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## A Unique Hyperfine ESR Spectrum in Mouse Neoplasms Analyzed By Computer Simulation.\* (31585)

M. J. BRENNAN, T. COLE, AND J. A. SINGLEY

*Michigan Cancer Foundation, and Ford Motor Company Scientific Laboratories, Detroit and Dearborn, Mich.*

Electron spin resonance (ESR) spectra have been demonstrated in many intact plant and animal tissues. We have recently undertaken a detailed mapping of the ESR spectra occurring in various organs of genetically pure inbred strains of laboratory mice(1). This study parallels that of Nebert and Mason(2) but has concentrated on the region of  $g = 2.1 - 1.9$ , the region of organic free radical lines, where they recorded broader spectra. Our objective was to determine the characteristics and replicability of ESR spectra in normal adult tissues and to compare these with homologous tissues in various states of active cell-multiplication including the neonatal, regenerating, and neoplastic conditions.

We here report observations in neoplastic spleen and in the JAX C 1300 neuroblastoma. A unique spectrum showing nitrogen hyperfine splitting was found to occur in these tissues. This spectrum has been seen only in neoplastic tissues. Because of its unusual appearance and its association with the neoplastic state we have studied the phenomenon in detail.

*Experimental procedures.* BALB C+ mice and C<sub>3</sub>H+ mice, bred and carried in our own laboratories, maintained on Purina lab chow and housed in fiberglass cages, were used. Cedar shavings were used for bedding. Males and females 6 months old were used in the normal series and as transplant recipients for the 2 neoplasms. No strain differences in tissue signals were noted.

Animals were killed by CO<sub>2</sub> asphyxiation. As rapidly as possible, the tissue to be studied

was excised, minced into a Petri dish supported over ice chips, packed into signal-free fused-silica tubes of 3 mm inside diameter, and immersed in a liquid nitrogen Dewar. We estimate that time from excision to cooling to 1-5°C was in the order of 2 to 3 minutes and that samples had been taken to 77°K within 5 minutes of excision. Throughout these studies standard methods for cleaning glassware and surgical instruments were employed and the same set of surgical tools was used. Experiments with Ehrlich ascites suspensions showed little or no change in transplantation behavior of cells handled in this way and subsequently thawed and transplanted from that of control non-frozen suspensions of the same tumor.

The ESR spectrometer used in these experiments was a homodyne type(3) employing 100 kc/sec magnetic field modulation and operating at 9.6 Gc/sec. A cylindrical sample cavity operating in the TE<sub>011</sub> mode was used. The cavity and associated waveguide were enclosed in a Mylar bag which was immersed in a Dewar of liquid nitrogen. Sample temperature was determined by thermocouple to be within 3°C of the temperature of the liquid nitrogen during the recording of the spectra. Higher frequency spectra were observed by changing the microwave assembly, sample cavity, and klystron to components operating at 14 Gc/sec. The sensitivity of the apparatus was sufficient to provide a signal to noise ratio of 200 to 1 for the 0.00033% pitch in KCl standard sample (Sample type 904450, Varian Associates, Palo Alto, Calif.), microwave power 1 milliwatt and time constant 1 sec. ESR spectra were simulated on a GE-235 computer.

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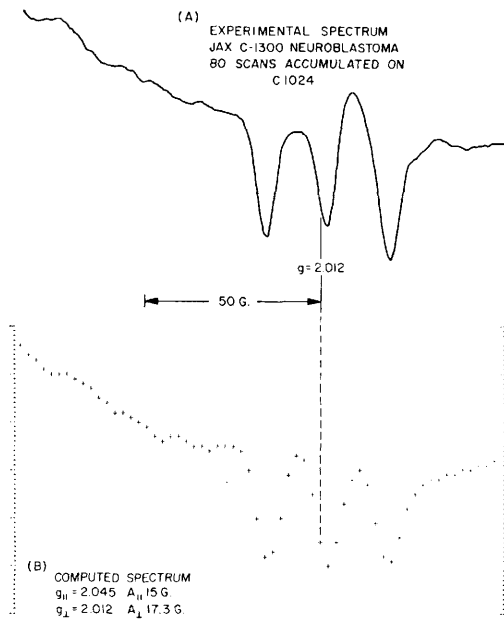


FIG. 1. (A) ESR spectrum of a sample of JAX C 1300 neuroblastoma observed at 77°K; (B) ESR spectrum computed with powder spectrum simulator program on GE-235 computer. Both spectra are first derivative presentations with magnetic field increasing to the right.

In order to improve signal to noise ratios and facilitate identification of the spectrum, several spectra were accumulated on a Varian C-1024 time averaging computer (Fig. 1A).

