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นิพนธ์ต้นฉบับ

Plasma ALS-Immunoglobulins Increased Haemolysis of Normal Red Blood Cells

อิมมูโนโกลบบูลินสกัดจากน้ำเหลืองของผู้ป่วยด้วยโรค ALS เพิ่มการแตกของเม็ดเลือดแดงปกติ

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บทคัดย่อ : **รัตนา สินธุภัคและคณะ. 2531. อิมมูโนโกลบบูลินสกัดจากน้ำเหลืองของผู้ป่วยด้วยโรค ALS เพิ่มการแตกของเม็ดเลือดแดงปกติ. วารสารวิจัยวิทยาศาสตร์การแพทย์ 2(2) : 69-75**

การแตกของเม็ดเลือดแดงปกติโดยน้ำเหลืองที่แยกตามขนาดโมเลกุล และวิธีเฉพาะที่แยกอิมมูโนโกลบบูลิน แสดงให้เห็นว่าคุณสมบัติในการทำลายเม็ดเลือดแดงเกิดจาก IgA และ IgG จากน้ำเหลืองของผู้ป่วยด้วยโรค ALS

Abstract : **Ratana Sindhuphak, Evert Karlsson, Lars-Olof Ronnevi, and Sebastian Conradi. 1988. Plasma ALS-Immunoglobulins increased haemolysis of normal red blood cells. Thai J Hlth Resch 2(2) : 69-75**

Haemolysis of normal red blood cells with plasma fractionated by gel filtration and with isolated immunoglobulins produced evidence for cytotoxic properties of IgA and IgG from ALS plasma.

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Introduction

Abnormal red blood cells (RBC) as indicated by increased haemolysis has been observed in amyotrophic lateral sclerosis (ALS) (Ronnevi and Conradi, 1981; Westall and Jablecki, 1981; Ronnevi *et al.*, 1982). This RBC fragility has been shown to depend on cytotoxic factors in ALS plasma (Conradi and Ronnevi, 1982 and 1985). In later experiments, plasma from a large number of ALS patients have been tested. Among plasma from more than 150 patients, significantly higher haemolysis was observed in all but two cases. The haemolysis assay is thus specific for ALS and a positive result is obtained in practically 100% of the cases after a few hours. This assay is also much more convenient than those with cell cultures in which sera from ALS patients are shown to destroy anterior horn cell (Wolfgram and Myers, 1972 and 1973). Roisen *et al.* (1982 a,b) have developed the latter method, and upon testing sera from ALS patients they found that more than 75% of the sera were toxic to anterior horn cells after several days.

In the present study we have investigated the capacity to induce haemolysis in different plasma fractions obtained by gel filtration and in plasma immunoglobulins (Igs) isolated by affinity chromatography. These experiments produced further evidence for a specific type of cytotoxic effect of ALS plasma and for this effect to be linked to plasma Igs.

Materials and Methods

Nine ALS patients were studied. The diagnosis was made clinically and confirmed by electromyography (Conradi and Ronnevi, 1985). The normal controls were registered blood donors. All blood samples were taken in the morning after more than 8 hours of fasting. Plasma samples from ALS were blood type (ABO and Rh) matched normal controls.

Plasma samples (2.0 ml) from two ALS patients and two normal controls were chromatographed at 4°C on Sephadex G-200 (Pharmacia). The absorbance at 280 nm was determined in consecutive fractions collected, and in selected fractions (see below) RBC testing was performed.

Plasma IgA, IgG and IgM from nine ALS patients and three controls was isolated by affinity chromatography. Columns were prepared by coupling antihuman Igs (Dakopatts AB) to cyanogen bromide activated Sepharose 4B (Axén *et al.*, 1967). About 8 mg of anti-IgA and IgG and 4 mg of anti-IgM was coupled to respective columns. Igs were adsorbed to the affinity columns directly from control or ALS plasma. After washing the columns with 0.1 M sodium phosphate buffer (NaPB) pH 7.4, the Igs were desorbed with 0.1 M acetic acid. The amount of Igs obtained was determined spectrophotometrically using the absorbance coefficients $A_{280}^{0.1\%}$ 1.34 for IgA, 1.38 for IgG and 1.33 for IgM (Anon., 1976), and freeze-dried.

In another experiment, plasma was subjected to Igs-elimination by two or three passages through a column of Protein A-sepharose 4B and the three anti-Ig columns in series. The non-absorbed fraction thus obtained was dialyzed (0.02 M ammonium acetate), freeze-dried and checked for the absence of Igs by agarose immunodiffusion.

