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Effects of polysaccharide fractions isolated from *Caltha palustris* L. on the activity of phagocytic cells & humoral immune response in mice with collagen-induced arthritis: A comparison with methotrexate

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Background & objectives*: The extracts from *Caltha palustris* L. have been shown to be beneficial for treating arthritis and rheumatism. In this study, the immunomodulatory effects of polysaccharide fractions B and C of *C. palustris* extracts were studied, using the collagen-induced arthritis (CIA) mouse arthritis experimental model. The aim was to determine the activity of blood phagocytic cells and humoral immune response in CIA mice treated with polysaccharide fractions from *C. palustris.

***Methods*: The effects of fractions B and C of *C. palustris* were explored by evaluating phagocytic activity of peripheral blood granulocytes and monocytes and humoral immune response in sheep red blood cell (SRBC)-immunized mice. The results were compared with methotrexate (MTX) treatment. Following the onset of CIA, DBA/1J mice were treated for 21 days with B or C fractions (10 mg/kg; i.p.) or MTX (every 48 h, 6.6 mg/kg; i.p.).**

***Results*: The results showed that fraction B reduced the level of interleukin (IL)-1β, boosted nitric oxide synthesis in murine peritoneal macrophages stimulated *in vitro* with lipopolysaccharide and enhanced the monocyte phagocytic activity. Exposure of SRBC-immunized mice to fraction B and MTX during the course of CIA resulted in decreased total anti-SRBC haemagglutinin titres.**

***Interpretation & conclusions*: Fraction B of *C. palustris* polysaccharides modulated macrophage function and exerted beneficial effects on the clinical course of CIA in mice. The results also suggested efficacy of fraction B was comparable to that of MTX treatment for certain parameters.**

Key words Antibodies - *Caltha palustris* - collagen-induced arthritis - interleukin-1 - nitric oxide - phagocytic cells

Rheumatoid arthritis (RA) is an autoimmune systemic inflammatory disease[1]. RA pathogenesis involves different pathways, in which pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumour necrosis factor-α (TNF-α) play a crucial role[2,3]. IL-1β has the strongest destructive effect on bone and cartilage tissues, and it impairs repair processes by inhibiting the synthesis of cartilage matrix proteins[4,5]. In RA, the joint synovial tissue is infiltrated by various inflammatory cells such as macrophages,

T-cells, B-cells, granulocytes and monocytes[6]. Macrophages are profusely present in the inflamed synovial membrane and cartilage and their activity contributes to joint inflammation, pannus formation and cartilage destruction[7,8]. Overexpression of TNF-α, IL-1β, and IL-6 is induced by infiltration or activation of macrophages[9]. Furthermore, a large number of synovial lining macrophages produces nitric oxide (NO) that promotes high expression of TNF-α and additionally increases synovitis frequency[10]. Another cellular population determining the severity and duration of RA, includes the B-cells. Their reactivity in RA patients is usually prominent, and they secrete a large amount of autoantibodies that can promote tissue destruction and release of autoantigens[11].

Collagen-induced arthritis (CIA) in mice is a well-known model of human RA, due to its numerous clinical and histological similarities to human RA[12]. This model has been extensively used to estimate prospective anti-arthritic substances[13].

Caltha palustris, a plant species from the *Caltha* genus (*Ranunculaceae* family), is a widespread plant in Europe, Asia and North America. Our previous experiments[14] confirmed that polysaccharide fraction B from *C. palustris* beneficially affected the course of CIA in mice. The presented results were comparable to those of methotrexate (MTX) treatment, especially for alleviating joint swelling severity, erythema and inhibition of CIA-induced peripheral blood leucocytosis. Moreover, fraction B exhibited immunomodulating activity toward thymic T lymphocyte subpopulations, lowered the percentage of splenic Tregs and reduced TNF-α concentration in peripheral blood, previously enhanced during the course of CIA[14]. In this study, we explored the effects of polysaccharide fractions B and C from *C. palustris* extract on the activity of blood phagocytic cells and humoral immune response in CIA, a mouse arthritis experimental model.

