ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Research paper Acta Neurobiol Exp 2009, 69: 73–78

Spinal cord hydrolysate ameliorate immunological reaction in experimental allergic encephalomyelitis

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The aim of this study was to use the hydrolysate of pig spinal cord proteins to induce oral tolerance in the animal model of sclerosis multiplex – experimental allergic encephalomyelitis. The female Lewis rats were fed with hydrolysate of pig spinal cord proteins in two doses for one week before immunization, which was induced by injection of guinea pig spinal cord homogenate. At the peak of clinical symptoms (the 13th day post immunization) the rats were sacrificed and the spleen removed. Splenocytes were suspended in a culture medium and placed in microculture plates. The cells were stimulated with homogenate. The cells were cultured for seven days. Proliferation of splenocytes was estimated by means of methyl-3H thymidine incorporation. In supernatants of cultures of splenocytes the level of cytokines INF-γ, IL-10, IL-4, and TGF-γ was measured. It was demonstrated that homogenate-induced splenocytes of hydrolysate-fed rats gave rise to low proliferation as compared to the controls used. The IFN-γ was inhibited in hydrolysate-fed animals. The hydrolysate of pig spinal cord proteins has a modulatory effect on the immune reaction, particularly on the orally-induced antigen-specific modulation of autoimmune response.

Key words: oral tolerance, sclerosis multiplex, experimental allergic encephalomyelitis

INTRODUCTION

Multiple Sclerosis (MS) is common disease in Western countries, which affects especially people between 20 and 40 years old. There are four different forms of MS: relapsing-remitting (RRMS), primary progressive (PPMS) and secondary progressive (SPMS) and progressive-relapsing (PRMS). The etiology of MS still remains unidentified but some of researchers suggest the reasons can be viruses (Zawada et al. 2006), which have structural homologies with myelin antigens (molecular mimicry), environmental factor/s, and/or genetic predisposition. As is known so far, MS is the autoimmune, inflammatory, demyelinating disease of the central nervous system (CNS) what is characterized by a demyelination of axons in inflammatory plaques (Hohlfeld and Wekerle 2004, Hauser 2005). When the myelin is lost the transmission of signals between nerves is slowed or even broken down. It is believed that MS is disease, with cell-mediated and humoral response which is directed against myelin proteins: myelin basic protein (MBP), myelin associated glycoprotein (MAG), myelin oligodendroglial glycoprotein (MOG) (Ewing and Bernard 1998). In the normal conditions the brain is isolated from peripheral immune system by the blood-brain barrier, but in the MS and EAE those cells have an access to the brain, what is mainly mediated by adhesion molecules and chemokines. It had been found that expression of Nerve Growth Factor receptor is increased in spinal cord in EAE (Oderfeld-Nowak et al. 2003) as well as expression of oligodendroglia progenitors (Oderfeld-Nowak et al.

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Received 28 October 2008, accepted 28 November 2008

2002). The activated, autoaggressive T cells have access to the brain. There after finding their specific antigen in CNS they become activated again. They start production of pro- and anty-inflammatory cytokines and recruit other immune cells such as: B lymphocytes and macrophages, which lead to the damage of myelin and axons (Lassman 2005). The migration of inflammatory cells into and within the brain involve the action of proteolytic enzymes, mainly matrix metalloproteases (Cuzner and Opdenakker 1999, Kwiatkowska-Patzer et al. 2004). At this time there are three types of disease-modifiers which are approved and used for treatment of MS. Those include different forms of interferon – beta-1b and beta-1a (known as an Avonex, Rebif and Betaseron, Betaferon), a copolymer of four amino acids termed glatiramer acetate (known as a Copaxone) and mitoxantrone. Immunomodulatory therapy may influnce synthesis of cytokines (Losy 2007). In spite of still growing number of available medicines and therapies for MS treatment no one can stop the disease processes (Steinman 2001, Lassman 2005, Cendrowski 2004). Induction of neurogenesis from oligodendrocytes progenitors (NG2) and promotion their defferentiation into oligodendrocytes and neurons gaves a great hope for future treatment for MS (Sypecka et al. 2007). One of the other methods used in MS treatment, but still in the pre-clinic tests, usually on EAE animal model of relapsing or chronic inflammatory demyelination of the CNS is oral tolerance (Kwiatkowska-Patzer et al. 2003). After injection of myelin basic protein with adjuvant, rats or mice develop an acute paralysis, but if we orally administrate them myelin antigen or antigens before immunization, they inhibit paralysis sign and histopathological infiltrates of EAE (Higgins and Weiner 1988, Kelly and Whitacre 1996). Oral tolerance is lack of respond on particular antigen and it is explain by three different mechanisms: clonal anergy, deletion and active suppression (Jewell et al. 1998). Those forms of oral tolerance are associated to the amount of fed antigen and timing of oral antigen administration. Multiple low doses of fed antigen reduce the clinical paralysis and histopathological EAE by active suppression. The suppressor cells are antigen specific T lymphocytes which appear to mediate suppression by secreting anti-inflammatory transforming growth factor beta (TGF-beta) interleukin-4 (IL-4) and interleukin-10 (IL-10). However higher doses of fed antigen induce clonal anergy or deletion (Kelly and Whitacre 1996). The aim of this study was to use the hydrolysate of pig spinal cord proteins to induce oral tolerance in animal model of sclerosis multiplex – experimental allergic encephalomyelitis.