The material was then dissolved in Krebs Ringer buffer to a final concentration of 0.04 g/l of IgA and 0.2 and 0.02 g/l, respectively of IgG and IgM (i.e., about 1 : 50 of the normal plasma concentrations of the respective Ig types). The non-adsorbed fractions were diluted to a final protein concentration of 1.5 g/l (i.e., about 1 : 50 of the total plasma protein concentration). All fractions were then tested for cytotoxicity with the RBC test in parallel with plasma diluted 1 : 50 (in Krebs Ringer solution) from the respective RBC donors.

The haemolysis test has recently been described in detail (Conradi and Ronnevi, 1985). Briefly, equal amounts of sample and blood type-matched control RBC are incubated under physiological pH and temperature conditions, and the degree of haemolysis (%Hb) is determined after 5.5 hours. Differences in % Hb between ALS patient Igs or non-adsorbed fractions and diluted control plasma were tested with Wilcoxon's matched-pairs signed-ranks test.

Results

Three protein peaks and one peak eluting with the total volume and containing low molecular weight (MW) substances (Fig 1 A-D) were seen in all chromatograms of ALS and control plasma. The haemolysis produced by the gel filtration fractions of ALS plasma was compared with that produced by the corresponding fractions of control plasma (Fig 2). The highest degree of haemolysis was found in the second fraction, where IgA and IgG eluted. A slightly higher haemolysis than with the controls was also obtained with the first fraction that contained IgM and with the low MW fraction.

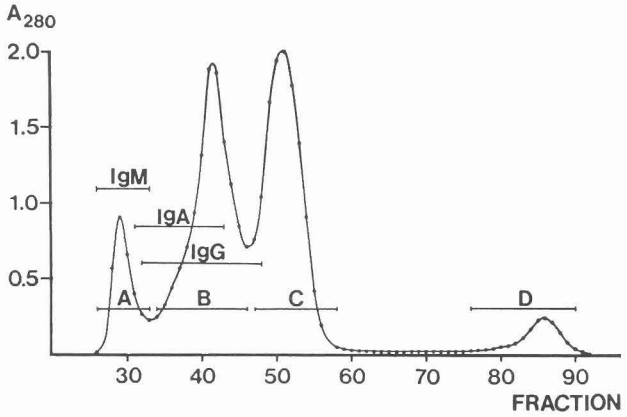


Fig 1. Gel filtration of 2.0 ml ALS plasma on Sephadex G-200 (2 cm^2 × 92 cm), in isoosmolar Krebs-Ringer buffer pH 7.4, at 5.0 ml per hour. A_{280} (absorbance at 280 nm) of the fraction (2.5 ml) was measured. Aliquots were taken for detection of IgA and IgG by immunodiffusion against anti-Igs in 1% agar in 0.1 M NaPB pH 7.4. The fractions (A-D) were subjected to erythrocyte incubation test.

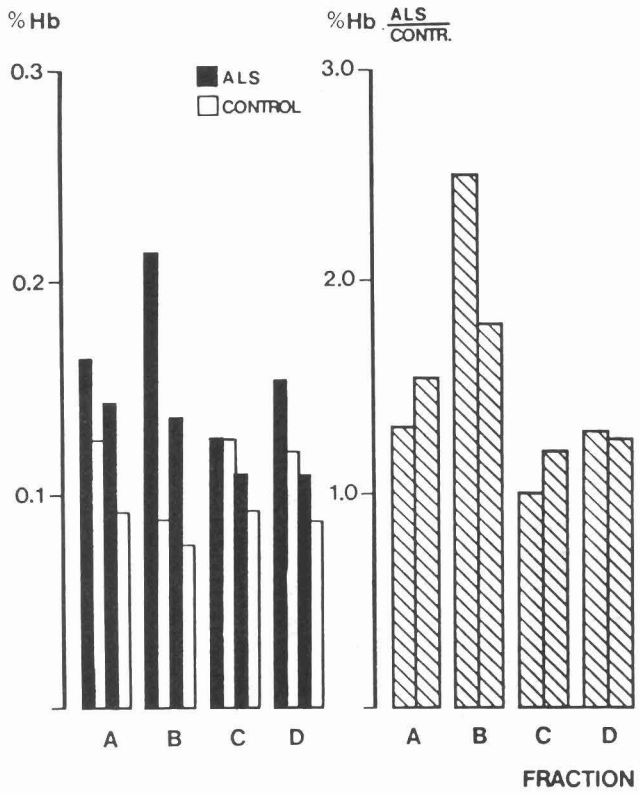


Fig 2. Haemolysis, expressed in % Hb (left) and the quotient % Hb ALS : Hb control (right), obtained after 5.5 hours incubation of normal control RBC with gel filtrations (A-D) from ALS and control plasma in two experiments.