**Results.** Table I lists the frequency of occurrence of this signal in the reticulum cell sarcoma and the JAX C 1300 tumors studied. The signal was detected most regularly in the neuroblastoma. It is clear from these preliminary observations that the signal may not be present or apparent in tumor samples and that it is not necessarily a systematic character of all neoplastic tissue. The signal did not become apparent in RCS spleen until extensive infiltration of the spleen by the leukemia cells had developed. However, the signal was

TABLE I. Incidence of 3-Line Spectrum in RCS and JAX C 1300 Neoplasms.

Type	No. of tumors	No. with 3 lines	Remarks
RCS (mouse)	13	5	>9 days after transplant
JAX C 1300 (mouse)	6	6	Both early and late post-transplant specimens

clearly not the result merely of pre-terminal or agonal events. In the JAX neuroblastoma it was observed not only in large late implants but in early ones as well. Implants of this neoplasm commonly contain necrotic regions but care was taken to limit the sampling to viable regions of the tumors in these studies.

**Discussion.** We have assigned this spectrum to a paramagnetic molecule bearing a considerable unpaired electron spin density on or near a  $N^{14}$  nucleus. A computer augmented study of the details of the spectrum leads us to propose  $g_{II} = 45 \pm .003$ ,  $g = 2.012 \pm .001$ , and a hyperfine coupling  $A_I = 17.3 \pm .5$  gauss,  $A_{II} = 15 \pm 3$  gauss. Also there is probably a heavy atom (at. wt. >20) sharing some unpaired electron spin density.

Our reasons for the above assignment of the 3-line spectrum are as follows:

1. The 3 lines are *not* due to 3 different radicals with a fortuitous series of closely spaced  $g$  values. If such were the case the interline separation would have been proportional to the magnetic field strength. A spectrum taken with a 14 Gc spectrometer showed no change in the interline spacing. Had the line separations been due to 3 different  $g$  values the interline spacing would have increased by 45%.

2. Three hyperfine components of equal intensity are produced when an electron spin interacts with a nuclear spin  $I = 1$  (number of hyperfine lines =  $2I + 1$ ). Nitrogen is the only element having a single abundant isotope of  $I = 1$ .

3. The size of the hyperfine splitting is within the range known to occur (4,5,6,7,8,9, 10) for  $N^{14}$  in various free radicals.

4. A computer program has been written which calculates ESR powder spectra for a magnetic molecule when the  $g$  and hyperfine tensor components and nuclear spin are given. A reasonably good fit to the experimental spectra can be obtained from this program by using the magnetic parameters given above. (Details of the computer program are available on request from the authors.) The first spectrum calculated by the computer for the parameters  $A_{II} = 15$  gauss;  $A_I = 17.3$  gauss;  $g_{II} = 2.045$ , and  $g_I = 2.012$ , did not show the broad line observed in the experimental spectrum. It should be noted that there is, in the

experimental spectrum, a line centered at  $g \approx 2.06$  of about 200 gauss width which gives a concave shape to the baseline. In subsequent calculations the computer program was modified to include a broad line (200 gauss width) centered at  $g = 2.057$  for the above  $g$  and  $A$  parameters. A close fit to the experimental spectrum occurred (Fig. 1B). We have found the fit of the computed spectrum to the experimental ones deteriorates notably for changes of the order of 3 gauss in  $A_{II}$  and .005 in  $g_{II}$  around the values given above. Changes of .5 gauss in  $A_I$  and .001 in  $g_I$  give noticeably poorer fit. It is not possible to prove that some drastic modification in our model could not produce a better fit. However, the close correspondence between experimental and computed spectra provides support for the proposed model.

5. Proximity of a heavy atom is inferred from the deviation of the  $g$  value from that of the free electron ( $g_{free} = 2.0023$ ). Deviations of the  $g$  factor from 2.0023 are due to the effect of spin orbit coupling. The spin orbit coupling in an atom increases rapidly with atomic mass. Thus when a radical contains only C, H, O, and N,  $g$  factors are always in the range of 2.000 to 2.007. In the present case where the smallest component of the  $g$  tensor is 2.012 there is strong reason to suspect the presence of a heavy atom near the site of the unpaired electron in the molecule. Since only nitrogen hyperfine structure is apparent, the heavy atom must have an isotopic composition consisting in large excess of isotopes with nuclear spin  $I = 0$ . Iron, sulfur and calcium are heavy tissue elements which fit this requirement.

6. This region of  $g$  values (2.010-2.050) is one in which lines associated with complexes containing  $Fe^{3+}$  and  $Cu^{++}$  have frequently been described. We have ruled out copper as the source of our spectrum due to the completely different characteristics of copper hyperfine structure from those which we have observed.

To the best of our knowledge, after an extensive review of published ESR spectra observed in whole tissues and in tissue fractions and homogenates obtained from plant and animal sources, the signal which we have studied

in these two mouse neoplasms is unique. Samples of several other transplantable mouse tumors have been tested. In the small number of samples observed to date, this hyperfine spectrum has not been clearly demonstrable in these other tumors.

