show that the new monoclonal antibody specifically detects equine IL-4 and can be used for flow cytometric analysis and ELISA to investigate this important Th2 cytokine in the horse.

Acknowledgements

This work was supported by the Dorothy Russell Havemeyer Foundation, Inc., the Morris Animal Foundation, and the Zweig Memorial Fund for Equine Research.

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