Material & Methods

Detailed information on animals, induction of CIA in mice, treatment and study design were as described in our previous study[14]. Briefly, the studies were conducted on male and female DBA/1J mice (8-10 wk old). Experiments were performed on non-immunized and sheep red blood cells (SRBCs)-immunized mice. The immunization was conducted on day 43 of the experiment with 0.2 ml of 10 per cent SRBC suspension (4×10^8 cells/mouse) administered intraperitoneally (i.p.). The sheep blood was collected (SRBC, Department of Physiology and Biostructure, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, Poland) into the Alsever's solution and kept at 4°C for at least three days. The SRBC suspension was prepared *ex tempore* in phosphate-buffered saline (PBS, Institute of Immunology and Experimental Therapy Polish Academy of Sciences, Wrocław, Poland).

Animals were divided into five groups: group 1 (negative control) - healthy animals, group 2 (positive control) - animals with induced CIA, group 3 - CIA mice treated with fraction C, group 4 - CIA mice treated with fraction B, and group 5 - CIA animals treated with MTX (methotrexate, Ebewe Pharma GmbH Nfg., Austria). Each control and experimental group comprised eight mice. The experimental protocol is presented in Fig. 1.

The study protocol was approved by the Local Ethics Committee in Wrocław, Poland (No. 95/2009). The experiments were conducted from August 2009 to July 2011, at Wrocław, Poland.

The plant material was collected from Trzebnica (Western Poland), identified and a voucher specimen (number WR-GN0058095) was deposited at the Herbarium of Museum of Natural History, Wrocław University, Poland. Polysaccharide fractions B and C from *C. palustris* extract were prepared and kindly provided by Professor Janina Kuduk-Jaworska as described previously[15]. Stock solutions of the plant extract fractions were prepared *ex tempore* by dissolving 2 mg of B or C fraction in PBS solution.

Cytokine assay and NO production: The mice were anaesthetized with isoflurane (Aerrane, Baxter, USA). Peritoneal exudate macrophages were acquired and cell culture procedure were conducted as described by Szczypka *et al*[16], except one modification that the medium was replaced after shorter incubation time, 18 h instead of 20 h.

Murine IL-1β level (pg/ml) in macrophage culture supernatants was established by using a commercial ELISA kit (Quantikine R&D Systems, Minneapolis, USA) in compliance with the manufacturer's instruction. Each sample was tested in duplicate.

NO released from murine peritoneal macrophages, stimulated *in vitro* with lipopolysaccharide (LPS) from *Escherichia coli,* was measured as nitrite using the procedure described by Szczypka *et al*[16]. The level of IL-1β, NO, and the phagocytic and oxidative burst

