Toxicology and Industrial Health 2009; **25:** 329–335
http://tih.sagepub.com

Study of new ways of supplementary and combinatory therapy of rheumatoid arthritis with immunomodulators. Glucomannan and Imunoglukán® in adjuvant arthritis

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We studied the anti-arthritic activity of glucomannan (GM) isolated from *Candida utilis* and of Imunoglukán®, a beta-(1,3/1,6)-D-glucan (IMG) isolated from *Pleurotus ostreatus*. Adjuvant arthritis (AA) was induced intradermally by the injection of *Mycobacterium butyricum* in incomplete Freund's adjuvant to Lewis rats. Blood for biochemical and immunological analysis was collected on experimental days 1, 14, 21, and 28. A clinical parameter – hind paw volume (HPV) – was also measured. The detection of IL-1 alpha, IL-4, TNF alpha, and MCP-1 was done by immunoflowcytometry. On day 28 – the end of the experiment – we determined spectrophotometrically: the total anti-oxidant status (TAS) of plasma samples along with thiobarbituric acid-reacting substances (TBARS) levels in plasma and we assessed the activity of gamma-glutamyl transferase (GGT) in hind paw joint homogenate. The experiments included healthy animals, arthritic animals without treatment, and arthritic animals with administration of glucomannan (GM-AA) in the oral daily dose of 15 mg/kg b.w. and of IMG (IMG-AA) in the oral daily dose of 2 mg/kg b.w. The progress of AA was manifested by all parameters monitored. Both substances had beneficial effects on HPV, TBARS levels, GGT activity, and TAS levels. For cytokine assessment, only IMG-AA samples were selected, considering the significant HPV improvement accompanied with the observed anti-oxidant action. IMG administration had a positive immunomodulating effect on all cytokine plasma levels measured, changed markedly due to arthritis progression. Thus, IMG may be considered as a candidate for combinatorial therapy of rheumatoid arthritis. *Toxicology and Industrial Health* 2009; **25**: 329–335.

Key words: *adjuvant arthritis; Candida utilis; glucomannan; immunomodulators; Imunoglukán®; Pleurotus ostreatus; rheumatoid arthritis*

Introduction

Rheumatoid arthritis (RA) is a common severe joint disease that involves all age groups. In general, the disease progresses and often leads to disability. It can shorten the patient's life span by 10 years. The cause of the disease is multifactorial, including genetic predisposition. It is characterized by typical chronic inflammation and initiated and maintained by autoimmune mechanisms. The disease is assumed to be triggered by a micro-organism in genetically predisposed subjects (Bauerová and Bezek, 1999).

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10.1177/0748233709102945

The pathogenesis of RA is associated predominantly with the formation of free radicals and pro-inflammatory cytokines at the site of inflammation. The inflammatory process develops in the tissue of the synovium; primary sources of reactive oxidative species (ROS) in RA are leukocytes, which are recruited to accumulate within the synovium. ROS can be produced by activated macrophages in the synovial membrane and by activated neutrophils in the synovial cavity. Macrophage-like synoviocytes, which normally phagocytize debris in the joint fluid, are intrinsically capable of secreting pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α. Fibroblast-like synoviocytes, which physiologically produce hyaluronic acid, synthesize matrix metalloproteinases, and prostaglandin E2 when stimulated by TNF-α and IL-1 (Firestein, *et al*., 1997; Firestein, 2003).

New ways of supplementary or combinatory RA therapy are of great importance. The aim is to find an alternative or additive to classical RA therapy with natural molecules without side effects possessing immunomodulatory, anti-inflammatory, and anti-oxidative properties. In recent decades, polysaccharides isolated from botanical sources (mushrooms, algae, lichens, and higher plants) have attracted a great deal of attention in the biomedical arena because of their broad spectrum of therapeutic properties and relatively low toxicity (Tzianabos, 2000). Plant and mushroom polysaccharides reveal immunomodulatory effect that depends on polysaccharide structure and molecular weight (low molecular weight – inhibition, high molecular weight – activation) (Schepetkin and Quinn, 2006).