The total amount of Igs and nonadsorbed fraction obtained from equal amounts of ALS patient and control is shown in Table 1. The highest increase in haemolysis was obtained with IgA and IgG (Fig 3). The values for these Igs were significantly ($P < 0.05$) higher than the control values. The non-adsorbed fraction that contained other proteins (low MW constituents were removed by dialysis prior to lyophilization) and IgM gave much smaller differences, which nevertheless were larger than control in most experiments. In parallel incubations of Ig fractions from ALS patients and controls (Fig 4), all patient fractions gave higher figures than their corresponding controls, but the difference for IgM was very small.

Table 1. *Amount of substance obtained by affinity chromatography.*

Substance (mg)								
ALS plasma					Control plasma			
No	IgA	IgG	IgM	Non-adsorbed	No	IgA	IgG	IgM
1	1.0	3.8	0.5	6.0	1	0.9	4.7	0.8
2	0.6	4.5	0.3	6.0	2	1.0	2.0	0.4
3	0.6	4.6	0.4	7.4	3	1.0	5.2	0.4
4	0.5	5.2	0.5	11.8				
5	1.4	3.1	1.1	15.0				
6	0.1	2.1	0.1	13.0				
7	1.2	4.0	0.8					
8	0.2	1.6	0.2					
9	1.1	3.9	0.8					

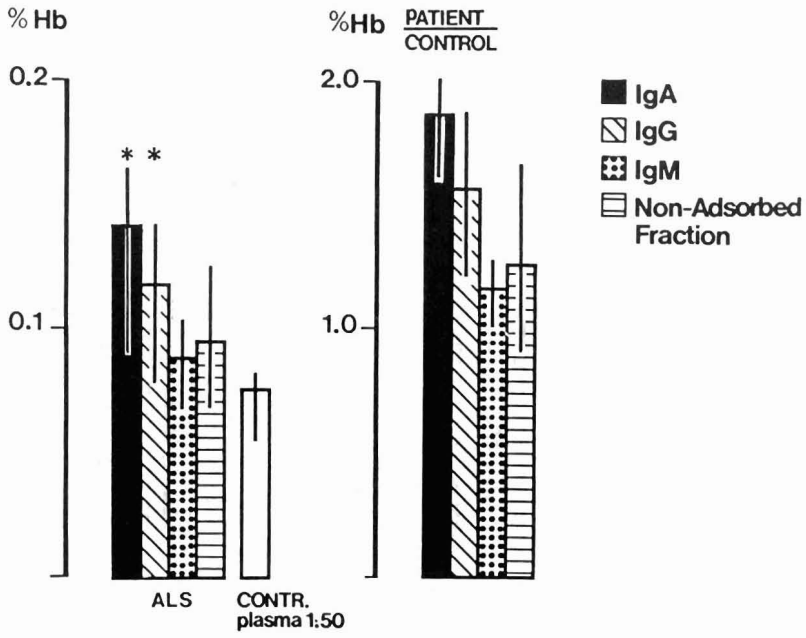


Fig 3. Haemolysis expressed in mean % Hb (left) and quotient % Hb ALS : % Hb control plasma 1 : 50 (right) obtained after 5.5 hours incubation of normal control RBC with Igs and non-adsorbed fractions isolated by affinity chromatography of plasma from ALS patients (n = 6). Significant ($P < 0.05$) difference as compared with control plasma.