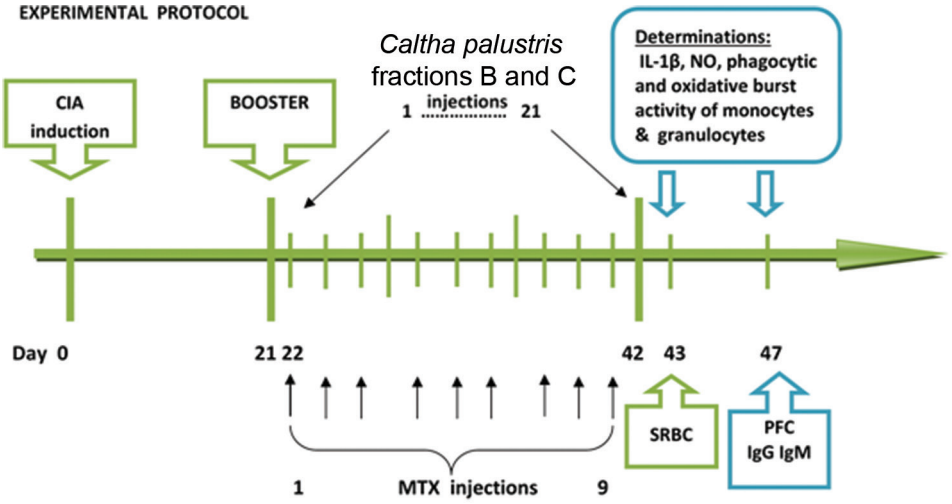

Fig. 1. Experimental protocol. Collagen-induced arthritis (CIA) induction - day 0 - intradermal injection 50 µl of type II collagen emulsified in complete Freund's adjuvant. Booster - day 21 - injection with 50 µl of type II collagen emulsion in incomplete Freund's adjuvant. Fractions B and C injections - intraperitoneal administration, 10 mg/kg daily, for 21 consecutive days. Control group animals were treated with phosphate buffer saline (PBS). Methotrexate injections - intraperitoneally, 3 times a week, every 48 h at a dose of 6.6 mg/kg at three weekly cycles. Sheep red blood cell (SRBC) immunization - 0.2 ml of 10 per cent SRBC suspension intraperitoneally, day 43. Determinations of immunological parameters were performed at two time points: day 43 and/or 47 of experiment.

activity of the blood monocytes and granulocytes were determined after finishing treatment at two time points: day 43 of the experiment (24 h after the last administration of fraction C and B and 72 h after the last administration of MTX) and day 47 of the experiment (5 days after the last administration of fraction B or C and 7 days after the last administration of MTX).

Phagocytic and the oxidative burst activity of blood granulocytes and monocytes: The blood samples were collected into the heparinised tubes (Equimed, Kraków, Poland) from retro-ocular arteries of isoflurane-anaesthetized mice. The animals were then euthanized by cervical dislocation. Commercial Phagotest and Bursttest (Phagoburst) kits were used following the manufacturer's instructions (ORPEGEN Pharma, Heidelberg, Germany). A flow cytometer (FACSCalibur, Becton Dickinson Biosciences, USA) with softwere - CellQuest 3.1f (Becton Dickinson, USA) was used to analyze the fluorescence. To determine the percentage of phagocytic blood granulocytes and monocytes (ingestion of one or more bacteria per cell), and their mean fluorescence intensity (MFI) (number of bacteria per cell) the Phagotest kit (fluorescein-labelled opsonized *E. coli* and necessary reagents) was used. The Bursttest was used to establish the leucocyte oxidative burst by measuring the percentage of blood granulocytes and monocytes producing reactive oxygen species (ROS) and their MFI (enzymatic activity). The measurements were made on days 43 and 47 of the experiment.

Plaque forming cells (PFC) assay: The mice were immunized i.p. with 0.2 ml of 10 per cent SRBC suspension (4×10^8 cells/mouse) on day 43 of the experiment. The animals were anaesthetized with isoflurane (Aerrane, Baxter, USA) and then sacrificed by cervical dislocation on day 4 after priming (day 47 of the experiment). Briefly, following euthanasia, the murine spleens were removed, cell suspension acquired and the number of splenocytes producing haemolytic anti-SRBC antibodies (PFCs) was determined according to a method previously described[17].

Determination of anti-SRBC antibodies in serum: The mice were treated for PFC assay as described above. The blood samples were taken from retro-ocular arteries of isoflurane anaesthetized mice. The number of anti-SRBC haemagglutinin titres in SRBC-immunized mice was determined on day 4 after priming (day 47 of the experiment)[17].

Statistical analysis: One-way ANOVA and *post hoc* analysis using Duncan's test were used to evaluate the differences between the groups using STATISTICA 10 software (StatSoft Inc., Oklahoma, USA).

Results

Effects of polysaccharide fractions B and C of C. palustris and MTX on IL-1β production by peritoneal macrophages in CIA mice: Synthesis and release of

IL-1β by murine peritoneal macrophages, stimulated *in vitro* with LPS from *E. coli* (2.5 μg/ml cell culture), was noticed on day 47. Administration of fraction B to the CIA mice inhibited (day 47) the stimulating effect of inflammation on the production and release of IL-1β by murine peritoneal macrophages stimulated *in vitro* with LPS from *E. coli*. MTX administration caused only a transient (day 43) induction of this stimulating effect (Fig. 2).