Cell wall polysaccharides D-glucans are branched polymers of D-glucopyranose, either with α- and/or β-linkage configuration. Both α- and β-glucans have been reported in micro-organisms, plants, and animals. The β-glucans, in particular, are the predominant carbohydrates, making up to more than 50% of the dry weight of the cell wall. The β-glucans (β-1,3-, β-1,4-, and β-1,6-glucose polymers) from diverse sources are different in their structure, chemical, physical, and biological properties, and as a consequence, in their immnunomodulatory effects. Moreover, they represent the conserved structure – pathogen-associated molecular pattern (PAMP) and are effective biological response modifiers, non-specifically enhancing the host immune system by multiple interactions within innate and adaptive mechanisms. The gamut of biological activities of glucans includes direct leukocyte activation, stimulation of phagocytosis, and oxidative burst (Sakurai, *et al*., 1992; Wakshull, *et al*., 1999) and stimulation of cell cytotoxicity (cytotoxic T-lymphocytes) (Cross, *et al*., 2001). The stimulated induction of ROS and inflammatory mediators, chemokines, cytokines, and nuclear transcription factors (Czop, 1986; Sakurai, *et al*., 1996; Adams, *et al*., 1997; Battle, *et al*., 1998; Majtan, *et al*., 2005) is selectively guided *via* β-glucan specific receptors on competent cells of the immune system, e.g., complement receptor 3, lactosylceramide, scavenger receptors, and dectin-1 (Brown, *et al*., 2001; Rice, *et al*., 2002).

Glucomannans (GMs), another yeast cell wall component, are functionally and structurally very similar to other yeast mannans, differing only in the presence of terminal glucopyranosyl units in the side-chains. However, their biological activities appear to be more similar to those of cell-wall glucans than to those of cell-surface mannans, possibly due to the terminal glucosyl units (Kogan, *et al*., 1993). Potent anti-mutagenic, anti-clastogenic, and bioprotective activities of *Candida utilis* GM against chemical compounds with different modes of action were documented (Vlckova, *et al*., 2004). The protective effect of GM *in vivo* was studied after p.o. or i.p. administration in the model of cyclophosphamide-induced mutagenicity prior to cyclophosphamide injection (Chorvatovicova, *et al*., 1999). A recent study described GM due to its efficient action as an anti-mutagen, anti-clastogen, DNA-break inhibitor or inducer, and as cytotoxic/cytostatic effect enhancer. Several possible mechanisms of the observed bioprotectivity, including free radical scavenging, have been suggested (Miadokova, *et al*., 2006).

Prokopova, *et al*., (1993) were the first to describe a therapeutic effect of simple carbohydrates on rat adjuvant arthritis (AA). Methyl-α-D-mannopyranoside, mannooligosaccharides, and yeast mannans inhibited the development of rat AA. While a mannan from *Candida albicans* inhibited both the inflammation and destructive arthritic changes, a mannan from *Saccharomyces cerevisiae* showed a lower effect. However, acetolysate of *S. cerevisiae* mannan as well as simple methyl-α-D-mannopyranoside inhibited both inflammation and destructive arthritic changes to a similar degree as the mannan isolated from *C. albicans*. The effect, which was not

dose-dependent, was indicative of a possible immunoregulatory mechanism of mannans.

As to immunomodulators with anti-oxidant activity, new ways of supplementary or combinatory RA therapy have been investigated. Protective anti-oxidant and anti-inflammatory activities of carboxy-lated (1-3)-beta-D-glucan isolated from *S. cerevisiae* were reported in AA in Lewis rats (Kogan, *et al*., 2005). GMs from *C. utilis* were evaluated in the same model. The anti-arthritic activity for cell-wall GM was associated with anti-oxidant activity *in vivo* (Bauerova, *et al*., 2006; Mihalova, *et al*., 2007).

In our experiment described in this paper, the objectives were to assess the potential anti-arthritic activity of GM from *C. utilis* and of Imunoglukán®, a beta-(1,3/1,6)-D-glucan (IMG) isolated from *Pleurotus ostreatus*, in the model of AA in rats.

