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:ALTE RATIONS OF ARGINASE ACTIVITY IN SCRAPIE-INFECTED MICE AND IN AMYOTROPHIC LATERAL SCLEROSIS

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Summary. — We followed the dynamics of arginase activity, the ultrastructural changes, and accumulation of the scrapie agent in the CNS of scrapie-infected mice. The arginase activity has been shown to increase 5-fold within the first 3—4 months of the incubation period followed by subsequent fall at its end. The peak of increased arginase activity coincided with appearance of multilayer membranes, whereas the decrease of this activity was associated with the greatest development of *status spongiosus*, synaptic degeneration, accumulation of five-layer membranes as well as with maximal accumulation of the scrapie agent in the CNS. Examination of 100 patients with different forms of amyotrophic lateral sclerosis (ALS) revealed a 2—8-fold increase of arginase levels in the cerebrospinal fluid (CSF) as compared with the background levels in the comparative group of control patients. Similar enzyme alterations in scrapie — and in ALS — are discussed in terms of possible resemblance of their pathogenesis.

Key words: *scrapie; amyotrophiclateral sclerosis; arginase*

Introduction

The classification of slow CNS infections as a specific group of diseases caused by unconventional viral agents, prions (Prusiner, 1982) requires thorough studies of biochemical aspects of their pathogenesis. The subacute transmissible spongiform encephalopathies include diseases of man (Creutzfeldt-Jacob disease, kuru and Herstmann-Streisler syndrome) and of animals (scrapie, mink transmissible encephalopathy, chronic atrophy of reindeer). The infection with scrapie agent adapted to mice is regarded for a suitable model to study the regularities inherrent to the group as whole (Kimberlin, 1976). A number of chronic diseases of human CNS with unknown aetiology is referred to belong to the slow CNS infections (Gajdusek and Gibbs, 1977). Studying the pathogenesis of amyotrophic lateral sclerosis (ALS) Zavalishin *et al.* (1987) demonstrated decreased levels of arginine in the biological fluids of these patients and the occurrence of

arginase in the cerebrospinal fluid (CSF) of patients with ALS. As known, the arginase activity in the brain determines the accumulation apart from urea, of other biologically active compounds, in particular, methylated derivatives of arginine which stabilize the structures of biomembranes in CNS cells. The arginase activity also provides for the rate of guanidine compound synthesis, particularly, γ-guanidine of butyric acid and creatine. This is especially important for the CNS cells as guanidine compounds do not penetrate through the haematoencephalic barrier. The product of arginase reaction, ornithin, in turn, is a precursor of a number of biologically active compounds: γ-aminobutyric acid, polyamines and other compounds vital for the functioning of CNS cells. Therefore, it seemed worthwile to follow the arginase activity in murine CNS cells in the course of experimental scrapie as compared with certain virologic and morphologic indices of the infectious process, and to compare the data obtained with the kinetics of arginase activity in CSF of patients with various types of ALS.

Materials and Methods

Animals. Four-week-old BALB/c mice weighing 12—14 g were infected intracerebrally (i.c.) with the scrapie agent (C-506 strain) using a 10% brain suspension with a stock titre of 5.7 log LD_{50} per 0.03 ml. Control mice received a 10% suspension of normal mouse brain; another control was the group of intact animals. One, 2, 4, 5, or 6 months later at random chosen mice were killed and their brains were used to assess the accumulation of the scrapie agent for electron-microscopic examination and evaluation of arginase levels; altogether 170 mice were investigated. The scrapie agent was titrated by i.c. infection of BALB/c mice, the results were evaluated considering the number of animals exhibiting typical clinical signs of disease. The titre of the agent was estimated according to the method of Reed and Muench (1938), the infected animals were followed for up to 11 months.

Electron microscopic examination. For electron microscopy specimens of the frontal lobe of cerebral cortex and cerebellum of mice were fixed in 2% glutaraldehyde in cacodylate buffer (pH 7.2), subsequently fixed in 2% solution of glutaraldehyde in the same buffer, dehydrated in a graded series of alcohols and embedded in Epon-Araldite. Ultrathin sections were cut using LKB-1800 or Reichert ultramicrotome contrasted with uranylacetate and lead citrate and examined using a JEM-100B electron microscope, operated at 80 kV.

