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Is the Identification of Antibodies Against the Nervous Tissue an Indicator of Brain Injury?

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Summary

Using a modification of the ELISA method, auto-antibodies against the own nervous tissue have been identified in the serum of laboratory rats. The prevalence of the IgM class of antibodies suggests their physiological significance. Antibody levels are higher in females than in males. Brain hypoxic injury brings about a shift in the spectrum of antibodies towards the IgG class. It may thus serve as an indicator of brain impairment caused by a lack of oxygen.

Key words

Brain • Auto-antibody detection • ELISA method • Brain damage indicator

Introduction

Hypoxic damage of the brain resulting from perinatal asphyxia is a frequent cause of a non-progressive neurological deficits in children, which may concern a delay in mental development, seizures, spasticity, choreoathetosis and ataxia (for review see Trojan and Šťastný 1988, Trojan and Gross 1990, Volpe 1992). Such posthypoxic changes may be caused by various reactive oxygen products formed after reperfusion. They may inhibit proteosynthesis, destroy cell membranes and ultimately bring about the loss of whole neuronal populations (Watson *et al.* 1996). The damaged nervous tissue may become a source of autoantigens.

Production of antibodies against the brain tissue after exposure to a low oxygen atmosphere was described earlier. We found particularly high production of antibodies against prosencephalon, which suffers most heavily under hypoxic conditions (Trojan and Ježková 1979). Here we present our latest results obtained by the ELISA method. This very sensitive examination enables to identify individual isotypes of antibodies.

Method

Animals and experimental hypoxia

Antibodies were detected in the serum of laboratory rats of the Wistar strain of our own breed. Adult rats (3-month-old) were exposed to a single trial (one or eight hours) of altitude hypoxia (7000 m) or to intermittent hypoxia (7000 m) for 3 weeks in 5 days (8 hours a day, 5 days per week). Newborn rats were exposed to the same scheme of altitude hypoxia.

In the group of adult animals exposed once to altitude hypoxia for one hour, antibodies were determined

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60, 120 min, and 5 days after the exposure (group A). In the group of animals exposed to hypoxia once for 8 h, the determinations were performed 5 days after the exposure (group B). Animals of the group exposed to intermittent hypoxia were tested 5 days after the last exposure (group C). In animals exposed to intermittent hypoxia since birth till the 18th day, antibodies were also assessed 5 days after the last exposure.

Antigen preparation

Samples of the following three regions of the brain were analyzed: cerebral cortex, subcortical structures (basal ganglia, corpus callosum, capsula interna) and the cerebellum. The tissues were first homogenized using a glass pestle in physiological saline, then the sample was washed three times in phosphate buffer saline (PBS) in order to remove immunoglobulins of body fluids, and PBS was added to a final concentration of 100 mg per 5 ml of fluid. To release intracellular antigens, cells were lysed by repeated freezing and thawing in the presence of proteolytic inhibitor phenylmethylsulfonyl fluoride (PMSF) in the concentration of 5 μg per 1 ml of the sample. Samples were then divided into aliquots and stored at -20 °C.

ELISA

Rat cerebral antigen, processed as described above, diluted 1:100 in PBS was bound by physical adsorption upon polystyrene microtitration plates (GAMA-ČR). An attempt to bind the antigen covalently using glutaraldehyde was not successful. The binding process took two hours at 37 °C and then progressed till the next day at 4 °C. The next day, after two washings in PBS, followed by two washings in PBS + 0.05 % Tween 20, and after one hour of blocking of the binding sites by Tween 20, the tested sera were added in the volume of 50 μl per well. Fetal calf serum (FCS, 5 % in PBS) was used as a diluent. After testing different degrees of dilution, we used the dilution 1:200 to prove the presence of antibodies. The plates with tested sera were incubated overnight at 4 °C. After new washing, antigen-bound antibodies from the tested sera were detected by anti-immunoglobulin antibodies against IgG, IgM and IgA labeled by biotine (Immunotech). Anti-immunoglobulin antibodies were diluted (1:1000) in PBS with FCS (5 %). Samples were incubated for one hour at room temperature. The binding of biotinylated antibodies was detected using peroxidase-labeled streptavidine (dilution, dilutant and incubation conditions were analogous to the preceding step) and developed by a substrate reaction (orthophenylendiamine and hydrogen peroxide). The reaction was stopped by 2M H_2SO_4 and absorbancy was measured at 492 nm.

SDS-PAGE

Antigens in amounts of 80-100 μg in the sample buffer were separated on a gradient polyacrylamide gel (5 to 15 %) in the presence of sodium dodecylsulphate after which protein staining was carried out by Coomassie Brilliant Blue (Laemmli 1970).