Effects of polysaccharide fractions B and C of C. palustris and MTX on NO production by peritoneal macrophages in CIA mice: Fraction B increased the NO output production by murine peritoneal macrophages stimulated *in vitro* with LPS from *E. coli* on both days (43 and 47). The strongest effect was observed on day 43 of the experiment. MTX suppressed NO production in peritoneal macrophages (Fig. 3).

Effects of polysaccharide fractions B and C of C. palustris and MTX on phagocytic activity of peripheral blood granulocytes and monocytes in CIA mice: Transient rise in the percentage of phagocytic granulocytes was perceived on the day 43 of the experiment in CIA mice. No effects on the phagocytic activity of monocytes were noticed in CIA mice (Table I).

No significant changes in the phagocytic activity of monocytes and granulocytes were observed on day 43, after administration of any fractions (Table I). On day 47, the exposure to fraction B increased the percentage of phagocytic monocytes, and it was accompanied by increased fluorescence intensity of these cells.

On day 43, a transient drop in the percentage of phagocytic granulocytes was noticed in MTX group. However, on day 47, a significant enhancement in the percentage of phagocytic monocytes and fluorescence intensity of these cells were observed.

Effects of polysaccharide fractions B and C and MTX on oxidative burst activity of peripheral blood granulocytes and monocytes in CIA mice: Reduced percentages of granulocytes producing ROS and lower enzymatic activity (fluorescence intensity) of monocytes were noted in CIA mice on day 43 of the experiment. However, reduced oxidative burst activity of granulocytes (and MFI of these cells) and monocytes was found on day 47. Administration of fraction B resulted in higher percentage of ROS producing monocytes, and this corresponded to increased fluorescence intensity of these cells. Fraction C did not significantly affect the burst activity of granulocytes and monocytes.

MTX administered to CIA mice strongly reduced the percentage of ROS producing granulocytes with augmented enzymatic activity, but only on day 43 (Table II).

Effects of polysaccharide fractions B and C and MTX on primary humoral immune response in SRBC-immunized mice during the course of CIA: The primary humoral immune response in SRBC-immunized mice was

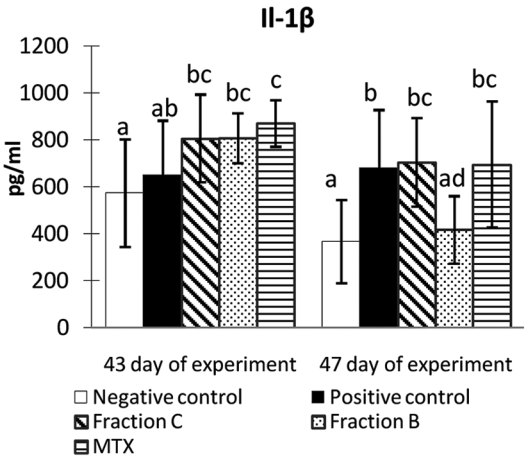

Fig. 2. Effects of polysaccharide fractions B and C, and methotrexate on interleukin-1β (IL-1β) production by murine peritoneal macrophages in mice with collagen-induced arthritis. Values are presented as mean±standard deviation; n=8 mice for each group tested by ANOVA *post hoc* Duncan test. Data with different superscript letters indicate significant differences (P<0.05).

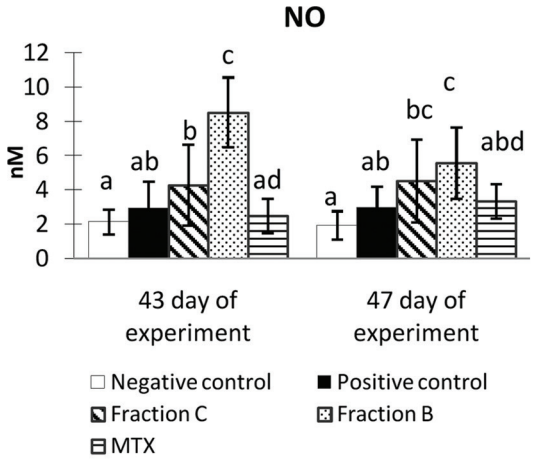

Fig. 3. Effects of polysaccharide fractions B and C of *C. palustris*, and methotrexate on nitric oxide (NO) production by murine peritoneal macrophages in mice with collagen-induced arthritis. Values are presented as mean±standard deviation; n=8 mice for each group tested by ANOVA *post hoc* Duncan test. Data with different superscript letters indicate significant differences (P<0.05).