METHODS

Animals

Female Lewis rats, six weeks old were used to experiments. Induction of EAE was made by injection of guinea pig spinal cord 50% homogenate with complete Freund Adjuvant and Mycobacterium tuberculosis into hint pad. Rats were weighted and examined for clinical signs. Animal care procedures were conducted in conformity with Intramural Committee and Institutional guidelines in accordance with National and International laws and policies.

Induction of oral tolerance. Examined rats were fed with hydrolysate of pig spinal cord (5 mg/kg or 20 mg/kg) every other day one week before immunization. The hydrolysate of pig spinal cord proteins was prepared in dr Lipkowski laboratory (Lipkowski et al. 2000).

The clinical course was observed and evaluated in a five grade scale. Scores were graded as follows: 0 – no symptoms, 1 – limp tail, 2 – hind leg weakness, 3 – hind leg weakness and incontinence, 4 – paraplegia and weight loss, 5 – death. At the peak of clinical symptoms (the 13th day post immunization) the rats were sacrificed and the spleen removed. Brain, spinal cord and serum were frozen.

Spleen cells were prepared by grinding through a wire mesh, depleted of red cells with osmotic lysis, washed in phosphate-buffered saline (PBS) and suspended in RPMI-1640 with 2 mM glutamine, supplemented with 10% of fetal calf serum (FCS, Gibco, Paisley, UK). Cell viability determined by trypan blue uptake was over 90%.

Cytokines levels (IFN-gamma and IL-10) were tested in the supernatants of spleen cells (2×10^6/ml) cultured for 48 h at 37°C in Costar plates in a volume of 1 ml medium at a humidified atmosphere containing 5% CO_2. The guinea pig spinal cords samples were mixed at 1:10 ratio in RPMI-1640, centrifuged to remove debris and filtered 0.22 μm filter. The spleen cells were either stimulated with this solution added to the culture medium at a final concentration of 1:100 (previously tested to be optimal both for cytokines synthesis and lymphocyte proliferation) or medium

alone. After the incubation time the plates were centrifuged and supernatants were tested for IL-10 and IFN-gamma concentrations by means of an enzyme-linked immunoabsorbent assay (ELISA) using commercially available ELISA-sets (Becton-Dickinson). Sensitivity of the ELISA was 10 pg/ml for both cytokines. A microtiter plate ELISA reader (Labsystem Multiscan-PLUS) with simultaneous computer data analysis (Serocalc, Merlin) was used. Results are presented in pg/ml.

Lymphocyte proliferation

Spleen cells were obtained as described above, washed in PBS and suspended in RPMI-1640 with 10% FCS at a concentration of 1×10^6 /ml. The cells were stimulated for 5 days with guinea pig spinal cord solution (prepared as for cytokines induction) or medium alone (control). The cultures were set up in triplicate (2×10^5 cells /well), in a sterile 96-well flat-bottomed culture plates (Falcon), in a humidified atmosphere containing 5% CO_2. The cultures were pulsed with 0.5 μCi/well methyl-3H thymidine (Amersham) for the last 16 h of incubation and harvested onto glass-fiber filters. Thymidine incorporation was determined by liquid scintillation counting (LKB instruments, Stockholm, Sweden). The results were expressed in cpm as the mean value of triplicate determinations.

