ESR Spectra of Normal Human Serum after Treatment with Complement Activating Agents*

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We describe the appearance of a free-radical signal in the ESR spectrum of normal human serum incubated with several complement activating agents. The intensity of this signal is dependent of dose of activating agents, time and temperature. Signals elicited by different complement activators differ in morphology and kinetics. Inhibition by treatment with EDTA and the presence of the signal in activated C6-deficient rabbit serum suggest that the convertase forming steps of complement activation (C2 to 5) could be the source of free-radical containing molecules.

Introduction

Commoner's [1] pioneer work opened the way to the use of electron spin resonance (ESR) techniques in studying biological phenomena. Since then a growing body of experimental evidence has been accumulated showing correlations between the concentration of free-radicals and the activities of biological substances [2, 3], thus confirming earlier proposals of the involvement of free-radicals as intermediates in metabolic enzymatic processes [4]. Radicals formed during biological reactions give rise to ESR spectra typical of those seen in a pool of organic paramagnetic species in frozen solutions [4, 5]. In the strong free-radical signal generally slight or no hyperfine structures can be resolved. The strong influence exerted by the highly populated hypomobile molecular domain of these organic free-radicals averages the hyperfine structures so that such "solid state" spectra generally have much broader lines than the corresponding "liquid phase" ones [5].

In this study we present the results of investigations of ESR spectra of normal human serum (NHS) after treatment with complement (C) activating agents. The C-system represents a well-known amplification mechanism of immune and non-immune responses [6]. Its activation triggers an enzymatic cascade that leads to partial proteolysis of some components which originate short-lived and highly reactive fragments [7].

C-activation was achieved by different activators: inulin (polymer of fructo-furanose), cobra venom factor (CVF) (supposed to be the activated third component of C snake [8]), and zymosan (bacterial cell walls).

Experimental

Serum samples: blood samples from healthy male and female donors were obtained by venotomy. Serum collected from the clotted samples and free of hemolysis was stored at −80 °C until used. All donors gave explicit informed consent. ESR absorption derivative spectra were recorded with a Varian E-4 Spectrometer at 77 K, a frequency modulation of 100 KHz, and an amplitude modulation of about 5 gauss. Scans of 4000 gauss width were over 16 min with a time constant of 3 sec. Serum samples were placed in a 4.8 mm inner diameter glass cylinder, open at both ends, and snap frozen in liquid nitrogen. Frozen samples were transferred in a suitably shaped dewar tail (Varian E-246) filled with liquid nitrogen, by mildly warming the external surface of the glass cylinder and pushing the sample out with a wire rod. The diameter of the cylindrical frozen sample was chosen so as to minimize any vibration in the dewar and its length was such that the useful volume in the resonance cavity was completely filled [9].

Results and Discussion

Fig. 1 a shows an ESR spectrum of NHS. Two main signals are evident: the former at $g = 2.05$ is attributable to ceruloplasmin bound rameic ions (Cp-Cu^{2+}), the latter at $g = 4.3$ is due to transferrin bound Fe^{3+}. In Fig. 1 b the spectrum of an inulin treated serum is shown. A new signal at $g \simeq 2$, just after that of Cp-Cu^{2+}, is evident. Other activating