Immunoblot

Antigens present in the suspension of the cerebral cortex, subcortical structures and cerebellum were divided by electrophoresis, transferred on a nitro-cellulose membrane and after blocking of free binding sites by 1 % skimmed milk, the membrane was incubated with the tested rat serum diluted at 1:20, then with the biotinylated antibody to rat immunoglobulins (anti-RatIgGMA-biot, Immunotech, diluted at 1:1000). Detection was performed by peroxidase-labeled streptavidine diluted at 1:1000. Aminoethylcabazole was used as substrate.

We also used a more sensitive method of specific detection of antibodies, i.e. enhanced chemiluminiscence (ECL). This is a light emitting non-radioactive method for the detection of an immobilized specific antigen, which is directly or indirectly conjugated with a peroxidase-labeled antibody. Luminol oxidized by hydrogen peroxide is in the excited state which then decays to the ground state *via* a light emitting pathway. When chemical substances based on phenol are present, such radiation becomes more intensive and the duration of light emission is prolonged. The maximum light emission is at a wavelength of 428 nm which can be detected by a short exposition upon an autoradiographic film sensitive to the blue part of the spectrum (Whitehead *et al.* 1979).

Statistics

The results were expressed as the means ± S.E.M. Student's unpaired t-test was employed for comparison of values in individual groups.

Results

Analysis of brain antigens (SDS-PAGE)

The electrophoretic analysis revealed great heterogeneity of the used antigens. Samples containing the cortex, subcortical structures and cerebellum did not

qualitatively differ after the electrophoretic separation. They contained a large number (not less than 20) of well separable protein fractions of molecular weight 15 to 65 kDa.

No difference in the protein composition of the brain tissue was found between control and experimental animals (Fig. 1).

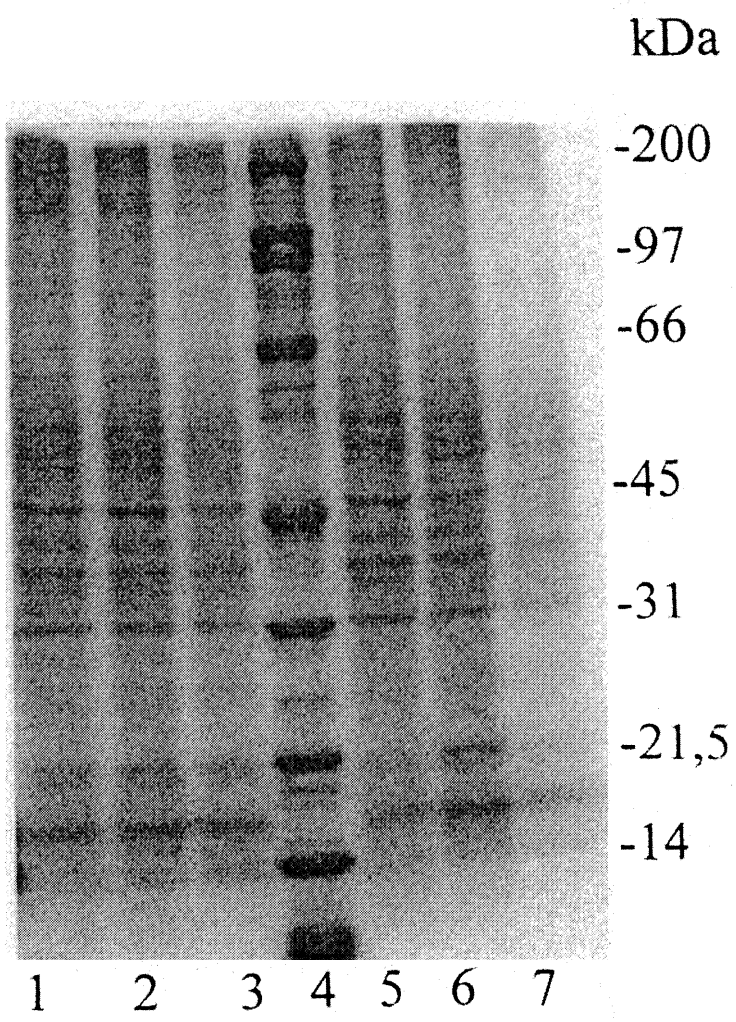


Fig. 1. *Electrophoretic analysis (SDP-PAGE) in the gradient gel. Trace 1: homogenate of the cerebral cortex of control rats, 2: homogenate of the subcortical structures, 3: homogenate of the cerebellum, 4: standards of molecular mass (14 to 200 kDa), 5: homogenate of the cerebral cortex of rats exposed to hypoxia, 6: homogenate of subcortical structures of the rats exposed to hypoxia, 7: homogenate of the cerebellum of rats exposed to the hypoxia.*

Evaluation of the antibodies against brain tissue in the serum of control rats

In the serum of control animals, which were not exposed to hypoxia, relatively high amounts of auto-antibodies against the brain tissue were detected when all three tissue samples were used as antigens (cerebral cortex, subcortical structures, cerebellum). These auto-antibodies belonged to the IgG, IgM and IgA class. The highest share in the spectrum of natural auto-antibodies in the rat serum belonged to immunoglobulins of class M. Serum antibody levels were higher in female than in male rats. There were no major differences among the levels of antibodies against antigens from individual brain regions. Antibodies against cerebellar antigens had the lowest values, while those against the cortex and subcortical structures were comparatively high. For this reason, only antigens from cortical homogenates were used in the subsequent study (Fig. 2).

