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Further studies on incubation conditions for ALS-immunoglobulinsand human erythrocyte acetyl-cholinesterase

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The incubation conditions for the reaction of ALS-and control-Igs on the activity of human erythrocytes AChE were studied in detail. The optimum incubation conditions were chosen to preserve the biphasic Arrhenius plots occurring normally with control-Igs, and straight plots with ALS-Igs. Prolonged incubation changed the Arrhenius plots to the same pattern in both ALS and control. Precipitation of the incubation mixture with anti-IgG specific for γ-chains decreased the enzyme activity in ALS and also changed the Arrhenius plots to the normal biphasic. The results confirm the possibility of autoimmunity in ALS, and erythrocytes AChE may possibly be one of the antigens in this disease.

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ได้ศึกษาถึงรายละเอียดในปฏิกิริยาของอิมมูโนโกลบูลินจากผู้ป่วยด้วยโรค ALS และจากกลุ่มควบคุม กับเอ็นไซม์แอเซททิลโคลีนเอสเทอเรสจากเม็ดเลือดแดงของคน การอินคิวเบทที่เหมาะสม เมื่อแสดงผลโดยใช้ Arrhenius plots พบว่ากราฟที่ได้จากอิมมูโนโกลบูลินในกลุ่มควบคุม เป็น biphasic (เส้นตรงสองเส้นมาพบกัน) ส่วนอิมมูโนโกลบูลินจากผู้ป่วยด้วยโรค ALS ให้กราฟเส้นตรงเส้นเดียว การเพิ่มเวลาของการอินคิวเบทเปลี่ยน Arrhenius plots ของทั้งกลุ่ม ALS และกลุ่มควบคุมให้อยู่ในรูปแบบเดียวกัน เมื่อตกตะกอนของสารที่อินคิวเบทข้างต้นด้วย anti-IgG จำเพาะต่อ γ-chains ทำให้การทำงานของเอ็นไซม์ในกลุ่ม ALS ลดลง และเปลี่ยน Arrhenius plot เป็น biphasic เช่นเดียวกับกลุ่มควบคุม ผลการทดลองนี้ยืนยันถึงความเป็นไปได้ของภูมิคุ้มกันในตัวเอง ในผู้ป่วยด้วยโรค ALS และเอ็นไซม์ แอเซททิลโคลีนเอสเทอเรสจากเม็ดเลือดแดง อาจเป็น แอนตีเจนตัวหนึ่ง

Amyotrophic lateral sclerosis (ALS) is a progressive degenerative disease that affects the motor neurons of spinal cord, the cranial nerve nuclei and the motor cortex. The affected cells degenerate and eventually disappear. The lateral corticospinal tracts degenerate and become sclerotic. It usually ends fatally within two to three years. Death is mostly due to paralysis of the respiratory muscles or to infections of the respiratory tract, e.g. pneumonia. One typical symptom of ALS is fasciculation, a spontaneous muscle contraction. Fasciculation is also a sign of acetylcholinesterase (AChE) inhibition. AChE is common to erythroycyts and nerves.

Plasma from patients with ALS contains cytotoxic activity which induces an increased in haemolysis of normal human erythrocytes the vitro.[1,2] This phenomenon is in all probability induced by binding of immunoglobulins (Igs), of the IgG and IgA classes, to the erythrocyte surface.[2,3] The haemolytic effect of ALS plasma is decreased in the presence of synstigmine (neostigmine) or excess AChE, supporting the hypothesis that in this disease specific Ig binds to AChE on the red blood cell membranes.[4] We have recently determined that plasma-Igs of ALS-patients interact with erythrocyte AChE.[5] In the present study we have demonstrated in more detail the incubation conditions for ALS-Igs and human erythrycytes AChE as well as the precipitation of IgG after incubation. The correct incubation condition is the main question of this study.

Materials and Methods

Plasma Plasma from 15 ALS-patients and from the same number of normal healthy controls were used. Criteria for the ALS-diagnosis have been presented earlier.[1] Samples were taken as early as possible in the course of disease and the patients were as a rule able to walk and talk. Blood was obtained by antecubital vein puncture in both fasting patients and controls.

