Determination of Superoxide Dismutase Activity by the Polarographic Method of Catalytic Currents in the Cerebrospinal Fluid of Aging Brain and Neurologic Degenerative Diseases (43160)

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Abstract. **The activity of the superoxide dismutase was measured by the polarographic method of catalytic currents in the cerebrospinal fluid of patients with age-related neurologic degenerative diseases, namely, amyotrophic lateral sclerosis and Alzheimer's disease, and of a reference group of normal subjects. The superoxide dismutase activity was found to increase with age in reference subjects ($r = 0.81$) while no significant correlation was found in amyotrophic lateral sclerosis and Alzheimer's disease patients. The activity mean values were significantly lower ($P < 0.01$) in patients with neurologic degenerative diseases than in the reference subjects. The changes of superoxide dismutase activity in the aging brain and in age-related neurologic degenerative diseases are discussed.** [P.S.E.B.M. 1991, Vol 196]

Free radical reactions have been reported to play a significant role in the aging brain and in age-related degenerative processes of the central nervous system (1), and may provide a reasonable explanation for the clustering of degenerative diseases in the terminal part of life span. In this context, the continuous increase of the concentration of superoxide dismutase (SOD) in rat brain tissue with age (2) should be considered. This enzyme has been suggested to play a protective role against the superoxide ion-mediated oxygen toxicity. The SOD increase may be the expression of a self-protective mechanism from oxygen-generated radicals operating in normal aging brain. Although much attention has been given to intracellular copper- and/or manganese-containing SOD, the concentrations of which are relatively high (3), there is little information on the presence of these enzymes in extracellular fluids, where their concentrations appear to be of orders of magnitude lower than in cells (4). However, the low extracellular concentrations of SOD appear to be significant if the high value of the kinetic rate constant of the reaction between SOD and the superoxide ion is taken into account (5). Since cerebrospinal fluid (CSF) can reflect the enzyme concentration in central nervous system (6), we decided to measure the SOD activity in cerebrospinal fluid of patients with amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD) and in age-matched reference subjects to study the possible role of oxygen-reactive species in neurologic degenerative diseases.

ALS is a devastating neurologic disease, characterized by atrophy or by cell loss of motor neurons (7). Premature loss of motor neurons in ALS has also been suggested to represent an age-related phenomenon. AD is a disorder of the later decades of life characterized by dementia. The pathologic picture shows an abnormal concentration of senile plaques, which results from degeneration of nerve endings, and neurofibrillary tangles (8).

The polarographic method of catalytic currents (9) was used to measure the SOD activity. This method was set up to meet with the requirements of the CSF analysis and appears to be particularly suitable to assay biologic fluids in the light of its high sensitivity and lack of interferences.

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Materials and Methods

The chemicals used were of the highest available purity; the solutions were prepared in twice-distilled water.

Patients. Cerebrospinal fluid and serum samples were collected from 11 patients with ALS, 10 patients with AD, and a reference group of 14 "normal" subjects. The reference group was composed of subjects selected from patients with tension headaches, uncharacterized dizziness, or mild psychoneurotic diseases. The reports of physical and neurologic examinations of the reference subjects, the routine blood analyses, and paraclinical investigations intended to detect a possible organic origin of the specific symptoms were normal.

Nine ALS patients had clinical evidence of both upper and lower motor neuron involvement, whereas two patients had a progressive disease confined to bulbar and spinal lower motor neurons. The diagnosis of ALS was made independently by two or more neurologists after in-patient investigations comprising, when required, electrophysiologic studies, computed tomographic scan of the brain, magnetic resonance imaging, and myelography.

Clinical diagnosis of AD was made according to the diagnostic criteria adopted by the Diagnostic and Statistical Manual of Mental Disorders (DSM III) Criteria and by the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and related Disorders Association Criteria for probable AD (10, 11).

All of the subjects considered were hospitalized, and were not treated with drugs until the lumbar puncture. Routinely, CSF analysis included cell count, total proteins, CSF to serum ratio of albumin content (12), IgG index, and agarose isoelectrofocusing (13).

As regards the number of cells present in the CSF samples, it was always lower than 3 cells/mm^3. The blood-brain barrier integrity in the samples analyzed was assessed from the CSF to serum ratio of albumin content (12). In none of the CSF samples was an intrathecal synthesis of IgG present.