Biochemical studies. The arginase activity in the murine brain was assessed by a slightly modified technique (Khokhlov *et al.*, 1985) as reported previously. One unit of activity was defined as the amount of the enzyme forming 1 μmol/l of urea for 1 min. The calculations were made for 1 mg of protein. The procedure of assessing the arginase activity in the CSF of patients with ALS has been previously reported (Zavalishin *et al.*, 1987). The results were presented in I.U. per 1 l. One hundred patients who entered to the Department of Neuroinfections, Institute of Neurology of the AMS of U.S.S.R., were examined. ALS was diagnosed clinically, the age of patients varied within the range of 21—70 years; 74% of patients were males. The comparative group included 50 patients with other different lesions of CNS (neck myelopathy, spinal amyotrophy, spinal cord tumours, polyneuropathy). Another control group consisted o 100 healthy individuals.

The results were evaluated by Student's test determining statistically significant differences in arginase activity of the cpmpared groups.

Results

Experimental scrapie infection

The arginase activity was evaluated in its dynamics during the incubation period of infection lasting for 6 months under the experimental conditions

Table 1. The kinetics of arginase activity and the accumulation of scrapie agent in CNS of mice experimentally infected with a scrapie agent

Group	Date after infection, months	Arginase level, unit/1 mg protein, M ± m	Significance of the difference	Titre of the scrapie agent lg LD_{50}/0.03 ml
1 Intact mice	—	0.146 ± 0.004		—
2 Inoculation of the normal brain	—	0.110 ± 0.005	$99 > p_{1-2} > 95$	—
3 Inoculation of scrapie agent	1	0.228 ± 0.035	$p_{2-3} > 99$	5.0
4 ,,	2	0.297 ± 0.033	$p_{2-4} > 99$	7.0
5 ,,	3—4	0.521 ± 0.054	$p_{2-5} > 99$	7.5
6 ,,	5	0.260 ± 0.046	$p_{2-6} > 99$	8.0
7 ,,	6	0.152 ± 0.043	$99 > p_{2-7} > 95$	> 8.0

used. It is evident from Table 1 that 1 month post-infection (p.i.) a statistically significant increase in the arginase concentration occurred in the brain of scrapie-infected mice. At subsequent intervals up to 4 months, the activity of the enzyme increased while by months 5 and 6 it decreased; however, even by month 6, i.e. at the onset of clinical symptoms, it was significantly higher than in controls (Fig. 1). It should be noted that the kinetics of arginase concentration in CNS did not correlate with accumulation of scrapie agent in murine CNS as the latter was rising during the whole incubation period (Table 1) with a peak coinciding by the fall in the arginase activity. In relation to this it appeared of interest to analyse the dynamics of morphological changes in the CNS at the same dates of infection. The following indices of pathological process were taken into account during electron microscopic examination of the CNS of infected mice: development of *status spongiosus*, synaptic degeneration, presence of multilayer membranes and of five-layered membranes. The respective lesions are presented in Fig. 2. The rate of this development is shown in Table 2 which also lists the results of quantitative evaluation showing the extent

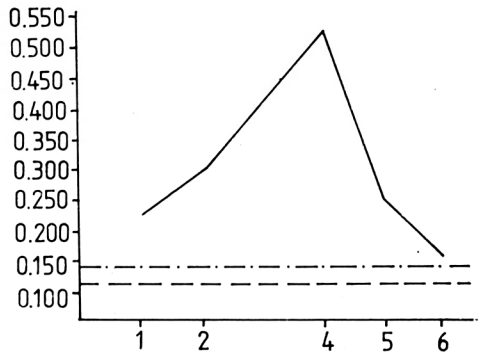

Fig. 1

The kinetics of arginase activity in the brain of scrapie-infected BALB/c mice (1) scrapie-infected mice; (2) mock-infected mice brain; (3) intact mice
Abscissa: the date following mouse infection (months); ordinate: arginase activity in mouse brain (units/mg protein).