Isolation of Igs Plasma was fractionated by gel filtration and affinity chromatography. Using Utrogel AcA 34 and Protein A-Sepharose 4B as described earlier.[5] The Ig concentration was determined spectrophotometrically using the extinction coefficient $A_{280}^{0.1\%} = 1.38$.[6] As the Ig-preparation contained on average 90% IgG (5 times more IgG than IgA in plasma), protein A binds 95% of IgG and 30% of IgA;[7] the use of this extinction coefficient introduces a negligible error ($A_{280}^{0.1\%}$ for IgA = 1.34)

Preparation of AChE This preparation has recently been described in detail.[5] Briefly, red blood cells were rewashed twice with three volumes of 0.1 M sodium phosphate buffer (NaPB) pH 7.4 and frozen as 1 ml aliquots. They were solubilized immediately with Triton X-100 to 1% concentration before use and centrifuged with a Beckman Microfuge (4-5 min at about 10,000 g). The supernatant was used.

Assay of AChE activity The assay has been described in detail,[5] which was in short : Thiocholine from hydrolysis of the substrate acetylthiocholine iodide (1 mM) reacted with 4,4 - dithiopyridine (0.2 mM) producing 4-thiopyridone. The change in absorbance at 324 nm ($\triangle A_{324}$) was recorded.[8] It was linear for at least 1-2 min and the enzyme activity was expressed as $\triangle A_{324}$/min. Assays were run in duplicates.

AChE activity in the presence of Igs

Three different types of experiments were carried out.

1. *Incubation conditions* Igs, prepared as described above, from ALS-patients and controls were incubated with solubilized AChE in 0.1 M NaPB pH 7.4 containing 0.1% Triton X-100. The concentration of Igs were 1 mg/ml. The incubations were carried out at different temperatures and for varying lengths of time.

At 37°C, the incubation times 0-0.5, 1-2 and 16-18 (overnight) hours were studied.

The incubation at 22-24°C (room temperature, summer) was studied at 4, 16-18 and 48 hours.

At 16-18°C (room temperature, winter), the incubation times 16-18 and 48 hours were used.

Incubation at 2°C (cold room) was carried out for 48 hours.

Assays at the enzyme activity were then performed at 26, 28, 30,.....and 40°C., when 850 ul 0.1 M NaPB pH7.4 (0.025% Triton X-100) and 100 ul (2.1 mM) chromophor were thermostated to the desired temperature in a quartz cuvette. First a 50 ul sample was then added, allowed to stand for 5 min for temperature equilibration before the 50 ul substrate (21 mM) at the same temperature was added. The activity, $\triangle A_{324}$/min, was determined. Then the Arrhenius plot can be done by plotting between the natural log (ln) of $\triangle A_{324}$/min and 1/T (absolute temperature).

Precipitation of IgG

2. Samples of the same composition as above were incubated at 37°C for 1.5 hour. After incubation, the incubation mixture was divided into two parts. One part was precipitated with equal volume of anti-IgG specific for the γ-chains or for the Fc fragment (Dakopatts AB, Sweden) in 0.1 M Nacl, containing 1.2 mg/ml of anti-IgG. Human serum albumin (HSA) (1 mg/ml) was added to maintain a high protein concentration after the precipitation to minimize adsorption of the enzyme to the

test tube. After standing for 30 mins at room temperature, the sample was centrifuged in a Beckman Microfuge, and 100 μl aliquots were taken for assay of AChE activity at 37°C.

The remainder of the sample to which no anti-IgG was added was diluted with 0.1 M NaCl to the same enzyme concentration; 100 μl aliquots were taken and assayed at the same time and under the same conditions as the sample to which anti-IgG has been added.

3. Two pairs of control and ALS-Igs were incubated and precipitated with samples of the same composition as described above. The assay for AChE activity was done at 26, 28, 30 and 40°C

Duplicates were used in all enzyme assays. $\triangle A_{324}$/min was always in the range 0.3-0.6.

Results

Enzyme activity in presence of Igs

Incubation conditions The results are shown in Table 1 and Fig 1. It was found that the shape of this plot changed with the temperature and the duration of incubation of the enzyme samples.

Table 1. Incubation conditions between red blood cell acetylcholinesterase and immunoglobulins.

