BACTERIAL LIPOPOLYSACCHARIDE ENHANCED IMMUNOLOGICAL RESPONSIVENESS IN EXPOSED RATS

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Abstract: In order to investigate the effects of the bacterial lipopolysaccharide (LPS) on the brain and serum protein content, LPS was stereotaxically infused into the substantia nigra (SN) of rats at different dosages (3 µg, 10 µg or 250 µg/kg). The results showed that 7 days after neurosurgery there is no variations in brain protein content, while in serum protein content, the amount of gamma-globulins significantly increased compared to sham-operated control rats. The results suggested an immunologic response in the injected rats exposed to the bacterial lipopolysaccharide.

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra (SN) and the presence of Lewy body inclusions in residual neurons. In recent years, increasing evidence has strongly suggested a role for inflammation in the brain in the pathogenesis of PD (Sun et al., 2003). Bacterial endotoxin lipopolysaccharide (LPS) is one of common toxins produced by Gram (-) bacteria, including *Escherichia coli*. This agent can elicit a multitude of pathophysiological effects, including inflammation, macrophage activation, fever, and septic shock (Burrell, 1994). The blood-brain barrier can become leaky as a result of sepsis (Brandtzaeg et al., 1989), allowing LPS to enter the cerebrospinal fluid.

Previous studies have demonstrated that bacterial LPS exposure, that mimics Gram (-) bacterial infections, could cause a significant loss of dopamine (DA) neurons in the substantia nigra (SN) of rat. LPS by inducing DA neurons degeneration serve as an important agent for elaboration of an experimental PD model. Along with DA neuron loss are the α-synuclein positive Lewy body-like inclusion formation and innate immunity dysfunction manifested by increase in number of reactive microglia, increase in pro-inflammatory cytokine levels, and blood-barrier leakage (Wang et al., 2009). LPS administration, upon first exposure, engages the innate immune system leading to the development of the acute phase response (APR; Heumann and Roger, 2002). The APR occurs as a result of immune activation, consisting primarily of the peripheral release of pro-inflammatory cytokines by circulating macrophages and monocytes. After the first exposure to LPS animals develop an adaptive immune response and the activity of the innate immune system is diminished. As a result of this, upon secondary LPS exposure, tolerance develops and a decrease in both physiological and behavioral measures of sickness is often observed (Franklin et al., 2003). In other study (Makela et al., 1983) an injection of bacterial lypopolisaccharide (LPS) into mice caused a considerable increase in the serum concentration of IgM and IgG (total Ig rose three-to four-fold in 7 days) and a corresponding increase in the concentration of antibodies.

The present study was conducted to determine the effects of LPS-induce immune challenge on brain and serum protein content of rats, with relevance for Parkinson’s disease.

MATERIALS AND METHODS

Animals

29 male Wistar rats weighing 300 ± 50 g at the start of the experiment were used. The animals were housed in a temperature- and light-controlled room (22°C, a 12-h cycle starting at 08:00 h) and were fed and allowed to drink water ad libitum. Rats were treated in accordance with the guidelines of animal bioethics from the Act on Animal Experimentation and Animal Health and Welfare Act from Romania and all procedures were in compliance with the European Council Directive of 24 November 1986 (86/609/EEC).

Stereotaxic injection of LPS into SN of rats

All surgical procedures were conducted under aseptic conditions, under sodium pentobarbital (45 mg/kg b.w., i.p., Sigma) anesthesia. Rats were mounted in the stereotaxic apparatus with the nose oriented 11° below horizontal zero plane. For injection of LPS into the SN of rats, the following coordinates were used: 5.5 mm posterior to bregma; 2.0 mm lateral to the midline; 7.4 mm ventral to the surface of the cortex (Paxinos and Watson, 2005). Rats were divided into different dosage of LPS (3 µg, 10 µg or 250 µg/kg). LPS was prepared as a stock solution of 1 µg/µl in sterile saline solution. After each injection, the needle was left in situ for an additional 5 min to avoid reflux along the injection track. The sham-operated rats were injected with same volume of sterile saline solution. 7 days after neurosurgery, all rats were anesthetized, rapidly decapitated and whole brain were removed. The temporal lobes were collected. Each of brain tissue samples was weight and homogenized with a Potter Homogenizer coupled with Cole-Parmer Servodyne Mixer in