Level of interleukine-10 (IL-10) was measured in tissue (brain and spinal cord) homogenates and in the serum using Western blot method. Proteins were divided using electrophoresis in polyacrylamide gel under constant voltage (150V). Protein transfer onto nitrocellulose membrane was performed under constant current (350 mA) for 2 hours. After transfer nitrocellulose was blocked in 5% non-fat milk for 1 hour in a room temperature. Blocked nitrocellulose, after washing in PBS-Tween, was incubated with first antibody (mouse monoclonal anti-rat IL-10, R&D) overnight in 4°C. Nitrocellulose was washed in PBS Tween, and then incubated with second antibody (ECL Anti-Mouse IgG, Horseradish Peroxidase-Linked, Amersham) diluted in 5% non-fat milk, for 1 hour, in room temperature. Washed in PBS-Tween nitrocellulose, was incubated with chemiluminescence reagent (ECL, Amersham) for 1 minute. Then, film was applied on a nitrocellulose, and after 2 hour exposition in

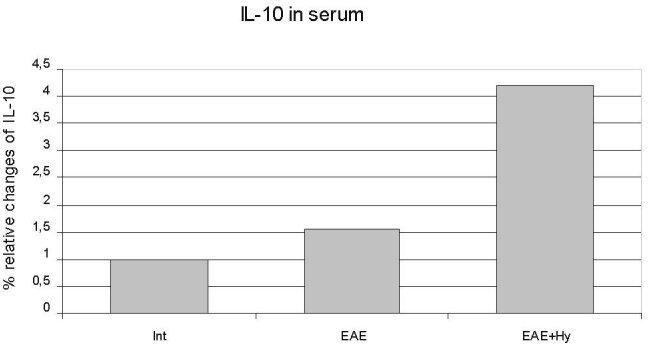

Fig. 1. Percentage changes of interleukine-10 (IL-10) level in rats serum after evoking EAE (EAE), and after oral administration of spinal cord hydrolisate to EAE animals (EAE + Hy), in comparison to Intact animals (Int). IL-10 level in Intact animals is 100%. It is densitometric analysis.

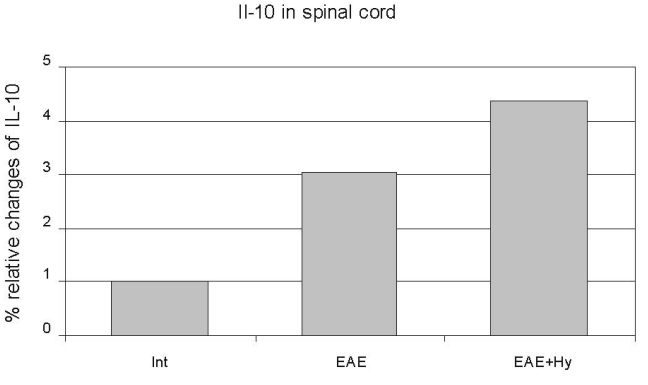

Fig. 2. Percentage changes of interleukine-10 (IL-10) level in rats spinal cord homogenate after evoking EAE (EAE), and after oral administration of spinal cord hydrolisate to EAE animals (EAE + Hy), in comparison to Intact animals (Int). IL-10 level in Intact animals is 100%. It is densitometric analysis.

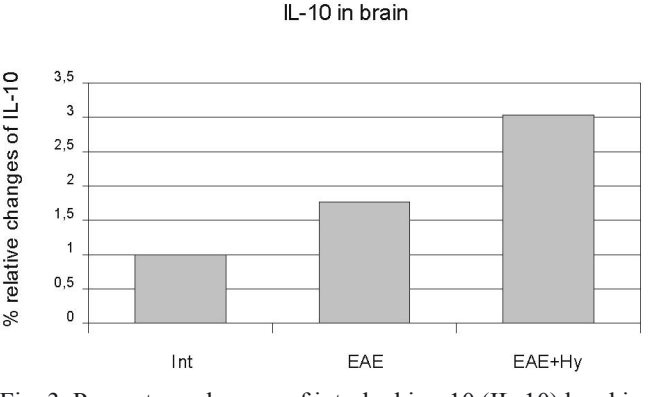

Fig. 3. Percentage changes of interleukine-10 (IL-10) level in rats brain homogenate after evoking EAE (EAE), and after oral administration of spinal cord hydrolisate to EAE animals (EAE + Hy), in comparison to Intact animals (Int). IL-10 level in Intact animals is 100%. It is densitometric analysis.