Temperature	Incubation (hours)				
°C	0-0.5	1–2	4	16–18	48
37	A	B		C	
22-24			D	E	F
16-18				G	H
2					I

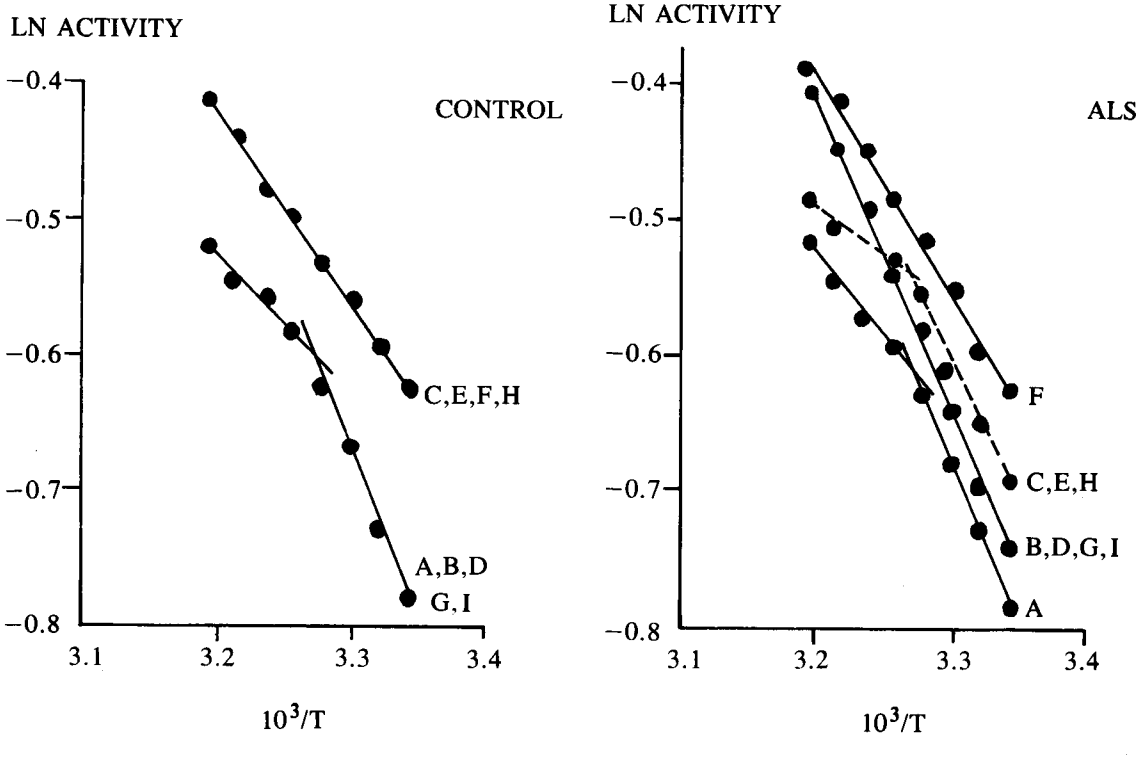

Figure 1. Arrhenius plots of the erythrocyte AChE activities after incubation for control and ALS-Igs.

Enzyme alone or in the presence of control Igs gave a biphasic plot when the assay was done directly after the preparation of the samples (A in Fig 1, Control). A similar plot was also obtained with AChE and control Igs after 2 hrs of incubation at 37°C or 4 hrs at 22-24°C, 16-18 hrs at 16-18°C and after 48 hrs at 2°C (B, D, G, I in Fig 1, Control). After a more intense incubation the Arrhenius plot was a straight line for the whole temperature range 26-40°C (C, E, F, H in Fig 1, control).

The results of the incubation of AChE with ALS-Igs are shown in Fig 1. A short incubation time (A in Fig 1, ALS) rendered a biphasic Arrhenius plot. A straight line was obtained after 1-2 hrs at 37°C, 4 hrs at 22-24°C, 16-18 hrs at 16-18°C and after 48 hrs at 2°C (B, D, G, I in Fig 1, ALS). Under these conditions a biphasic plot was obtained with the enzyme alone or with enzyme and control Igs. The biphasic plot obtained after half an hour of incubation (A in Fig 1, ALS) showed that the full effect of the ALS-Igs had not yet taken place. With an incubation longer than optimum, a biphasic Arrhenius plot was obtained (C, E, H, in Fig 1, ALS) followed once more by a straight line at the longest incubation time (F in Fig 1, ALS). Thus the sequence of events occurring with normal Igs was repeated in the presence of ALS-Igs but the time course of coversions in enzyme activity was somewhat changed. The plot was still biphasic after 16-18 hrs at 22-24°C or 37°C, where as it had already changed to a straight line in the controls (C, E in Fig 1, control).

