Changes of Tissue Water Proton Relaxation Rates During Early Phases of Chemical Carcinogenesis

(blood serum/azo-dye/aromatic amines/spleen iron)

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ABSTRACT Water proton spin-lattice relaxation rate (T₁) was determined on tissues of rats experiencing early phases of chemical carcinogenesis. Rats were fed a fast acting carcinogen, 3'-methylidimethylaminoazobenzene, and a slower acting carcinogen, 2-acetylaminofluorene, for up to 4 weeks. T₁ of blood serum and liver tissue was significantly higher than those of controls after 4 weeks of 3'-methylidimethylaminoazobenzene feeding. This was not the case for 2-acetylaminofluorene. The blood serum T₁ increase reflected the onset of liver nodulation (assumed to be preneoplastic). Liver T₁ values increased as the degree of nodulation increased. Blood serum T₁ correlated inversely with protein content and directly with water content. Liver T₁ values correlated with water content, but this was not true for spleen T₁ values. Spleen T₁ values were significantly lower than controls at the earliest sampling date for each carcinogen; one week for 3'-methylidimethylaminoazobenzene and 4 weeks for 2-acetylaminofluorene. The spleen T₁ decrease paralleled an increase of iron detectable by electron spin resonance in this tissue. Spleen T₁ decreases are probably not unique to chemical carcinogenesis.

Damadian (1) first observed that tumorous rat tissue had larger tissue water proton spin-lattice relaxation (T₁) values than the corresponding normal tissue. This basic observation has been repeatedly confirmed in various tumors of the many animals (1-10), including humans (11-14), tested. In fact, it was shown to be true in vivo by Weisman et al. (2), who made measurements on the tails of live mice. We (15) demonstrated, using ascites tumor development in mice, that the blood serum of cancer-developing animals reflects tumor development by increased T₁ values, and that conditions other than tumor development will result in T₁ increases. Since a major portion of human tumors have been attributed to environmental carcinogens (16, 17), it seemed particularly necessary to test the possibility of using nuclear magnetic resonance (NMR) to detect early changes associated with chemically induced carcinogenesis in a model animal study. Hazlewood et al. (4) had shown that preneoplastic nodules of a murine mammary tumor had elevated T₁ values over those of the normal tissue. We report here that significant T₁ changes occur in the liver, spleen, and blood serum of rats experiencing the very early stages of chemical carcinogenesis.

Abbreviations: T₁, tissue water proton spin-lattice relaxation; 3'-MeDAB, 3'-methyl-4-dimethylaminoazobenzene; AAF, 2-acetylaminofluorene; NMR, nuclear magnetic resonance; ESR, electron spin resonance.

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MATERIALS AND METHODS

Albino male rats (75-100 g) of the Wistar Strain were obtained from National Laboratory Animals (St. Louis). They were fed the carcinogens 3'-methyl-4-dimethylaminoazobenzene (3'-MeDAB) and 2-acetylaminofluorene freely at a level of 0.66% (w/w) in a basal synthetic diet for 3'-MeDAB (18) and AAF (19) liver carcinogens, respectively. The 3'-MeDAB is a fast acting carcinogen, producing liver tumors in rats by continuous feeding during 8-10 weeks (see ref. 20, for instance), whereas AAF requires a longer time, i.e., on the order of 15 weeks. Three rats receiving the 3'-MeDAB diet were killed each time after 1, 2 and 4 weeks of feeding, and at each time two rats receiving the 3'-MeDAB control diet were killed also. Three rats receiving AAF diet and three rats receiving the AAF control diet were killed after 4 weeks of feeding. The animals were killed by being placed in a 100% CO₂ atmosphere for 45 sec; then blood was withdrawn from the beating heart. The serum was separated from the blood by 15 min of centrifugation. The liver and spleen were taken rapidly from the animal, rinsed with H₂O, blotted dry, and then wrapped in aluminum foil. All samples were stored at -20°C until about 3 weeks after the first samples were taken, at which time they were thawed and placed on ice. T₁ values were determined by a method described (15). About 0.3 g of liver or spleen snipped from the margin and 50 μl of blood serum constituted the sample sizes. Measurements were done at 2°C ± 1° at 8 MHz. This frequency discriminated between tumor and normal tissue better than at higher frequencies (10). Independent studies showed that tissue storage of up to 3 weeks at -20°C did not alter the measured T₁ values. Blood serum protein was estimated colorimetrically and by measurement of 280 nm absorbance, with bovine serum albumin as a protein standard. Tissue water content was determined by drying the tissue samples at 110°C until constant weight was achieved. Water content of blood serum was determined by drying the samples over sulfuric acid for 48 hr. The range in values obtained was from 90 to 95%. Electron spin resonance (ESR) spectra of the oven-dried spleen tissue was determined at helium temperatures (10-15 K). Prominent transitions were observed at g values of 6, 4.3, and 2. The incident microwave power was 5 mW.