Material and methods

GM and Imunoglukán®

The yeast strain *C. utilis* was used as a biological source of GM. The strain CCY 29-38-18 was obtained from the collection of yeast and yeast-like micro-organisms (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia). GM was isolated from cell wall glycoproteins using extraction with 2% KOH and purification with Fehling reagent, as described previously (Kogan, *et al*., 1988). Imunoglukán®, a beta-(1,3/1,6)-D-glucan (IMG), was isolated from *P. ostreatus* and donated by the manufacturer (Pleuran®, Bratislava, Slovakia)

AA induction and experimental design

After approval by the local ethics committee, AA was induced in male Lewis rats (Breeding Farm Dobrá Voda, Slovakia), weighing 150–170 g each, by a single intradermal injection of heat-inactivated *Mycobacterium butyricum* (MB) in incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA). In each experimental group, 6–8 animals were used. The experiments included healthy intact animals as reference controls (Control), arthritic animals without any drug administration (AA), and arthritic animals with the administration of GM (GM-AA) in the oral daily dose of 15 mg/kg b.w. and of IMG (IMG-AA) in the oral daily dose of 2 mg/kg b.w. The glucans tested were administered from day 1, i.e., the day of immunization, to the experimental day 28.

Clinical, biochemical, and immunological analysis

We monitored one basic clinical parameter: change in the hind paw volume (HPV). The HPV increase was calculated as the percentage of increase in the HPV on day 28 in comparison with the beginning of the experiment.

Blood for biochemical analysis was withdrawn from the retroorbital plexus before MB injection (Control) and on experimental days 14, 21, and 28. The measurement of biochemical parameters was performed as described below: total anti-oxidant status (TAS) in plasma was measured by the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) assay (Rice-Evans and Miller, 1994). Thiobarbituric acid-reacting substances (TBARS) in plasma were measured spectrophotometrically at 535 nm (Brown and Kelly, 1996). The activity of cellular gamma-glutamyl transferase (GGT) in hind paw joint tissue homogenates was measured by the method of Orlowski (Orlowski and Meister, 1970) as modified by Ondrejickova (1993). All these biochemical parameters were determined on day 28. The detection of plasma IL-1α, IL-4, TNF-α, and MCP-1 was done by the flowcytometric (Cytomics FC 500, Beckman Coulter Inc. Fullerton, USA) fluorescent bead-based multiplex assay Rat Cytokine Flow Cytomix Multiplex (Bender Med System, GmbH., Austria) on experimental days 14, 21, and 28.

Statistics

The experimental values are given as means ± SEM. The statistical significance of differences between means was established by Student's *t*-test and P values < 0.05 were considered statistically significant, $P < 0.01$ very significant and $P < 0.001$ extremely significant. The arthritis group was compared to healthy control animals (*), the treated AA groups were compared to the untreated AA group (+).

Results and discussion

Nowadays, new ways of supplementary or combinatory RA therapy are of great importance. In our experiments, we have studied two natural compounds – GM and IMG in AA. Both glucans have a significant lowering effect on the basic clinical parameter – the change in the hind paw, which

was dramatically increased by the arthritis process, and that more than five times in comparison to healthy controls (Figure 1A). Similarly, a lowering effect, comparable for GM and IMG, was also observed for GGT activity assessed on day 28 (Figure 1B). The effect of GM and IMG on TAS in plasma (Figure 2A) and on plasmatic TBARS (Figure 2B) was, however, different. The anti-oxidative effect of IMG was evidently more pronounced than that of GM. These results are in agreement with the study of Bobek and Galbavy (2001) concerning the efficacy of IMG on the anti-oxidative status of the rat organism. They reported reduced precancerous lesions in the colon, together with improved glutathione peroxidase activity in erythrocytes and liver, as well as significantly reduced glutathione levels in the colon. Moreover, the administration of IMG in experimental colitis in rats indicated that IMG enhanced the anti-oxidant defense of the colonic wall against the inflammatory attack and played a possible role in the treatment of ulcerative colitis (Nosalova, *et al.*, 2001; Bobek, *et al.*, 2001).

The good anti-oxidative and anti-inflammatory effect of IMG gave us the impulse to study in a more complex way its effect on the course of the main cytokines/chemokines in AA. The course of plasma levels of pro-inflammatory cytokines TNF-α (Figure3A) and of IL-1 α (Figure 3B) in arthritis is very similar, with the maximum on day 14 and with decreasing levels for days 21 and 28 in comparison to day 14. The daily administration of IMG suppressed significantly these levels for all days monitored. Moreover, the observed inhibitory effect of IMG became stronger with time. These obtained results are of importance as TNF-α controls the gene expression of various cytokines and chemokines in different cell types engaged in the host immune response to infection and triggers the cascade of cytokines acting in the inflammatory response. The efficient biological activities of TNF-α include direct activation of T- and B-lymphocytes, macrophages, and natural killer cells, release of acute-phase proteins, and endothelial cell activation. The activated monocyte or macrophage represents the primary source for TNF-α, especially after IFN-γ priming. TNF-α is a key regulator of other pro-inflammatory cytokines such as IL-1 α, IL-6, and IL-8. IL-1 α is a further pro-inflammatory cytokine similar to TNF-α in