Enzyme activity after precipitation with anti-IgG

The Igs from 15 ALS-patients and 15 control, the same samples as used above, were incubated with AChE. After precipitation with anti-Igs specific for the γ-chains, the enzyme activity of the supernatant was measured and compared to the activity before precipitation. Duplicated measurements of each sample were made. The values for $\triangle A_{324}$/min were in the range 0.33-0.56. As seen from table 2 there was a decrease in activity with all ALS-samples. The average decrease was 5.0 ± 3.0% (SD) with a range of 2.1-13.5%. With controls the activity was higher in 5 cases, the same in 2 and lower in 8. The values varied from an increase of 3.5% to a decrease of 2.3%, and the average was an increase of 0.6 ± 1.5% (SD).

Table 2. Change in activity of acetylcholinesterase after precipitation with anti-IgG specific for γ-chains.

Sample	Change in % (− = decrease, + = increase)	
	ALS	Control
1	− 3.25	+ 1.80
2	− 5.18	− 0.38
3	− 2.20	+ 0.58
4	− 2.90	− 0.62
5	− 3.61	0
6	− 5.71	+ 1.53
7	− 3.00	+ 3.50
8	− 4.69	0
9	− 5.84	+ 0.25
10	− 5.45	− 2.25
11	− 13.53	+ 2.80
12	− 10.56	+ 1.74
13	− 4.90	− 0.73
14	− 2.09	+ 0.73
15	− 2.82	− 0.61
Mean with Standard deviation	− 5.0 ± 3.0 (− 5.04 ± 3.02)	+ 0.6 ± 1.5 (+ 0.56 ± 1.45)

Similar experiments were also done by adding anti-IgG specific for the Fc fragment. No precipitation occurred and the differences in activity before and after addition of anti-IgG were practically the same in the ALS as in the control samples. For ALS, the average was an increase of 1.2 ±1.7% (n = 7, SD) (range −1.1 to + 2.4%) and for controls an increase of 0.6 ± 1.3% (n = 7, SD) (range −1 to + 2.7%). These values were also similar to the control values obtained after precipitation with anti-IgG specific for γ-chains.

Arrhenius plot after precipitation with anti-IgG

Two samples of Igs (ALS and control) were incubated with AChE. One pair was incubated overnight at 16-18°C and the second for 2 hours at 37°C. Before precipitation the two controls had biphasic and the two ALS samples straight line Arrhenius plots. After precipitation, all samples gave biphasic plots (Fig. 2).