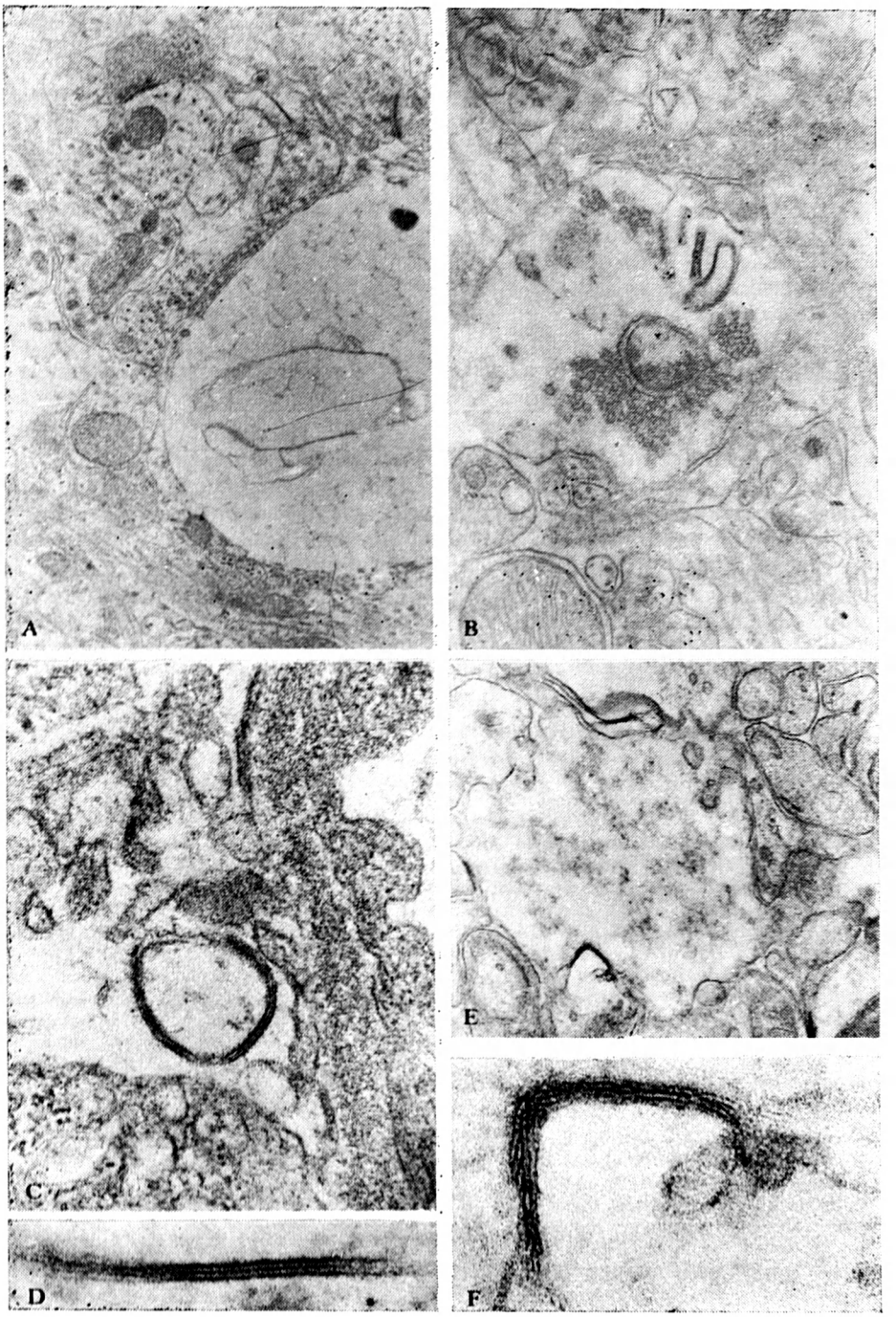
A
B
C
D
E
F

Table 2. The kinetics of ultrastructural changes in the CNS of scrapie-infected mice

Date after infection, months	The alterations considered			
	Status spongiosus	Synaptic degeneration	Five-layer membranes	Multilayer membranes
1	1+	0	0	0
2	4+	1+	0	3++ 3+++
3	4+ 2++	0	0	1++ 5+
5	5+ 1++	1+	2+ 1++	1+
6	1+ 5++	4+ 2++	2+ 4++	0

Footnote. The Table presents the number of mice with the alterations detected in each of 6 animals examined;
+ designates weak alterations occurring rarely, less than in 20% fields of view; ++ designates weak alterations occurring less than in 30% fields of view; +++ designates well expressed alterations occurring in more than 50% fields of view.