Table I

Comparison of proliferation of splenocytes from hydrolysate-fed and non-fed Lewis rats (in cpm)

Experiment	Non-fed EAE	Hydrolysate-fed EAE + H	Control NT	% of suppression
1	18 421	1 342	448	95.1
2	1 233	418	400	97.9
3	2 708	232	453	91.5
4	5 960	3 522	851	47.8
5	7 414	3 617	338	53.7

Proliferation was stimulated by homogenate in concentration (1:8)

Table II

Changes in the level of INF γ (in pg/mL)

Experiment	Control	EAE	EAE/H	% of suppression
8		1 063	266	75.0
9		730	100	86.3
19 (48 h)	17	2 164	1 036	52.6
19 (72 h)	288	3 871	2 593	35.7
16		3 555	2 328	34.5
25		950	465	51.1
26	28	410	30	99.4

Table III

Amount of cytokines secreted by splenocytes from hydrolysate-fed and non-fed Lewis rats (in pg/mL)

Cytokines	Splenocytes	Hours after antigen stimulation		
		48	72	96
IL-10	Non-fed	254	520	121
	Hydrolysate-fed	283	293	169
IFN-γ	Non-fed	2 164	3 871	410
	Hydrolysate-fed	1 036	2 593	184

a closed cassette, was evoked. Intensity of a stripes was measured using image analysis software (GelExpert, Nucleo Vision).

RESULTS

The proliferation of splenocytes has been suppressed by 40–94% in rats fed with hydrolysate of pig spinal cord (Table I). The level of interferon gamma in splenocyte culture supernatant was diminished in rats fed with hydrolysate of pig spinal cord (Table II). The level of interleukine-10 in splenocyte culture supernatant was slightly increased in some experiments. Table III presents comparison of the level of interferon gamma and interleukin 10 in culture supernatant after 48, 72 and 96 hours. Other cytokines, interleukine 4 and tumor growth factor (TGF-β) were found in trace amounts.

Determination of interleukine-10 by Western blot method in rat tissue (brain and spinal cord) and in serum showed 3–6-fold increase in rats fed with hydrolysate of pig spinal cord in comparison to the control (Fig. 1–3).

DISCUSSION

Oral tolerance is a method to diminish the immune response. Orally administered autoantigen induce tolerance *via* the active suppression or clonal anergy (Friedman and Wiener 1994). Important factors dictating which mechanism predominates are the dose of fed antigen, frequency of feeding and the mode of antigen presentation. Low doses induce the active suppression, high doses favor clonal anergy. The regulatory cells that mediate active suppression act *via* the secretion of suppressive cytokines such as TGF-β and IL-4. Antigen stimulate gut-associated lymphoid tissues (GALT) and generate a Th2 type response (Mowat et al. 1996). Initial clinical trials of oral tolerance in multiple sclerosis, rheumatoid arthritis and uveitis gave positive effect (Whitacre et al. 1991, Weiner et al. 1994, Pryce et al. 2005). Our proposal to use the hydrolysate of spinal cord (mixture of neuropeptides) to induce oral tolerance to myelin antigen seems to be promising for amelioration immunological response. In our studies the small doses of hydrolyzate were given prior to animal immunization. In fed animals some data indicate on active suppression mechanism – decreased splenocytes proliferation and decrease of interferon gamma.

CONCLUSION

Results indicate that the mixture of neuropeptides in the hydrolisate of spinal cord given orally diminished immunological response to myelin antigen. This is probably caused by active suppression involving decrease splenocytes proliferation and interferon gamma level. It might have a clinical implication in SM management.

ACKNOWLEDGEMENT

This work has been supported by Polish Grant 2PO5A 17525.

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