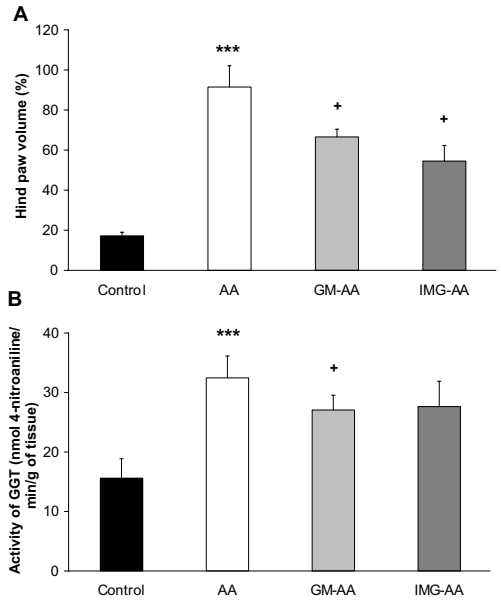

Figure 1 Parameters of inflammation – HPV (A) and activity of cellular GGT assessed in hind paw tissue homogenate (B). Comparison of the effect of GM and IMG administration in AA. The experimental values are given as means ± SEM. The statistical significance of differences between means was established by Student's *t*-test. The arthritis group was compared to healthy control animals (*), the treated AA group was compared to the untreated AA group (+). $^{***}P < 0.001$: extremely significant; $^{*}P < 0.05$: significant.

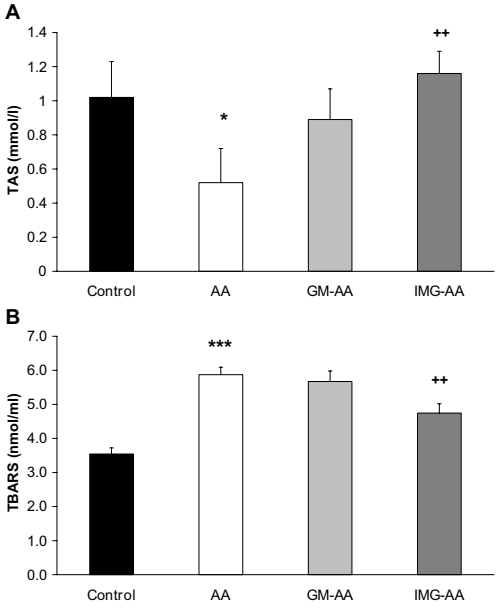

Figure 2 Parameters of oxidative stress – total anti-oxidant status (A) and level of TBARS in plasma (B). Comparison of the effect of GM and IMG administration in AA. The experimental values are given as means ± SEM. The statistical significance of differences between means was established by Student's *t*-test. The arthritis group was compared to healthy control animals (*), the treated AA group was compared to the untreated AA group (+). $^{*}P < 0.05$: significant, $^{**}P < 0.01$: very significant; $^{***}P < 0.001$: extremely significant.

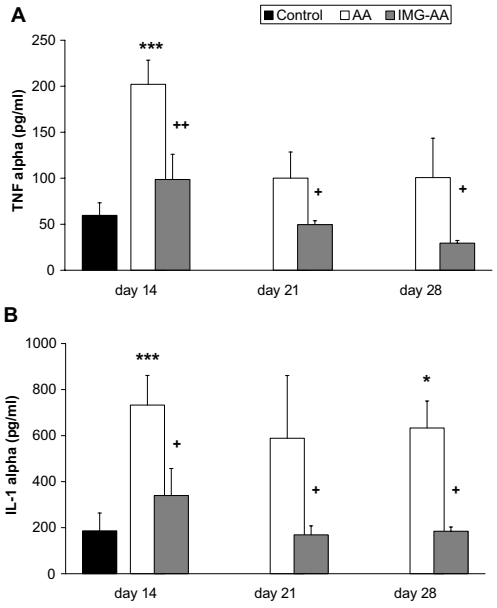

Figure 3 Pro-inflammatory T_H1 cytokines – level of TNF-α (A) and IL-1 α (B) in plasma. Effect of IMG treatment in time profile. The experimental values are given as means ± SEM. The statistical significance of differences between means was established by Student's *t*-test. The arthritis group was compared to healthy control animals (*), and the treated AA group was compared to the untreated AA group (+). $^{*}P < 0.05$: significant, $^{***}P < 0.001$: extremely significant; $^{+}P < 0.05$: significant, $^{++}P < 0.01$: very significant.