However, we have learned from H. Kon (personal communication) that similar lines were recently seen by him in a preparation of boiled guinea pig synaptosomes, admixed at a ratio of approximately 15 to 1 with mitochondria. The JAX C 1300 transplantable tumor was originally designated as a neuroblastoma at Bar Harbor. The Stansly virus(11) by which the transplantable reticulum cell sarcoma which we obtained from Dr. Stansly was originally induced, is an oncogenic virus with neurocytotoxic effects(12).

*Summary.* A unique triplet signal has been found in two mouse neoplasms during ESR studies of various mouse tissues. A virus-induced reticulum cell sarcoma of the spleen and the JAX C 1300 neuroblastoma both manifest this hyperfine spectrum. This spectrum has been assigned to a nitrogen containing free radical with the unpaired electron vicinal to a heavy atom. Specifications for this triplet signal are  $g_I = 2.012$ ,  $g_{II} = 2.095$ , and  $A_I = 17.3$  gauss,  $A_{II} = 15$  gauss. Analysis of the ESR signal was facilitated by the use of a computer simulation of the experimental spectrum.

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**The Ontogeny of Complement Activity. Complement Titers in the  
Developing Chick Embryo During Graft-Versus-Host Reactions.\*  
(31586)**

HENRY GEWURZ,<sup>†</sup> MARY ANN SOUTH,<sup>‡</sup> AND ROBERT A. GOOD<sup>§</sup>

*Pediatric Research Laboratories, Variety Club Heart Hospital, and the Department of Microbiology,  
University of Minnesota*

This report summarizes our experience with titrations of total complement (C') activity in the developing chicken embryo undergoing a graft-versus-host (GVH) reaction. Using a rapid convenient assay for total C' activity in chicken serum, it was found that (a) C' activity is present in the embryonic chick, and (b) C' titers are elevated rather than decreased during the GVH reaction in the embryonic chick.

*Material and methods. GVH reaction in the chick embryo.* GVH reactions, based on the methods of Simonsen(1) and Cock and Simonsen(2), were produced as previously described(3). In each of these experiments 0.1 ml of heparinized whole blood from an adult White Leghorn chicken was injected into the vitelline circulation of each of a group of 2 to 6 13-day embryos of this same outbred strain. Five days later, on the 18th day of incubation, following exsanguination by direct puncture of the vitelline circulation, the eggs were opened, the spleens removed, and the weight of each individual spleen recorded in milligrams.

*Collection of serum.* Freshly drawn whole blood was allowed to stand at room tempera-

ture for ½ hour and at 0 to 4°C for 2-4 hours. Serum was collected by centrifugation and assayed immediately for total C' activity. Although adult chicken serum can be frozen and stored for subsequent C' assay, fetal chicken serum sometimes shows precipitation on freezing and is thus unsatisfactory for subsequent study.

*Preparation of indicator cells.* The washed rabbit erythrocyte was used as the indicator particle in this reaction. Rabbit blood was collected in sodium EDTA. The erythrocytes were washed twice in 0.01 M EDTA in veronal-buffered saline and twice in the veronal-buffered saline with supplemental Mg<sup>++</sup> and Ca<sup>++</sup>, (GVB<sup>++</sup>), as described by Mayer(4). The suspension was standardized to 2 × 10<sup>8</sup>/ml cells in GVB<sup>++</sup>, the buffer in which the lytic reaction was to be performed. The rabbit erythrocyte is approximately twice the size of the sheep erythrocyte, and contains proportionately more hemoglobin.

*Preparation of chicken-antiserum against rabbit erythrocytes.* This antiserum was prepared by immunizing a group of adult White Leghorn chickens with washed rabbit erythrocytes according to the schedule of Nelson(5). The animals were given 1.0 ml of a 20% suspension of these cells intraperitoneally on day 1, another 1.0 ml of this suspension intravenously on days 3, 5, 7, and 9, and 1.0 ml of a 50% suspension intraperitoneally on day 11. Serum was harvested on day 17; it had an agglutinating titer of 81,920 when tested by adding 0.1 ml of this serum to 0.1 ml of a washed 2% suspension of rabbit erythrocytes. The serum was heated at 56°C for 30 minutes to destroy C' activity.

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<sup>†</sup> Postdoctoral Fellow, USPHS Cardiovascular Graduate Training Grant 5T1 HE-5222; present address: Nat. Inst. of Dental Research, NIH, Bethesda, Md.

<sup>‡</sup> Special Fellow, Nat. Inst. of Allergy and Infect. Dis.

<sup>§</sup> American Legion Memorial Heart Research Professor of Pediatrics and Microbiology.