Evaluation of antibodies in the serum of adult rats exposed to hypoxia

In rats exposed to one-hour hypoxia, antibodies against the brain tissue of classes IgG, IgM and IgA were detected. Higher levels of antibodies of the classes IgG, and IgM were not significant, while a significant decrease of IgA antibodies was found using a normal brain tissue as the antigen ($P=0.01$). After 8 hours of hypoxia, a highly significant increase of IgG ($P=0.0007$) and IgM antibodies ($P=0.0014$) was found using the normal brain tissue as the antigen.

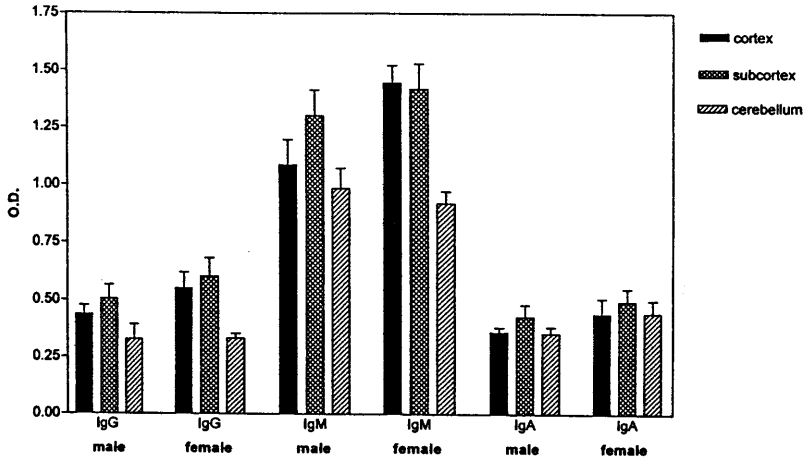
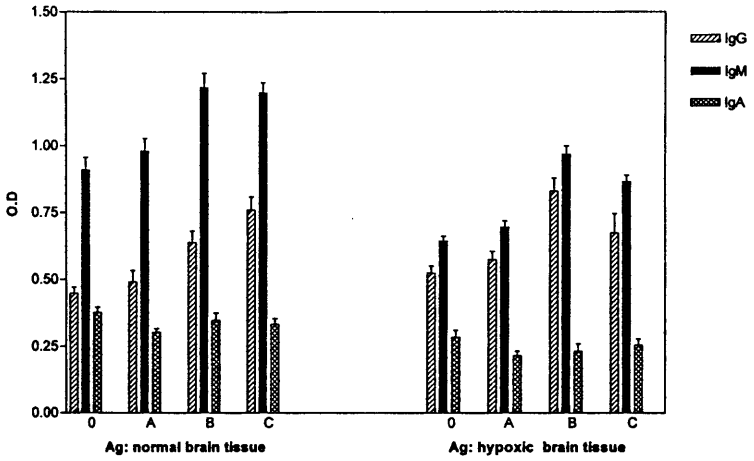


Fig. 2. *Autoantibodies of all immunoglobulin classes to brain tissue detected in the sera of control adult rats using the ELISA method. Activity of antibodies is expressed as the optical density (n = 5). Homogenates of the cerebral cortex, subcortical structures, and cerebellum were used as the antigens.*

Fig. 3. *Autoantibodies of all immunoglobulin classes to normal brain tissue and to hypoxic brain tissue detected in the sera of the adult rats exposed to hypoxia using the ELISA method. Activity of antibodies is expressed as the optical density (n = 6-8). Homogenate of the cerebral cortex was used as the antigen. O – control rat serum, A – 1-hour hypoxia, B – 8-hour hypoxia, C – intermittent hypoxia.*