RESULTS

The results obtained are shown in Tables 1, 2, and 3. Table 1 shows that blood serum T₁ values of animals fed 3'-MeDAB were different from controls only after 4 weeks of feeding; but this was not true for AAF. Liver T₁ values showed the same
tendency as blood serum except the difference between the animals fed 3'-MeDAB and control animals after 4 weeks of feeding was only marginally significant. There was an increase in Ti as the degree of nodulation increased (see footnote to Table 1). In contrast to liver and serum, spleen Ti values differed highly significantly from control tissue even after 1 week of 3'-MeDAB feeding. The same was true after 4 weeks of AAF feeding, the first and only sampling date for this carcinogen. Ti values of spleen from carcinogen-fed rats were lower than control tissue. This is in contrast to that of the liver and blood serum Ti values, which increased due to carcinogen feeding.

Table 2 shows that the Ti value of serum and liver correlated with water content, but this was not true of spleen Ti values. The spleen Ti values were a surprise and, hence, we made an estimation of the paramagnetic species content of the tissue, anticipating that this would explain the results. ESR spectra of the oven-dried spleen tissue were made at liquid helium temperatures. The most prominent features of the spectra at this temperature are g = 6, 4.3, and 2 transitions. The g = 6 signal is a measure of ferric high-spin heme iron, whereas g = 4.3 is a measure of ferric nonheme iron, i.e., iron in the rhombic configuration (21). The g = 2 signal is a mixture of a free radical component along with a heme and also nonheme iron component. The correlation coefficients in Table 2 indicate that the Ti values of spleen correlate best with the g = 4.3 signal and that the g = 2 signal correlates best with the g = 6 signal, i.e., the ferric high-spin heme iron contributes strongly to the g = 2 signal. The relative magnitudes of the ESR signals obtained are presented in Table 3.

**DISCUSSION**

The data show that there is an increase in blood serum Ti when carcinogen feeding causes the liver to pass from normal into the preneoplastic nodulation stage. The nodulated livers had higher than normal Ti values, and the Ti values increased as the degree of nodulation increased. The liver Ti increase correlated with water content, which has been shown to be true for other tissues (7, 9, 10). The blood serum Ti increased as the water content increased and, therefore, as the protein content fell. The reason that there is an increased water content of liver and blood serum is not known. Diminished protein synthesis, if true, would only partially explain the results.

Our data show that the carcinogen diet, at the earliest sampling date (the first week for 3'-MeDAB and the fourth week for AAF), caused a decrease in spleen Ti values. The Ti decrease of spleen occurred long before liver nodulation oc-
curred. An increase of paramagnetic species in the tissue would be expected to decrease T1 values. ESR measurements showed that, in fact, there was an increase in iron content in the spleen tissue that had lowered T1 values.

One interpretation of the increased iron content of the spleen is that the carcinogens either alter the spleen morphology, i.e., microcirculation, such that an increased number of erythrocytes is phagocytized by spleen macrophages, or the carcinogens alter or damage the erythrocyte membranes such that spleen macrophages "call" the red cells from the circulating blood. These mechanisms have been shown to be operative under certain conditions; for example, in Gaucher's disease (22), spleen microcirculation is altered and in the case of "noxious" agent-induced erythrocyte damage (23), the red cell membranes are assumed to be damaged. An increased iron content could also be explained by erythropoiesis. Erythropoiesis occurs during metastases of some cancers, but we would rule it out here since the iron accumulation occurred very early in chemical carcinogenesis. Also, erythrocyte breakdown products have been observed in the spleen of rats fed azo-dye (24). Thus, rather than interpreting the spleen T1 decrease as a unique manifestation of chemical carcinogenesis, we think it is merely due to "noxious" chemical action on the erythrocytes or spleen per se.

Our observation here that the blood serum of rats experiencing the early phases of chemical carcinogenesis is increased, extends in a sense our previous findings that tumor development is reflected by T1 increases in blood serum (15). Blood serum is convenient to monitor. This, and the fact that the T1 perturbations showed up in the preneoplastic nodulation stage, suggest the potential use of blood T1 determination as a possible screening technique.

We are not completely sure that the nodules observed after 4 weeks of azo-dye feeding are preneoplastic. However, the results obtained in a detailed study by Acros et al. (25) of liver tumor formation induced by 3'-MeDAB feeding imply that 25% of the livers would develop tumors by seven months if the carcinogen feeding was stopped at four weeks. It seems justified to conclude, then, that our results show that there is an increase in blood serum T1 long before bona fide liver tumors are present. There is a need for the work presented here to be extended to other chemical carcinogens on other model animal systems, and to keep in mind its potential usefulness in detection of human preneoplastic lesions.

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