bidistilled water (1g tissue/10ml bidistilled water). Samples were centrifuged 15 min at 3000rpm. Following centrifugation, the supernatant was separated and pipetted into tubes. Also, whole blood was collected and incubated for 1h at room temperature and than serum was separated, by centrifugation 15 min at 3000 rpm.

SDS-PAGE analysis of soluble proteins

SDS-PAGE was performed using the discontinuous buffers system of Laemlli. Gradient 5-15% or 5-20 % gels were casted fallowing the procedure described by Ausubel, 2002, using a MIDI Gradient gel Mixer, Roth, Germany and a TV400YK (Scie-Plas,UK) electrophoresis apparatus.

The rat serum was diluted 1:10 with distillated water prior of use. 10 µl of diluted serum was mixed with SDS-loading buffer containing 100 mM mercaptoethanol (final concentration) and boiled for 10 min. on a Thermomixer (Eppendorf, Germany).

The brain extract were obtained as described above. Approximately 125 µg of proteins were mixed with SDS-loading buffer, boiled for 5 min. and loaded on each lane.

The gel was run at 55 mA/gel until the blue dye run out (usually overnight) and afterwards was stained using Coomasie Brillant Blue R 250 0,25% in 45% methanol, 10% acetic acid for about 2 hours. The destining was done in 30% methanol, 10% acetic acid until the background was completely de-stained. The gels were kept in 10% acetic acid until photographed.

Gel densitometry and molecular weight determination

The stained gels were photographed and quantified using ImageQuant TL from GE Healthcare. For quantitation a calibration curve was constructed by loading on gel in parallel with the samples 2,5; 5 and 10 µg bovine serum albumine (BSA). In the conditions used, the calibration curve had an regression coefficient of 0, 98. Molecular weight determination was done by running in parallel with the samples a wide range molecular weight protein marker from Sigma. The marker was used to fit a curve from which the molecular weight of the unknown proteins was determined.

Target proteins were recognized according to their molecular weight: an unknown negative control at 78 kDa, serum albumin at around 61 kDa (Peters, 1962), immunoglobulin heavy chain at about 51 kDa and light chain at about 21 kDa (Mathews, 2000). Samples were run in several repetitions (6-8). Each repetition was densitometrated and mean, standard deviation and confidence level for mean was calculated according to common statistical methods.

RESULTS AND DISSCUSIONS

The aqueous brain extracts from LPS treated rats (250 µg) and sham-operated rats control were resolved on 5-20 % gradient gels. About 20 protein fractions could be exponentiated by Commasie staining, but no significant and repetitive differences could be observed (Fig.1, A). All samples were rather uniform regarding their concentration, with the most abundant proteins not exceeding 12 % of total protein in both sample and control (56 kDa – 12% of total protein, 44 kDa – 9% of total protein and 6,6 kDa – 10.5% of total proteins (Fig.1, B).

Regarding the electrophoresis of serum proteins, one aspect must be highlighted. Unlike the older methods using disk electrophoresis or acetate gels, we used SDS-PAGE in both denaturing and reducing conditions. Because of this, the electrophoretic profile of separated proteins is slightly different. More precisely, the gamma-globulins do not migrate as a single high-molecular weight band, but are separated in two distinct fractions (Marshall, 1984).

In both the sample (LPS treated rats, 3 µg and 10 µg) and the control (sham-operated rats) around 20 fractions could be separated and visualized by Commasie staining. Unlike the brain extracts, the electrophoretic profile of serum proteins shows a much greater heterogeneity (Fig. 2), with 4 predominant protein fractions making 60 – 70 % of total protein content. According to their molecular weight, three out of these 4 proteins were recognized as being albumin (61 kDa), immunoglobulin heavy chain (51 kDa) and light chain (21 kDa). The forth band, an unknown protein of 78 kDa was arbitrary considered as a control.