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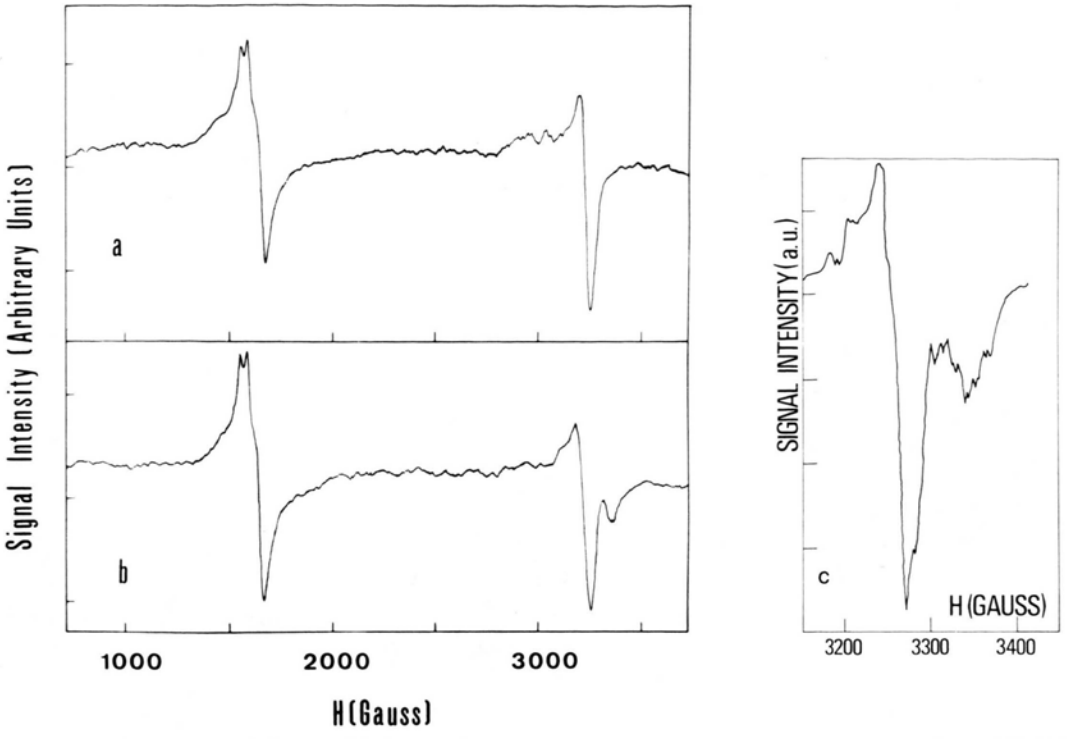

Fig. 1. a) ESR spectrum of normal human serum: signal at $H = 1640$ G is due to Transferrin bound Fe^{3+} ions, signal at $H = 3210$ G is attributed to Ceruloplasmin (CP) bound Cu^{2+}. b) ESR spectrum of a serum sample incubated at 37 °C for 10 min with inulin (1.5 mg/ml). A new signal at $g \simeq 2$ is evident just after that CP-Cu^{2+}. Inulin is currently considered a pure, though not powerful, activator of an alternative pathway of C-activation [6]. c) Detail at $g \simeq 2$ of the multistructured signal due to free radicals.

substances, like levamisole, zymosan and CVF, are equally effective in supporting the appearance of this new signal.

Saturation analysis (Fig. 2) shows that the intensity of the Cp-Cu^{2+} signal, due to the short spin-lattice relaxation time, does not reach a steady state at microwave power much higher than that sufficient to completely saturate the complement induced signal. This higher field signal is completely saturated at a microwave power of 5 mW, indicating that the spin-lattice interaction is weak in this system. This result and the g value strongly support the hypothesis that the paramagnetic centers responsible for this signal are free-radicals.

The multistructured aspects of this higher field signal suggest that different radical species contribute to its complex and broad ESR line (Fig. 1c).

Dose-response (Fig. 3) and time-course analyses after activation with inulin make it possible to show that 1.5 mg/ml inulin and 10 min at 37 °C represent the more amenable experimental conditions. On the other hand, maximal signal levels are obtained with a 1:3 dilution of serum, more concentrated samples being inhibitory. All of these data are in agreement with C activation studied with other methods [10]. It can be shown experimentally that the intensity and the morphology of this signal depend on the activating substance and hence on the mode of involvement of the enzymatic cascade (Fig. 4). Infact the CVF enters in the C-enzymatic cascade at C3 level [8] and thence does not require the intervention of C early components, while inulin induces convertase forming steps after the assembly of a functional binding site for the appropriate components.

Further evidence that this signal derives from activation of the C enzymatic reaction comes from the different origin and chemical structure of the substances capable of eliciting it, the only unifying factor among them being their ability to activate complement. Moreover, effects produced by two dif-

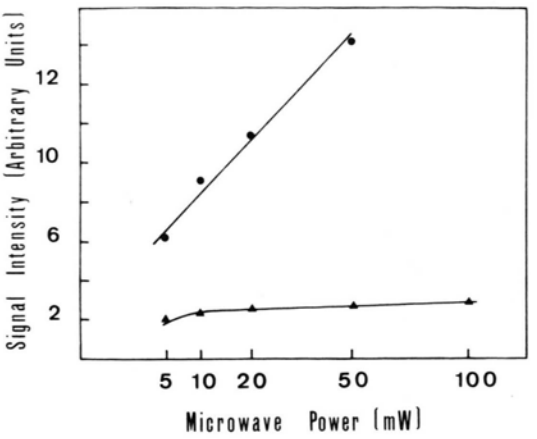

Fig. 2. (●) Peak-to-peak amplitude of Cp-Cu^{2+} ESR signal. (▲) Peak-to-peak amplitude of ESR signal at $g \simeq 2$. The root squared values of microwave field power are reported on the abscissa. Different behaviour of these two signals with increasing microwave field power demonstrates their different nature.