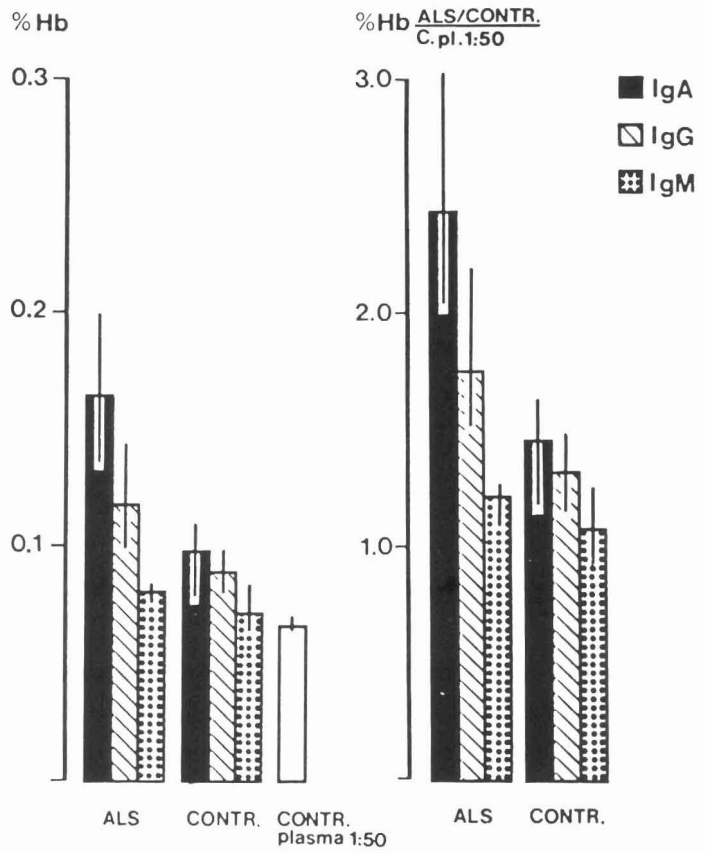


Fig 4. Haemolysis expressed in mean % Hb (left) and the quotient % Hb ALS/control : % Hb control plasma 1 : 50 (right) obtained after 5.5 hours incubation of normal control RBC with Igs isolated by affinity chromatography of plasma from ALS patients and controls (n = 3)

Discussion

The increased haemolysis provoked by patient plasma is mostly probably caused by plasma factors with an effect that is superimposed on the normally occurring nonspecific haemolysis during prolonged extracorporal incubation (Conradi and Ronnevi, 1985). The present experiments with fractionation on Sephadex G-200 was used to estimate the molecular size range of substances in ALS plasma that mediate haemolysis. The greatest differences were seen in the two fractions where Igs elute and particularly in the fraction where most of the IgA and IgG is found (Fig 1). When comparing the respective Ig fractions and fractions supposed to comprise the remaining macromolecular content of plasma after IgA, IgG and IgM elimination (non-adsorbed fraction) from ALS plasma to similarly diluted control plasma, a significant capacity to provoke haemolysis in excess of the control plasma effect was retained in the IgA and IgG fractions. In this context the possible elimination of both non-specific and ALS-specific RBC-noxious substances during the preparation of the non-adsorbed fraction must be considered. The degree of non-specific adsorption of cyanogen bromide activated Sepharose 4B is reported to be low (Cuatrecasas *et al.*, 1968), and the reduction in biological activity of active molecules, e.g., enzymes should be small (Axén *et al.*, 1969; Gabel and von Hofsten, 1970). Since several, and possibly interacting, mechanisms may be involved in the process of haemolysis at RBC exposure to ALS plasma, the increased capacity of isolated ALS Igs to induce haemolysis might well represent a different effect than that occurring with whole plasma. Therefore isolated Igs from ALS patients and normal controls were directly compared in the three additional experiments, and in all cases patient IgG and IgA gave a substantially higher haemolysis than did corresponding controls. It must therefore be concluded that plasma IgA and IgG from ALS patients have a specific property to provoke haemolysis of RBC in vitro. The type of relation observed between plasma

concentration and haemolysis may well be consistent with and underlying binding mechanism of antigen-antibody type, however, since at least two types of Igs were found to mediate the effect.

Immunological findings connected with ALS have also been reported, including descriptions of increased levels of circulating Igs and immune complexes (Tavolata *et al.*, 1975; Oldstone *et al.*, 1976; Antel *et al.*, 1979; Hoffman *et al.*, 1981). Recent studies have provided specific evidence for immune system involvement in ALS. Thus, Digby *et al.* (1982 and 1985) have reported the occurrence of both a decreased mitogen response and a decreased suppressor cell activity in ALS patients together with an increased binding of Igs from ALS patients to cultured spinal cord neurons. As a consequence further studies on the precise type of interaction between the ALS-specific antibodies and motorneurons seem, at present, highly relevant.

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