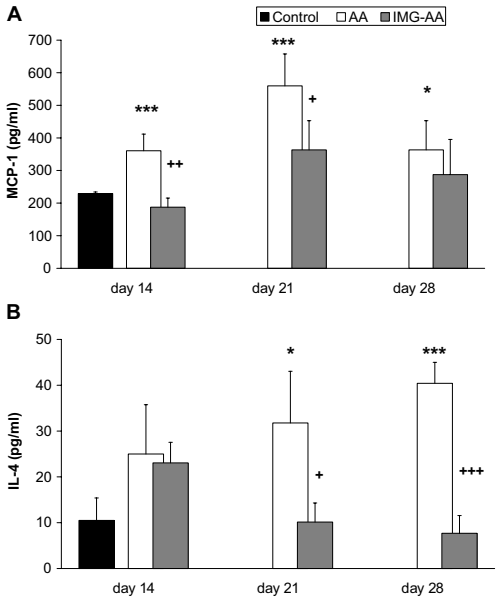

Figure 4 Levels of MCP-1 (A) and of the anti-inflammatory T_H2 cytokine – IL-4 (B) in plasma. Effect of IMG treatment in time profile. The experimental values are given as means ± SEM. The statistical significance of differences between means was established by Student's *t*-test. The arthritis group was compared to healthy control animals (*), the treated AA group was compared to the untreated AA group (+). $^{*}P < 0.05$: significant, $^{***}P < 0.001$: extremely significant; $^{+}P < 0.05$: significant; $^{++}P < 0.01$: very significant, $^{+++}P < 0.001$: extremely significant.

many aspects. It has a severe impact on different cell populations and exerts biological effects, e.g., increased synthesis of acute phase reactants. IL-1 α is secreted by monocytes/macrophages activated *via* TNF-α and/or bacterial endotoxin. Furthermore, IL-1 α markedly potentiates the toxic effect of TNF-α in animal experiments (Waage, *et al.*, 1991).

Further, we followed the course of MCP-l (Figure 4A). This chemokine is mainly expressed by macrophages in response to a wide range of cytokines, e.g., TNF-α and IL-1. In this experiment, the significant maximum of MCP-1 plasma level measured on day 21 and following decrease is in close association with kinetics of both TNF-α and IL-1α. According to the target cell specificity, MCP-1 was postulated to play a pathognomonic role in various diseases with monocyte cell infiltration. After IMG treatment, MCP-1 level was decreasing significantly on days 14 and 21. The course of MCP-1 level was found to be very close for treated and untreated arthritis animals. A completely different picture was revealed for IL-4 (Figure 4B). The level of this anti-inflammatory cytokine was increasing with time – the maximum was observed on day 28 in AA animals. IMG has probably an indirect inhibitory effect on this cytokine, which is time dependent. IL-4 is a pleiotropic cytokine produced by mature T_H2 cells and mastocyte- and/or basophil-derived cells. IL-4 has marked inhibitory effects on the expression and release of monocyte-derived pro-inflammatory cytokines, e.g. IL-1, TNF-α, IL-6, IL-8, and MIP-1α. It was shown to suppress macrophage cytotoxic activity, parasite killing, and macrophage-derived nitric oxide production (Vannier, *et al.*, 1992).

Conclusion

IMG had a more pronounced anti-inflammatory and anti-oxidant effect than the GM studied. Thus, it may be considered a candidate for supplementary and combinatory therapy of RA due to its optimizing immunomodulating effect
on the selected pro-inflammatory and anti-inflammatory cytokines and on MCP-1.

Acknowledgements

The work was supported by grants APVV-21-055205, APVV-51-017905, VEGA 2/0090/08 and COST B35.

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