of the development of submicroscopic changes. It is evident that within 1 month after infection the first signs of *status spongiosus* were recorded, their degree of manifestation was growing further on being the highest during the last stages of incubation. The course of spongiform changes and the ultrastructural pattern of vacuolization in neuropil showed no differences from those routinely observed in spongiform encephalopathy (Fig. 2A). Sinaptic degeneration (Fig. 2B) was manifested by oedema and swelling of synaptic endings, changes in the size, form, and distribution pattern of synaptic vesicles; this was recorded following 2 months p.i. but was most marked by the end of the incubation period. By this time (about 5 months) five layered membranes occurred (Fig. 2C, D) in the form of densely packed outer cell membranes or their outgrowths. These membranes were 17—19 nm thick, protein layers facing cytoplasm were 6 nm wide, the central protein layer was 3.5 nm lipid bilayers were about 2.5 nm (Fig. 2D). The intensity of their formation also continued to grow by the end of the incubation period. Somewhat different changes were noted in the rate of formation of so-called multilayer membranes with thick (up to 5 nm) protein layers of increased electron density, which number in the profile of a single membrane

Fig. 2

Ultrastructural changes in the scrapie-infected mouse brain

A — A large vacuole containing daughter vacuoles. Interrupted membrane encircles a large vacuole; 6 months p.i. × 15 000;
B — swelling of the presynaptical terminal, fusion and re-distribution of synaptic bubbles; 2 months p.i. × 26 000;
C — five-layer membranes encircling an astrocyte process; 6 months p.i., × 72 500;
D — enlarged five-layer membrane, × 143 000;
E — multilayer membranes formed on the membrane encircling swelled axon terminal. 2 months p.i., × 28 000;
F — Large magnification (× 160 000) of a multilayer membrane.

Table 3. The positive rate of arginase detection and its levels in the CSF of patients with ALS and in controls

Groups compared	Number of examinees	Incidence rate of arginase activity, registration, %	Arginase activity, units/l
1 Patients with amyotrophic lateral sclerosis	100	98	3.70 ± 1.20
2 Control patients (lesions of CNS other than ALS)	50	12 ± 5	1.10 ± 0.13 $_0p_{1-2} > 99$
3 Healthy controls	100	0	

occassionally exceeded 10 (Fig. 2E, F). Multilayer membranes were first recorded by 2 months following infection with a peak at that time interval. Later on their formation proved less intensive so that by the end of the incubation period their rate was negligible.

Clinical cases of human ALS

Arginase activity was examined in the CSF of patients, which had been clinically characterized in detail. The duration of disease in the majority of patients was 1—3 years and only in 8 patients it ranged from 5 to 7 years. The majority of patients (84) showed a clinically vivid picture of disease with varying spasmic atrophic paresis, paralysis, and bulbar disorders. Studies of arginase levels in the CSF of ALS patients and control patients and healthy subjects are presented in Table 3 indicating high level of arginase activity in 98% of patients with ALS. Arginase activity was found only in 12% of control patients in lower levels. No activity was detected in the CSF of healthy controls. When related to the extent of CNS lesions arginase levels were the highest in patients with predominant disorders of anterior horns and nuclei revealing clinical signs of disease (5.7 U/l), they were lower in patients with combined lesions of anterior horns, nuclear and conducting structures (4.10 U/l) and minimal (3.06 U/l) in patients with prevalence of pyramidal symptoms. In the case of malignant disease progression with rapid generalisation within a year the arginase activity was the highest (7.92 U/l), it was somewhat lower in subacute progression (6.07 U/l); it proved the lowest in the case of benign progression and slow development of the process (1.84 U/l). It should be noted that in several cases the advanced stages of ALS characterized with severe and spread amyotrophy the arginase activity was significantly diminished.

Discussion

Biochemical approach can be used to investigate the obscure common mechanisms of the development of lesions in slow neural infections caused by unconventional viral agents, or chronic CNS infections of unknown aeti-

ology; to the latter belongs ALS in particular. As known, an amino acid arginine has a vital role for the activity of neurons being present in functional proteins and participating in the formation of a number of biologically active compounds, in particular histones. It has been experimentally shown that arginine deficiency affects locomotory cells of the spinal cord (Newberne *et al.*, 1960); drastic reduction in arginine concentration was also noted in biological fluids of patients with ALS (Khondkarian *et al.*, 1978). CNS arginase concentration is apparently a limiting factor determining arginine levels taking into account the inability of the latter to penetrate through the haematoencephalic barrier (Davtjan, 1968; Shugalei *et al.*, 1977). That is why we aimed at present investigations to follow the kinetics of arginase activity in the CNS cells during experimental scrapie infection of mice and to compare it with the dynamics of the agent accumulation and the development of ultramicroscopic changes. The literature available does not indicate any changes in arginase activity in the CNS of animals infected with scrapie or any related agents inducing transmissible encephalopathy of man and animals.