Enzyme Determination. The activity of SOD was measured according to the polarographic method of catalytic currents (9) by an Amel polarographic unit model. The measurements were performed at −0.96 V versus a saturated calomel electrode in 0.1 *M* sodium borate solution (pH 9.8) equilibrated with air and containing 10^{-4} *M* triphenylphosphine oxide. A pH of 9.8 was chosen to minimize the contribution of the spontaneous dismutation of the O_2^-, catalyzed by H^+. The ionic strength of the solution was brought to 0.1 *M* by addition of KCl. This solution will be referred to as the polarographic solution. In the presence of a surfactant such as triphenylphosphine oxide, the superoxide ion is produced at the dropping mercury electrode by the monoelectronic reduction of molecular oxygen. In the presence of SOD, the superoxide ion is dismuted according to the equation:

$$2\ O_2^- \xrightarrow[k_1]{\text{SOD}} O_2 + H_2O_2$$

The O_2 generated at the electrode surface, adding to that diffusing from the solution toward the electrode surface, will cause an increase of the polarographic wave. The increase is correlated with the SOD concentration by the equation (14):

$$7.42/[i_d/(i_i - i_d) - 1.25] = C + k_1\, tg\ [\text{SOD}]$$

where i_d, i_i are the polarographic currents in the absence and in the presence of SOD, respectively, tg is the electrode drop time, and C is a constant, taking into account the small fraction of the superoxide ion which dismutes spontaneously in the reaction layer.

The addition of a large amount of CSF (up to 30% volume) into the polarographic cell was found to give a polarographic maximum (15), due to the adsorption and the deadsorption, at different potentials, of a CSF component on the electrode surface. This maximum interferes with the SOD determination (Fig. 1, A and B). The presence of 0.5% human plasma in the polarographic solution completely suppresses the maximum, while it has a negligible effect on the height of the polarographic wave (Fig. 1C). Under these conditions, CuSOD concentrations as low as 5×10^{-11} *M* can be detected in CSF.

Because of the low volume of the samples, the activity measurements were carried out in a small polarographic cell, total volume 0.6 ml, home-built, equipped with a dropping mercury electrode, a Pt counter electrode, a saturated calomel electrode, and with an automatic Hg discharging system.

The CSF, buffered at pH 9.8 by a suitable addition of 0.5 *M* Na_2CO_3, was injected into the polarographic cell after equilibration with air. Since the final concentration of the CSF in the polarographic cell was 30% v/v, the dilution effect was taken into account in the

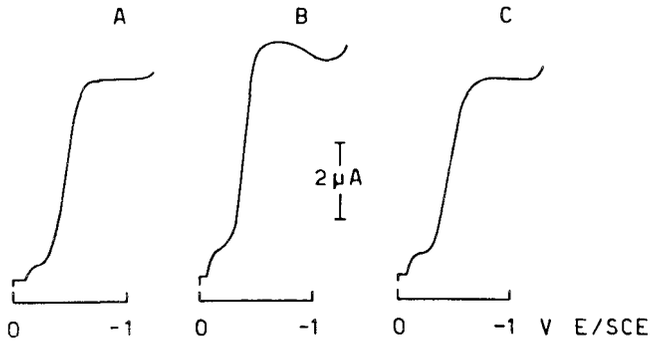

Figure 1. Effect of plasma on oxygen polarographic maximum due to CSF. (A) Polarographic solution. (B) As A plus 30% volume of CSF. (C) As B plus 0.5% of human plasma.

calculation of the SOD activity. The concentration of this enzyme was calculated from the activity measurements, assuming $K_1 = 2.3 \times 10^9\ M^{-1}\ sec^{-1}$, k_1 being the second order kinetic rate constant of dismutation of the superoxide ion by CuSOD at 0.1 M ionic strength (16). The SOD content of the serum was also measured by this method.

To test the effect of ascorbate and albumin on SOD activity measurements, CuSOD was assayed polarographically in the presence of increasing amounts of the above-reported compounds. No significant interferences by albumin up to 1 g/liter of albumin and by ascorbate up to 1 mM were found. These concentrations are about one order of magnitude higher than those present in CSF. Furthermore, 10^{-3} M diethyldithiocarbamate does not interfere with the polarographic oxygen wave while it strongly inhibits the CuSOD activity (17).