Table I. Effects of polysaccharide fractions of *Caltha palustris* and methotrexate on phagocytic activity of peripheral blood granulocytes and monocytes in mice with collagen-induced arthritis

Day	Experimental group	Phagocytic granulocytes (%)	MFI	Phagocytic monocytes (%)	MFI
43	Group 1 - Negative control	87.83±4.90	45.01±7.15	47.57±9.42	46.99±3.31
	Group 2 - Positive control	92.18±2.08	45.08±10.06	40.91±13.08	48.15±4.61
	Group 3 - Fraction C	88.71±5.84$^{\$}$	44.33±7.87	36.34±6.96*	48.86±7.44$^{\$}$
	Group 4 - Fraction B	91.02±2.46$^{\$}$	50.84±7.74	43.31±7.38	53.90±7.23$^{\$}$
	Group 5 - MTX	82.41±3.41$^{*\#}$	49.93±5.57	38.57±7.47	42.62±3.68$^{*\wedge\dagger}$
47	Group 1 - Negative control	78.11±8.84	62.99±7.79	44.25±8.64	36.58±3.43
	Group 2 - Positive control	76.38±6.25	56.49±8.06	50.09±7.97	37.28±3.90
	Group 3 - Fraction C	75.14±6.34†	58.69±7.89	54.01±9.12$^{*\dagger\$}$	41.87±5.37$^{*\#}$
	Group 4 - Fraction B	83.23±5.29$^{\wedge}$	61.97±7.77	62.37±3.88$^{*\#\wedge}$	41.04±2.37$^{*\#}$
	Group 5 - MTX	79.13±10.01	60.10±8.55	59.57±8.48$^{*\#\wedge}$	41.22±4.32$^{*\#}$

Values are presented as mean±SD; n=8 mice for each group tested by ANOVA *post hoc* Duncan test. $^{*}P<0.05$ versus negative control group; $^{\#}P<0.05$ versus positive control group; $^{\wedge}P<0.05$ versus fraction C; $^{\dagger}P<0.05$ versus fraction B; $^{\$}P<0.05$ versus MTX. MFI, mean fluorescence intensity; SD, standard deviation; MTX, methotrexate

Table II. Effects of polysaccharide fractions of *Caltha palustris* extract and methotrexate on oxidative burst activity of peripheral blood granulocytes and monocytes in mice with collagen-induced arthritis mice

Day	Experimental group	Granulocytes producing reactive oxidants (%)	MFI	Monocytes producing reactive oxidants (%)	MFI
43	Group 1 - Negative control	75.87±5.28	11.22±0.86	8.12±4.06	11.18±1.69
	Group 2 - Positive control	63.04±8.73	11.46±1.83	6.70±2.77	8.97±1.02
	Group 3 - Fraction C	57.2±10.40$^{*\$}$	11.60±0.72	7.00±2.10	9.33±0.99*
	Group 4 - Fraction B	59.13±5.73$^{*\$}$	12.06±1.91	9.57±4.13	10.10±0.87
	Group 5 - MTX	27.05±7.12$^{*\#\wedge\dagger}$	16.64±1.29$^{*\#\wedge\dagger}$	7.01±3.34	9.21±0.60*
47	Group 1 - Negative control	70.60±4.88	8.15±0.88	16.00±4.61	10.22±1.48
	Group 2 - Positive control	54.37±7.00	6.92±0.77	9.93±3.34	7.76±1.92
	Group 3 - Fraction C	58.25±10.49*	7.77±0.91$^{\#}$	11.53±4.20*†	8.39±1.21*
	Group 4 - Fraction B	58.81±9.57$^{*\$}$	7.87±0.77$^{\#}$	17.24±6.58$^{\#\wedge\$}$	9.82±1.58$^{\#}$
	Group 5 - MTX	49.85±11.15*†	7.10±0.80*	9.51±3.42*†	8.52±1.64*