Using bovine serum albumine (BSA) as standard for a calibration curve, the amount of each of these proteins could be measured by gel-densitometry. As it can be seen in table 1, injection of LPS (3µg and 10µg) in rats leads to a small decrease in the amount of serum albumin

and an increase in the amount of gamma-globulins (both the light and the heavy chains). This would indicate an immunologic response in the injected rats.

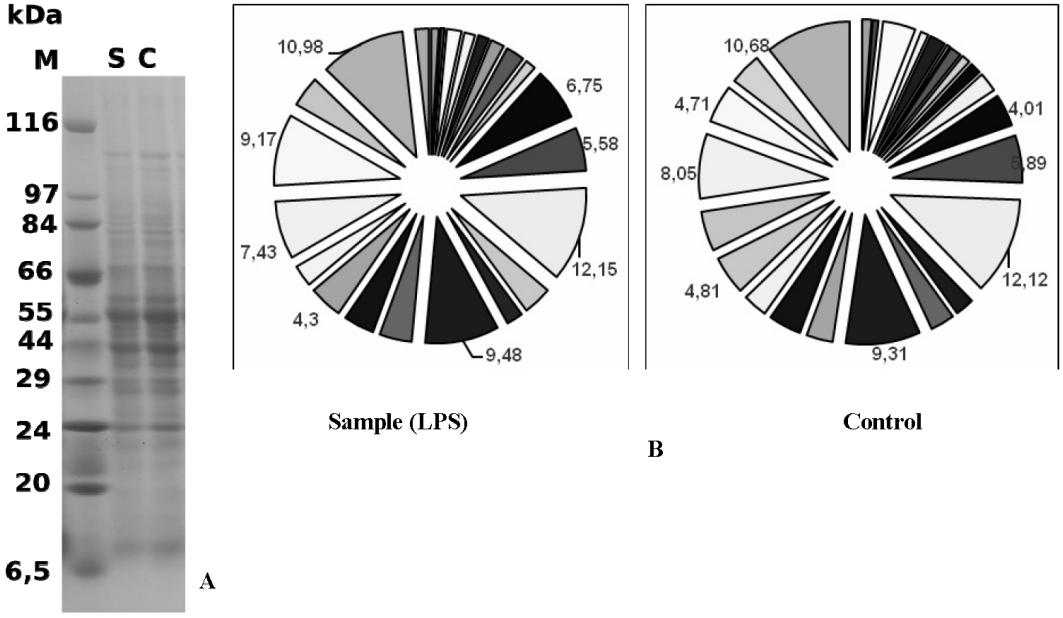

Fig. 1. Effects of LPS administration (250 µg) on rats brain protein content. A - electrophoretic profile of brain proteins; B- diagram of the percentage of total protein content resulted from gel densitometry of the same samples