To measure the contribution of MnSOD to the CSF total SOD activity, a sample of 4 ml of CSF was concentrated by a centrifugal microconcentrator with a 10,000 molecular weight cut off. The concentration of a sample of CSF by a microconcentrator resulted in a 50-fold enrichment of the SOD activity in the retentate. Since no significant residual SOD activity was measured in the retentate after 1-hr incubation at 37°C with 10^{-3} M diethyldithiocarbamate, it appears that the contribution of MnSOD to total SOD activity in the CSF is negligible. In fact, taking into account the value of the second-order kinetic rate constant of dismutation of the superoxide ion by MnSOD, which under our experimental conditions is $k = 1.1 \times 10^7\ M^{-1}\ sec^{-1}$ (18), the concentration of the MnSOD in CSF was calculated to be lower than 2×10^{-10} M. This result is in agreement with the previous findings which showed no cyanide-insensitive activity in CSF (4). All of the measurements were performed at 22°C.

Statistics. The regression lines of Figures 2–4 were calculated according to the method of least squares. The multiple comparisons of the data of Table I were carried out according to the Student-Newman-Keuls test (19).

Results and Discussion

The concentrations of total proteins, albumin, and SOD in CSF samples obtained from reference subjects and from AD and ALS patients are summarized in Table I. From this table the large variability of SOD concentration within each class is evident, while its average value in ALS and AD patients is about 65% and 35% lower than that of the reference subjects, respectively. Table I shows also that the average value of the total protein concentration in the CSF of AD and ALS patients is similar to that of the reference subjects. It should be noted that no significant difference in SOD mean concentration was found in the serum of the reference subjects (2.91 ± 0.38 nM) and of AD (3.11 ± 0.36 nM) and ALS patients (3.02 ± 0.47 nM).

The average value of CuSOD concentration in the CSF of reference subjects, reported in Table I, corresponds to a SOD-controlled first-order decay constant of the superoxide ion of 4.4 sec^{-1}. This value is close to the value of 4.2 sec^{-1}, reported by Marklund *et al.* (4), for the CSF of normal subjects. The CuSOD concentrations found in CSF are high enough to produce the decay of superoxide ion according to a first-order process. In particular, the half-life of superoxide ion in the

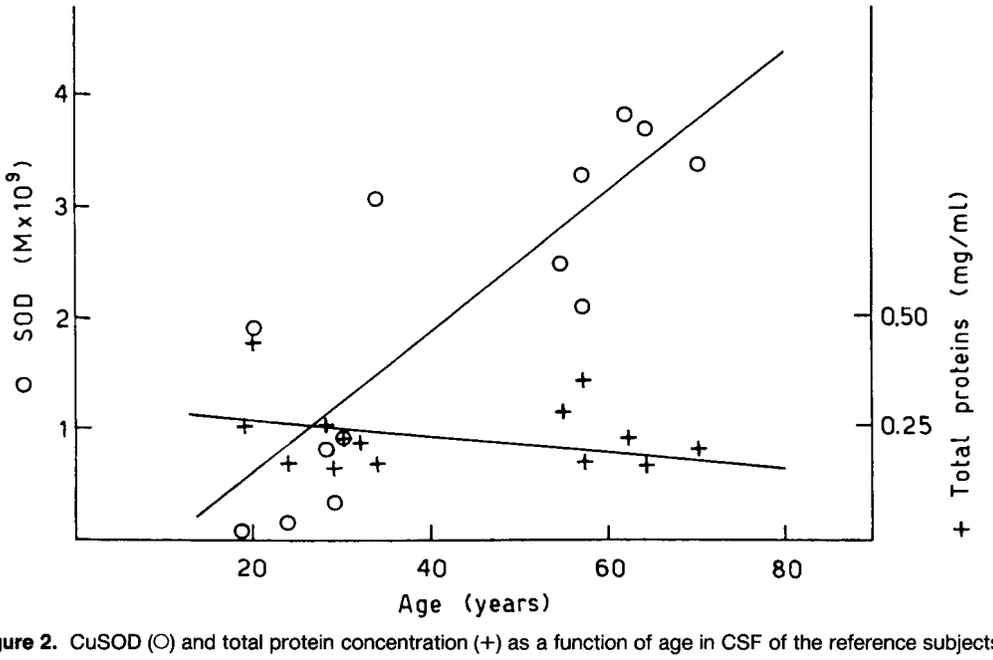

Figure 2. CuSOD (O) and total protein concentration (+) as a function of age in CSF of the reference subjects.