We demonstrated clear cut alterations in arginase activity of CNS in mice following experimental infection with srapie during the incubation period. The enzyme acitivity first increased being the highest by months 3—4 with subsequent decrease by the end of the incubation period, i.e. at month 6. Interestingly, the dynamics of multilayer membrane accumulation detected by us in CNS cells correlated (being a little ahead) with the kinetics of arginase activity. At the same time, the development of *status spongiosus*, synaptic degeneration and formation of five-layer membranes correlated with the increasing scrapie agent titre in CNS cells. Comparative analysis of the kinetics of arginase activity, of the accumulation of scrapie agent and of the expression of ultramicroscopic changes favours the assumption that the kinetics of enzyme activity as well as the number of multilayer membranes reflects the dynamics of neuronal response to the effect of the infectious agent. It seems apparent that by month 5 to the incubation period a shift occurred in this reaction as manifested by decreased arginase activity and less multilayer membranes in the presence of the elevated titre of scrapie agent but more marked development of *status spongiosus*, synaptic degeneration and growing numbers of five-layer membranes. The ultrastructure of the latter is very similar to that of gap junctions (Alberts *et al.*, 1987). The appearance of these junctions in the infected brain neuropil may be regarded as an adaptation reaction aimed at maintaining the metabolic cell cooperation, a disorder owing to the deterioration of electric and chemical conductivity in injured synapses.

The issue of the nature and origin of multilayer membranes and their role in cell pathology is another point. Beck *et al.* (1982) examined multilayer membranes in the brain of kuru-infected apes in a preclinical stage of the disease. We share the opinion of Beck *et al.* (1982) that the mechanism of the membranes' appearance can be related to the process of their "damming" due to the transport of the micro- and macromolecules in case of their excessive synthesis. Bearing this in mind one may suggest that arginase

accumulation detected in our experiments can be related also to disorders in axoplasmic flow and membrane permeability.

While discussing the possible role of arginase in the pathogenesis of scrapie we should take into account the opinion of some investigators that the major function of arginase is to regulate histone synthesis (Davtjan, 1968). It has been assumed that the disordered histone acetylation is one of early manifestations of scrapie infection (Caspary and Sewell, 1968). As known some biogenic polyamines are formed during arginine decarboxylation (White *et al.*, 1981). Of interest is the finding that in experimentally-induced scrapie infection the concentration of polyamines (spermine and spermidine) in the CNS of animals decreased at early stages of incubation and rose by the time of clinical manifestation of the disease with subsequent decrease (Giorgi *et al.*, 1972). A certain correlation between the kinetics of arginase activity and spermine and sperminide levels in CNS was found in scrapie-infected mice. There have been a number of reports on the kinetics of alterations in activity of certain enzymes in the course of slow infections in man and animals caused by nonconventional viral agents: oxidation-reduction enzymes, glycosil hydrolases, deposphorylating enzymes, acetylcholinesterase, acid phosphatase, monoaminooxidase, glutamate decarboxylase, adenylatecyclase, etc. (Robinson, 1969; Millson and Bountiff, 1973; Hunter and Millson, 1973; Federico *et al.*, 1980; Mackenzie, 1984; Igbal *et al.*, 1985; Rasenick *et al.*, 1986). However, it is difficult to evaluate the role of alterations in the enzymatic activity for pathogenesis since the primary character of these alterations has not been proved. Alterations in arginase activity in CNS cells in experimentally-induced scrapie-infection can be regarded as primary, as the augmented enzymatic activity preceeds the highest rate of agent accumulation and the development of major degenerative ultramicroscopic lesions.

Our second task was to compare the data on the kinetics of arginase activity in the course of experimental scrapie and in ALS. We have shown that in both cases the enzymatic activity was significantly increased (3—5-fold). The dynamics of alterations in arginase activity in patients with ALS requires further studies. However, the picture observed here was similar to that described by others. Of interest is to note that in case of Creutzfeldt-Jacob disease the curve depicting the activity of neuron-specific enolase in CSF of a patient proved similar to that showing the kinetics of arginase activity in scrapie (Wakayama *et al.*, 1987).

Thus, our results have demonstrated clear-cut alterations in the arginase activity in CNS of scrapie-infected mice and a high level of arginase activity in the CSF of patients with ALS. This finding suggests that changes in arginase activity may contribute to the development of both diseases indicating a certain similarity of their pathogenesis at least in biochemical terms.

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