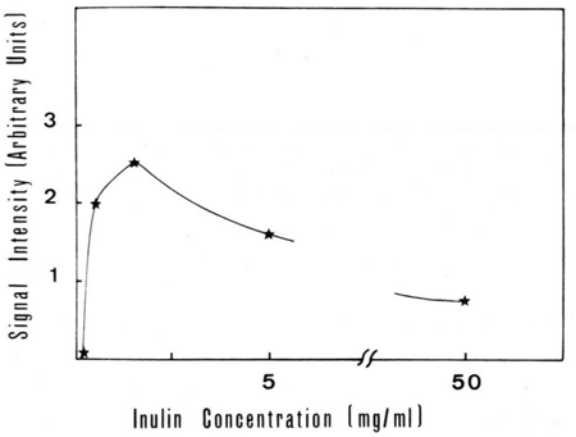

Fig. 3. Dose response analysis of peak-to-peak amplitude of ESR signal at $g \simeq 2$ of serum samples incubated at 37 °C for 10 min with inulin.

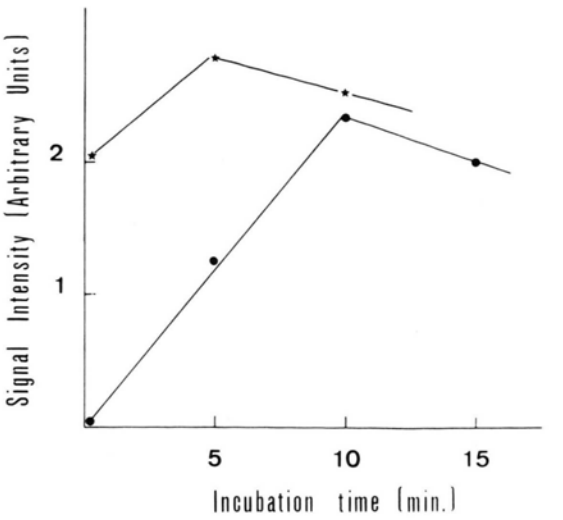

Fig. 4. Time course of the high field signal in serum treated with cobra venom factor (★) (Cordis Laboratories, Miami) or inulin (●). Note the higher intensity and the shorter latency of the signal induced by more efficient cobra venom factor. Inulin concentration: 1.5 mg/ml; CVF concentration: 0.1 units/ml.

ferent activators do not superimpose: *i.e.* serum already activated is unable to sustain the appearance of a second signal or a reinforcement of a previous one. Complement activation requires the presence of bivalent cations (Ca^{2+} and Mg^{2+}) which are essential for the assembly of functionally efficient enzymes [6]. If these essential components are chelated by adding adequate amounts of ethylendiaminetetraacetate (EDTA) inulin or zymosan treatment does not promote the appearance of the signal.

The appearance of high field signals in C6-deficient rabbit serum seems to exclude the necessity for an involvement of this component and the subsequently acting ones (C7–9). The presence of the signal in serum lacking Ca^{2+} but not Mg^{2+} (*i.e.*, containing excess Mg^{2+}-EGTA, Ethylmeglycolbis (*β*-aminoethylether) N,N′-tetraacetate) seems to rule out Cl. Our studies are mainly concentrated on the remaining C4, C2, C3 and C5 components. The interactions among these components best represent the cascade mode in which free-radical intervention is often claimed to take place [11]: there is active enzyme (convertase) formation from inactive precursors with the formation of highly reactive split-products from intact molecules (anaphylotoxins C3a, C5a) [6, 7].

The cell membrane is the main target of activated Complement components either for the transmission of messages through interaction with specific receptors or for damage of the membrane lipid bilayer with celle death. Some of the many different physio-pathological activities currently attributed to complement could in part be performed through free-radical formation mechanisms.

Acknowledgement

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