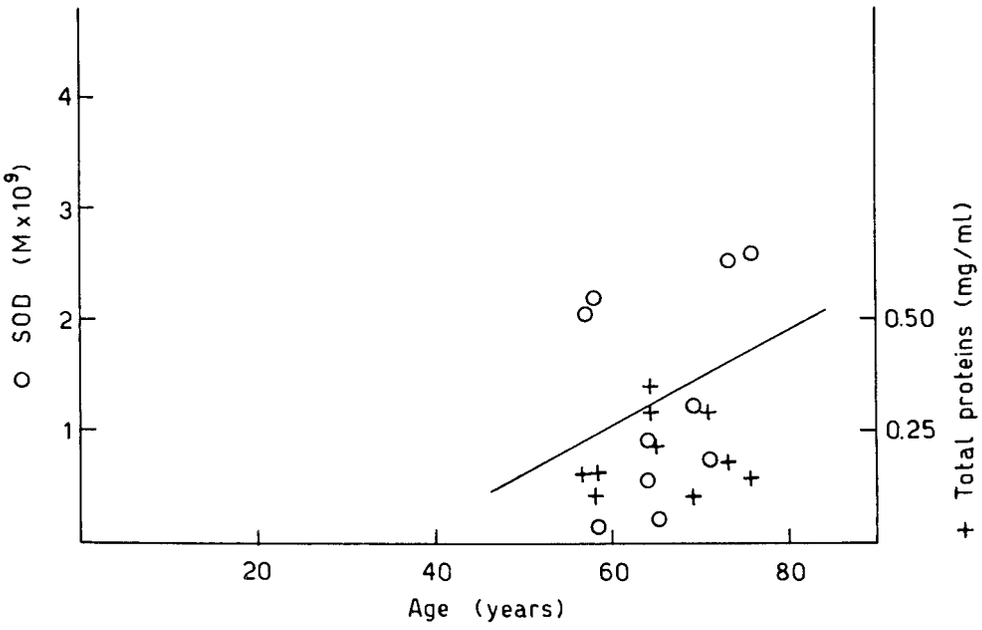

Figure 3. CuSOD (O) and total protein concentration (+) as a function of age in CSF of the AD patients. The regression line refers to CuSOD data.

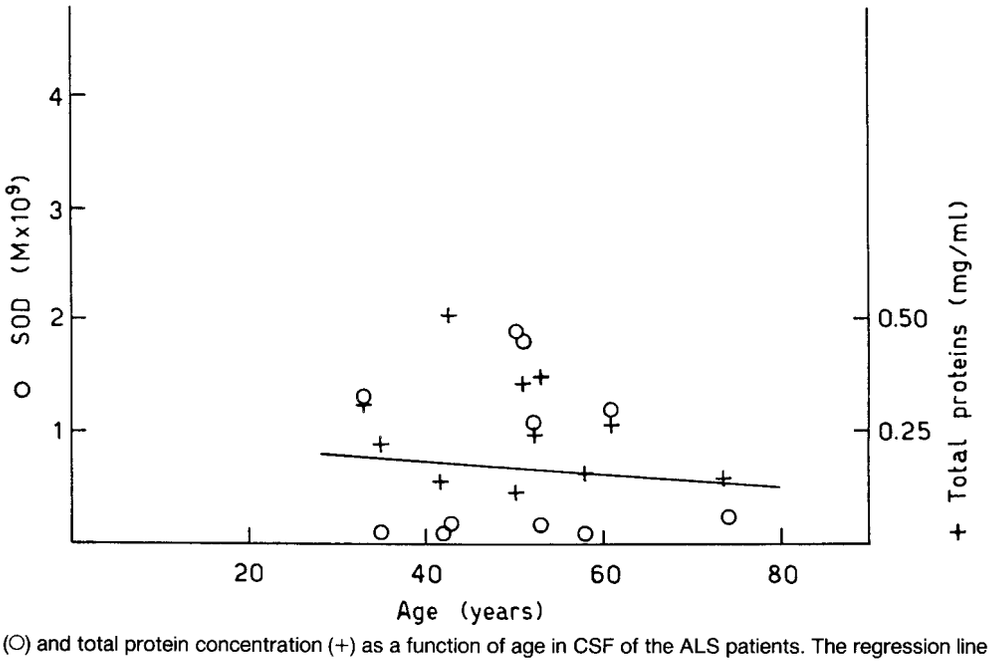

Figure 4. CuSOD (O) and total protein concentration (+) as a function of age in CSF of the ALS patients. The regression line refers to CuSOD data.

presence of 2 n*M* CuSOD (CuSOD mean value in the reference subjects, see Table I) is 0.15 sec. This value is close to the value of 0.16 sec, reported by Marklund *et al.* (4), for CSF of normal subjects.