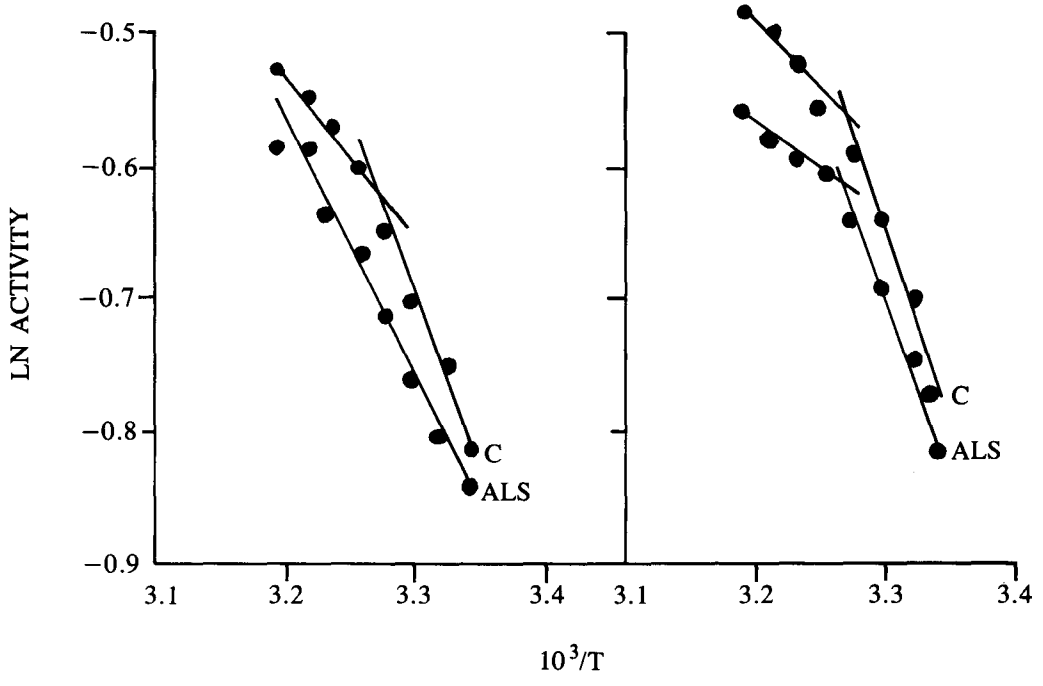

Figure 2. Effect of removal of IgG on Arrhenius plots obtained with control (C) and ALS-Igs. Incubation at 37° C for one hour and precipitation with anti-IgG specific for γ-chains to remove IgG.
Left : Before removal of IgG.
Right : After removal of IgG.

Discussion

Isolated Igs from plasma of ALS-patients were shown in a high proportion to induce a change in the enzyme activity of erythrocyte AChE as judged by Arrhenius plots.[5] Under certain well defined conditions regarding duration and temperature of incubation of the enzyme, presence of ALS-Igs induced a linear plot, whereas control-Igs gave a biphasic plot. An increased duration of the incubation in the control situation later also caused the biphasic plot to change into the linear shape. Solubilization of erythrocyte membranes and other membranes released proteolytic enzymes. The change of the enzyme plot into a straight line in the presence of control or no Igs indicates that the enzyme had lost the ability to undergo the conformational change (see below) that alters the activation energy. This was judges most likely to be due to a proteolytic change in the enzyme. The plot remained biphasic after 48 hrs at 2°C in the control situation which agrees with the fact that proteolysis is slower at a low temperature.

The rapid conversion of the enzyme activity into a straight plot in the presence of ALS-Igs was transient, since the biphasic plot reappeared at longer incubation. This excludes the possibitlity that these Igs had just accelerated the proteolytic effect on the enzyme. Instead, there must have been a direct Ig effect on the enzyme, since addition of anti-IgG to AChE incubated with ALS-Igs to give a straight line, restored the biphasic plot. The final (second) conversion of the enzyme to the straight line pattern in the presence of ALS-Igs at long incubation times was analogous to the control situation most likely to have been induced by proteolysis, and was delayed. Igs from ALS-patients must therefore have protected the enzyme from proteolysis for a time. It is suggested that the reappearance of the biphasic plot before the final conversin into the straight plot in the presence of ALS-Igs was due to proteolysis of the Igs.

Before precipitation with anti-IgG the Arrhenius plot was staight, indicating that most of the enzyme was in complex with ALS-Igs. If a large fraction of the enzyme

had been free, the plot would have shown a deviation from the straight line at the break point. Antiserum, as mentioned, precipitated on average only 5% of the enzyme activity, indicating that addition of anti-IgG dissociated most of the enzyme-Ig complex. Since the addition of anti-IgG specific for Fc fragment alone produced no differences in AChE activity, the γ-chain specific anti-IgG must have been directed aginst the antigen-binding part thereby inducing the edissociation of antigen-antibody complex on addition of such antiserum. This could mean that a great part of the autoantibodies had a low affinity and that they were rather easily removed from the enzyme. High affinity antibodies might have remained bound to the erythrocytes during preparation of the plasma and the samples could therefore be enriched with respect to low affinity antibodies.

This work points to the existence of immunological disorders in ALS. It is also supported by earlier immunological findings connected with ALS. Oldstone et al.[9] detected immune complexes in ALS-patients, and Digby et al.[10,11] showed that Igs from ALS-patients could bind to anterior horn cells. Gurney et al.[12] detected in ALS-sera Igs that inhibited the sprouting of mouse muscel neurons. Brown et al.[13,14] showed that antibodies from AlS-patients could bind to mouse spinal cord, cerebellum, brain and liver The size of the antigen was estimated at 70,000 daltons by SDS-electrophoresis, which probably gives only the subunit size. The value 70,000 agrees well with the subunit size of the globular forms of AChEs present in erythrocytes, spinal cord, cerebellum and brain. Kletti et al.[15] detected in ALS-sera IgG that bound to an antigen a protein doublet, the size of 140,000, which was present in both motor neurons and the dorsal root ganglia.

These results confirm the possibility of the autoimmune status in ALS, and erythrocyte AChE may possibly be one of the antigens in this disease.

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