Values are presented as mean±SD; n=8 mice for each group tested by ANOVA *post hoc* Duncan test. $^{*}P<0.05$ versus negative control group; $^{\#}P<0.05$ versus positive control group; $^{\wedge}P<0.05$ versus fraction C; $^{\dagger}P<0.05$ versus fraction B; $^{\$}P<0.05$ versus MTX.

reduced by CIA, as evidenced by the lower number of splenocytes producing haemolytic anti-SRBC antibodies (PFC) (Fig. 4) and the total anti-SRBC antibody titres in serum. The 2-ME-resistant anti-SRBC haemagglutinin titres did not change in the course of CIA (Fig. 5B). Exposure of CIA mice to fraction B and MTX resulted in lowering the total anti-SRBC antibody titres in serum (Fig. 5A). The suppressive effect of fraction B on the total anti-SRBC haemagglutinin titres in serum was equally strong in MTX samples.

Discussion

So far, treatment modalities for RA have been limited by their high cost and side effects, especially

immunosuppression and toxicity. Therefore, natural products have become a new therapeutic target for researchers. In the current study, the immunomodulatory effects of polysaccharide fraction B and C of *C. palustris* extracts were studied on the activity of phagocytic cells and humoral immune response in SRBC-immunized mice with CIA. MTX was used as a control treatment, due to the fact that it still remains as a standard in RA therapy[18].

Administration of fraction B inhibited the stimulating effect of inflammation on the synthesis and release of IL-1β by murine peritoneal macrophages stimulated *in vitro* with LPS from *E. coli*. The beneficial suppressing action of plant extracts on the synthesis of IL-1β in CIA mice was also demonstrated by Kim *et al*[19]. Red ginseng saponin extract (RGSE) from the root of *Panax ginseng* C., administered orally at a dose of 10 mg/kg, reduced the level of serum IL-1β and TNF-α in CIA mice. Moreover, in the *in vitro* studies on spleen cells isolated from RGSE-treated CIA mice, LPS-stimulated increase in cytokine levels was significantly inhibited. However, this suppressing effect on IL-1β production was quite different from most investigated plant polysaccharides as enhanced production of this cytokine was reported in several studies[20,21].

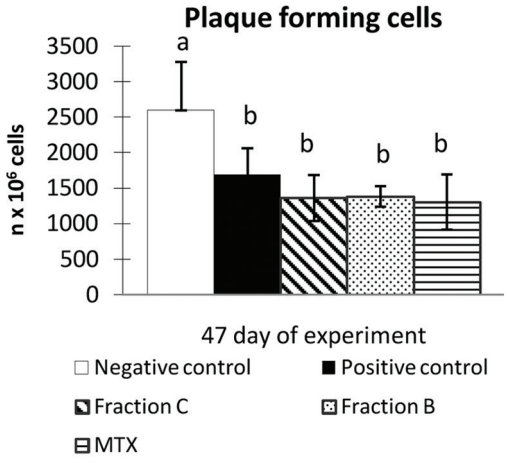

Fig. 4. Effects of polysaccharide fractions B and C, and methotrexate on the number of plaque forming cells in spleen in mice with collagen-induced arthritis. Values are presented as mean±standard deviation; n=8 mice for each group tested by ANOVA *post hoc* Duncan test. Data with different superscript letters indicate significant differences ($P < 0.05$).

Our results for MTX were consistent with the results, which showed a lack of MTX influence on IL-1β synthesis and release by mononuclear spleen cells obtained from MTX-treated CIA mice[22]. Another study also suggested that MTX might inhibit some IL-1β activities without affecting IL-1β production or secretion[23].