The large variability of SOD concentration (Table I) could be explained, at least partially, by the relation of SOD content to age. In fact, in the case of the reference subjects, the SOD concentration increases with age, correlation coefficient 0.81, while the total protein concentration appears to be fairly constant (Figs. 2–4). However, no significant relationship was found between SOD concentration and age in AD and ALS patients, which had the correlation coefficient 0.29 and 0.10, respectively. This lack of correlation may be due to the more narrow range of age of these patients and to the overlapping of the pathologic variability with the physiologic one. No correlation was found between age and the Ig index, albumin, and total protein for all three groups of subjects we have examined.

Since in the controls the SOD activity appears to be age dependent, the variability introduced by age was minimized by considering only the reference subjects

Table I. SOD and Total Protein Concentration in CSF of AD and ALS Patients and in Reference Subjects

	Reference subjects		ALS	AD
	Full age range	Restricted age range		
Sample size	14	8	11	10
Age range (years)	19–70	32–70	33–74	57–76
SOD[a] range (n*M*)	0.08–3.84	0.90–3.84	0.10–1.98	0.11–2.61
Mean ± SD (nM)	1.99 ± 1.41	2.89 ± 0.99	0.73 ± 0.76[b,c]	1.30 ± 0.99[c]
Total protein range (mg/ml)	0.17–0.47	0.17–0.29	0.11–0.41	0.10–0.35
Mean ± SD (mg/ml)	0.26 ± 0.09	0.21 ± 0.04	0.22 ± 0.12	0.20 ± 0.10

[a] The SOD concentration was calculated from activity measurements (see text).
[b] $P < 0.05$ versus full age reference group (Student-Newman-Keuls test).
[c] $P < 0.01$ versus restricted age reference group (Student-Newman-Keuls test).

in the same age range of ALS and AD patients. In particular, the SOD mean concentration in the CSF of eight reference subjects in the age range from 33 to 76 years was 2.89 ± 0.997 n*M*. The comparison of this value with the mean SOD concentration in ALS and AD patients (Table I) shows a significant difference in SOD concentration ($P < 0.01$). No significant SOD concentration difference was found between the AD and ALS classes.

The presence of SOD in CSF should be discussed in light of the CSF function and the rate of decay of the superoxide ion. CSF bathes the brain and spinal cord, thus it tends to reflect the chemical composition and metabolism of the central nervous system (6). Therefore, the difference between the SOD activities we have measured may reflect a parallel change in the SOD activity in the central nervous system. We previously demonstrated (2) an age-dependent increase in the SOD level in the rat brain. Such an increase could represent a self-protective response of the normal brain to an increased production of superoxide with age. Our findings on SOD concentration in CSF during aging in reference subjects suggest that a similar phenomenon may be hypothesized to occur also in the human brain. The significant decrease of SOD activity in CSF in AD and ALS patients could represent an accompanying nonspecific phenomenon due to the neural loss caused by the disease, as well as a decrease of the antioxidant capacity associated with the disease.

Finally, the SOD presence in CSF could not be trivial, since it increases the antioxidant properties of this fluid. In fact in the absence of SOD, the rate of decay of superoxide radicals is controlled by the H^+-catalyzed dismutation process, which depends on the superoxide generation rate. Assuming for the latter process an upper value of 2×10^{-8} M sec^{-1} (20), the half-life of the superoxide ions in CSF should be higher than 100 sec, in the absence of SOD. This value agrees with the very low decay rate of superoxide radicals found by Marklund *et al.* (4) in CSF, when SOD was inhibited by cyanide. However, the presence of SOD in CSF, at the concentration values reported in Table I, decreases the half-life of superoxide of three orders of magnitude.

Our findings suggest that opposed trends in the antioxidant activity occur in the aging brain and in neurologic degenerative diseases, namely, ALS and AD. Nevertheless, further work is necessary to demonstrate the possible role of free radicals in the pathogenesis of these diseases.

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