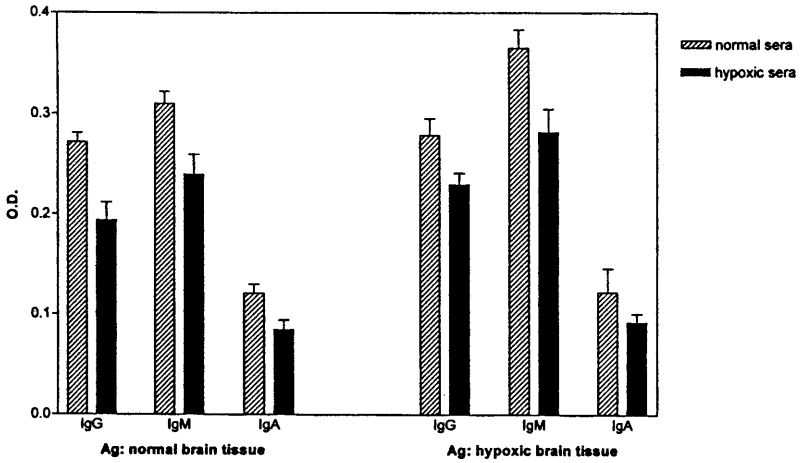


Fig. 4. *Autoantibodies of all immunoglobulin classes to brain tissue detected in the sera of young (23-day-old) rats using the ELISA method. Activity of antibodies is expressed as the optical density (n = 6 for normal rats, n = 12 for hypoxic rats). Homogenates of the cerebral cortex originating from control or intermittently hypoxic animals were used as the antigen.*

Repeated intermittent hypoxia also caused a significant increase of antibodies of both classes (for IgG P=0.0001, for IgM P=0.0007). However, hypoxia did not affect the level of IgA antibodies (Fig. 3).

Evaluation of antibodies in the serum of developing rats

In newborn rats, repeated exposition to hypoxia decreased the content of antibodies against the brain tissue. Using the normal brain tissue as an antigen, the

decrease in IgG class was highly significant (P=0.0005). The decrease was less significant for the IgM and IgA class antibodies (IgM P=0.0323, IgA P=0.0245) (Fig. 4).

Immunoblot

Possible differences in the heterogeneity of auto-antibodies against the brain tissue in normal rats and rats exposed to hypoxia were tested using the immunoblot. Both the classical immunodetection method using aminoethylcabazole as substrate and the more sensitive ECL method did not provide satisfactory results, although we used various techniques of blocking. The results cannot be interpreted because substantial non-specific reactions occur, both using biotinylated and peroxidase-labeled detecting antibodies.

Discussion

Hypoxic brain injury – perinatal in the infant population and of cerebrovascular origin in adults – are the major causes of neurological diseases. Search for new possibilities of an early and reliable diagnosis of brain damage caused by a lack of oxygen is essential. The immunological approach seems to provide the possibility of objective prognosis of such neurological states.

Our results have shown that, using sensitive methods, high amounts of auto-antibodies against the brain tissue can be detected in control rat sera. The prevalence of the class IgM antibodies is suggestive of their physiological significance. Hypoxic brain injury in adulthood is best reflected in changes of the levels of IgG antibodies. The enhanced formation of such antibodies after single or chronically repeated hypoxia may serve as an indicator of hypoxic brain injury.

The IgG antibodies in rats exposed to hypoxia since birth are mostly of maternal origin. The low production of organism's own antibody may thus be outweighed by the maternal supply. The reason for the decrease of maternal antibodies after hypoxia is still not known. One possibility may concern an increase of the catabolic rate of these maternal antibodies in hypoxic pups.

It has to be considered that chronic hypoxia in adulthood brings about not only a preferential elevation of some antibody isotypes (IgG) but it may also result in the appearance of new moieties, e.g. antibodies against stress proteins. Samples of sera were therefore analyzed not only by means of the ELISA method, which reveals total level of antibodies against a mixture of the brain antigen, but also using the immunoblot enabling to detect antibodies against individual fractions of the mixed brain antigen. Such an approach has not yet been successful because strong non-specific reactions occur. The next step in this research will be to use at least partly purified antigens.

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References

LAEMMLI UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685, 1970.

TROJAN S, GROSS J (eds): *Perinatal Hypoxia.* Charles University, Prague, 1990.

TROJAN S, JEZKOVÁ Z: Presence of antibodies as an indicator of brain damage (in Czech). *Čas lék čes* **118:** 267-270, 1979.

TROJAN S, ŠTASTNÝ F: Hypoxia and the developing brain. In: *Handbook of Human Growth and Developmental Biology,* Vol 1, Part C, CRC Press, Boca Raton, USA, 1988, pp 101-123.

VOLPE J: Perinatal hypoxic-ischemic brain injury. In: *Fetal and Perinatal Neurology*, Karger, Basel 1992, pp 46-59.

WATSON BD, GINSBERG MD, BUSTO R: Macroscopic indices of lipid peroxidation in cerebral ischemia/reperfusion: validity and sensitivity enhancement in term of conjugated diene detection. *Neurochem Int* **29:** 173-186, 1996.

WHITEHEAD TP, KRICKA LJ, CARTER TJ, THOREPE GH: Analytic luminescence: its potential in the clinical laboratory. *Clin Chem* **25:** 1531-1546, 1979.

Reprint requests

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