Our results showed that fraction B enhanced NO production by peritoneal macrophages stimulated *in vitro* with LPS from *E. coli*. Fraction B increased the percentage of phagocytic monocytes in the peripheral blood, which corresponded to increased fluorescence intensity of these cells, thus indicating a higher number of ingested bacteria per cell. Exposure to this fraction led to increased percentage of monocytes producing ROS, and their enhanced enzymatic activity. The polysaccharide fraction B has been shown to trigger monocyte phagocytic activity, burst activity and NO. Our results were in accordance with the studies which showed that most polysaccharides derived from higher

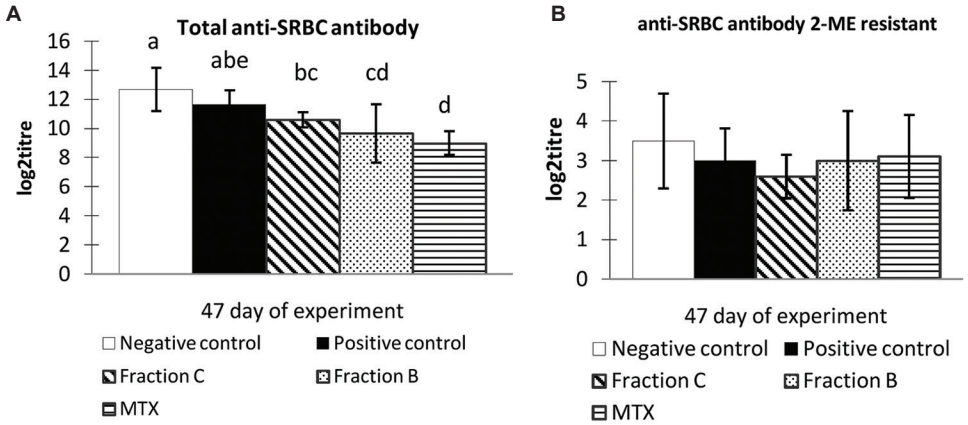

Fig. 5. Effects of polysaccharide fractions B and C, and methotrexate on total **(A)** and 2-mercaptoethanol-resistant anti-sheep red blood cell haemagglutinin titres **(B)** in the serum in collagen-induced arthritis mice. Values are presented as mean±standard deviation; n=8 mice for each group tested by ANOVA *post hoc* Duncan test. Data with different superscript letters indicate significant differences ($P<0.05$).

plants activated macrophages[24,25]. Unfortunately, this activation of macrophage function might pose a problem in the case of inflammation. Activation of T-cells and macrophages is a major pathogenic determinant of CIA[26]. However, our findings regarding NO production were obtained only *in vitro* and were limited to M1 phenotype of macrophage activation. To draw correct inference about the effects of polysaccharide fractions from *C. palustris* extract on the macrophage activity in CIA mice, especially the populations that infiltrate the synovium, further and more detailed experiments are required.

Our data also showed that MTX caused a transient drop in the percentage of phagocytic granulocytes and a decrease in the percentage of ROS producing granulocytes with augmented enzymatic activity (day 43 of the experiment). These findings confirmed the inhibitory effect of MTX on granulocyte phagocytic function[27]. Our data were also consistent with the results by Omata *et al*[28], who claimed that MTX reduced NO production by peritoneal macrophages from rats with adjuvant-induced arthritis. The polysaccharide fraction B and MTX showed inhibitory action of B-cell function. Park *et al*[29] showed that low molecular weight fucoidans from brown algae reduced the intensity of arthritis and the levels of collagen-specific IgG 2a[29].

In conclusion, our study showed that fraction B enhanced macrophage activity (boosted NO synthesis in murine peritoneal macrophages stimulated *in vitro* with LPS and enhanced the monocyte phagocytic activity), but reduced the level of pro-inflammatory cytokine IL-1β. In turn, humoral immune response was suppressed as fraction B showed inhibitory action on antibody formation. Taking into account the results of our present and previous experiments, it seems that at least part of the favourable effects of *C. palustris* polysaccharides on the clinical course of CIA in mice may be due to the modulation of macrophage function.

Acknowledgment

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***Conflicts of Interest*:** None.

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