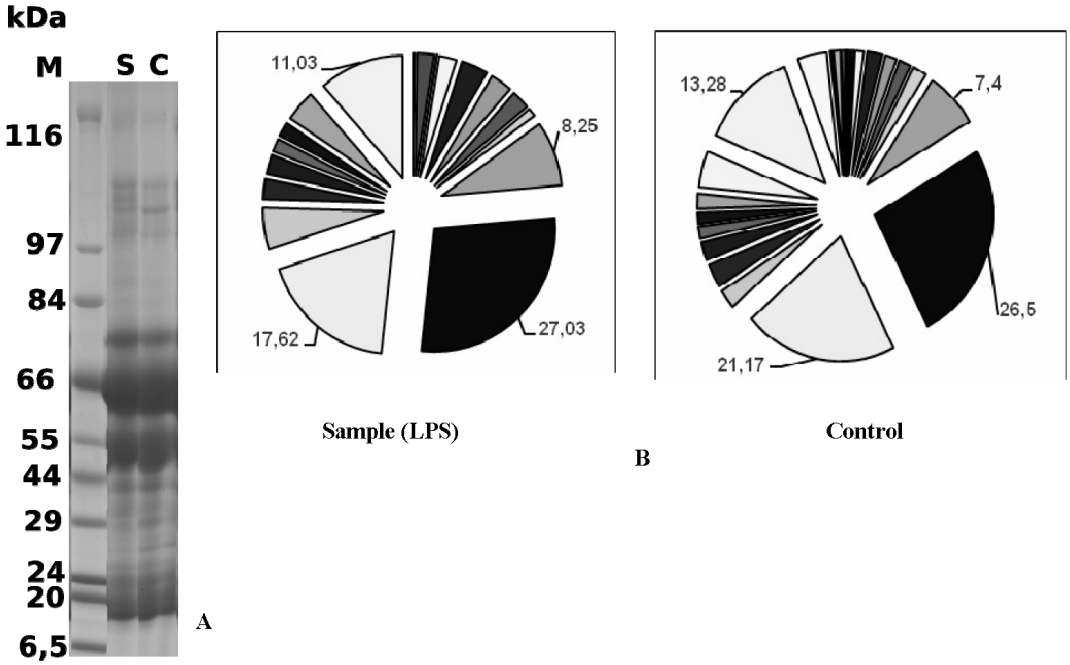

Fig. 2. Effects of LPS (3 µg and 10 µg) administration on rat serum protein content. A - electrophoretic profile of serum proteins; B- diagram of the percentage of total protein content resulted from gel densitometry of the same samples (LPS – 27.03% - albumin; 17.62% –gamma-globulins heavy chain; 11.03% - gamma-globulins light chain; Control – 26.5% - albumin; 21.17% - gamma-globulins heavy chain; 13.28% - gamma-globulins light chain.

Table 1. Effects of LPS administration on serum protein content

	LPS		**Control**		**p**
	Mean	**S.E.M.**	**Mean**	**S.E.M.**	
Unknown 78 kDa control	13.88	0.28	13.1	0.27	0.44
Albumine	46.74	0.32	48.86	0.49	0.0017
Gamma-globulins heavy chain	35.91	0.3	33.6	0.79	0.02
Gamma-globulins light chain	23.6	0.57	20.47	0.41	0,00415

CONCLUSIONS

On the basis of our results obtained by LPS administration, we can conclude that in the rats, stereotaxic administration of LPS induced immune responsiveness in rats.

REFERENCES

Ausubel, M.F., Brent, R., Kingston, E.R., Moore, D.D., Seidman, J.G., Smith, A.J., Struhl, K., 2002, Wiley John & Sons Inc
Brandtzaeg, P., Kierulf, P., Gaustad, P., Skulberg, A., Bruun, J.N., Halvorsen, S., Sorensen, E., 1989, *J Infect Dis*, 159: 195-204
Burrell, R., 1994, *Circ Shock*, 43, 137-153
Franklin, A.E., Engeland, C.G., Kavaliers, M., Ossenkopp, K.-P., 2003, *Psychopharmacology*, 170, 399-408
Heumann, D., Roger, T., 2002, *Clin. Chim. Acta*, 323, 59-72
Makela, O., Seppala, I.J.T., Vaara, 1983, *Annales de l'Institut Pasteur/Immunologie*, 134, 25-36
Mathews,K.C., van Holde, K.E., Ahern, K.G., 2000, *Biochemestry*
Paxinos, G., Watson, C., 2005, *Academic Press, Sidney*
Peters, T., Jr., 1962, *The Journal of Biological Chemestry*, 237, 1181-1185
Sun, S.G., Li, G., Cao, X.B., 2003, *Chin J Neurol (Chinese),* 36: 401
Wang, S., Yan, J.Y., Lo, Y.K., Carvey, P.M., Ling, Z., 2009, *Brain Res.*, 1265: 196-204

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