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# Alteration of Aliphatic Lipid Proton NMR Linewidths by Malignant Tumors in Guinea Pigs

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**Abstract**—*Water-suppressed proton nuclear magnetic resonance spectroscopy was used to observe plasma lipoprotein lipid methyl and methylene resonances from guinea pigs which had been injected with viable or heat-killed line 1 or line 10 tumor cells or sterile oil. It was shown that the widths of these resonances became significantly sharper as the number of tumor cells grew. Plasma from tumor-free control animals showed no change in the NMR linewidths. It is concluded that the changes observed reflect a specific host response to viable tumor cells, and in these models there is a reciprocal relationship between the number of viable tumor cells and the linewidths of plasma lipoprotein methyl and methylene resonances.*

## INTRODUCTION

WE HAVE REPORTED that the proton nuclear magnetic resonance linewidth of lipoprotein aliphatic lipid resonances obtained by water-suppressed proton NMR spectroscopy of plasma correlates with the presence or absence of malignant tumors, differentiates benign from malignant tumors, and differentiates cancer from non-cancer diseases in patients with a high level of certainty ($P < 0.0001$) [1]. Since this report appeared our observation has been confirmed in several publications [2–5] while others have failed to confirm it [6–9]. A water-suppressed proton NMR spectrum of plasma is essentially a spectrum of lipoprotein lipids as well as any small molecules (e.g. sugars) present in sufficiently high concentration [1, 10, 11]. The aliphatic methylene and methyl lipid resonances of the plasma from patients with malignant tumors are substantially narrower than the corresponding resonances from patients with benign tumors, non-tumor diseases, or normal controls.

We now report results from two well-defined guinea pig carcinomas of bile duct origin (line 1 and line 10), growing in syngeneic strain 2 guinea pigs [12–16]. The purposes of these studies were, first, to extend our clinical observation to an animal model and, second, to relate in a way not possible in the clinical setting, linewidth of plasma aliphatic lipid resonances to the tumor burden.

Both line 1 and line 10 grow as solid tumors when injected into the subcutaneous space or as ascites tumors when injected into serous cavities. Line 10 tumors grow progressively and metastasize whereas line 1 tumors induce an immune response and are rejected between 8 and 14 days [17, 18]. In this model, it was possible to sample each animal throughout the time course of the experiment.

## MATERIALS AND METHODS

*Collection of plasma*

Serial blood samples were collected from guinea pigs anesthetized with ketamine using citrade as anticoagulant. The blood was centrifuged and plasma was collected and stored at 4°C until the NMR spectra were obtained. Plasma cannot be frozen if it is to be used for NMR spectroscopy [19–22]. Strain 2 guinea pigs were purchased from Ribi Immunochem (Hamilton, Montana). The control and experimental group each consisted of six guinea pigs.

*NMR experimental procedure*

All spectra were collected in a Bruker AM360 spectrometer. The NMR probe was detuned (about 2 MHz) to eliminate radiation damping. In this procedure, the 90° radio-frequency pulse was set to 20 ms. Field shimming on the proton free-induction decay (FID) (without pre-saturation) was continued until the water linewidth was less than 4 Hz. In

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properly shimmed, properly prepared samples the linewidth of the resonances of citrate, or if applicable EDTA, should be less than 2.2–2.5 Hz. If water linewidths exceed 4 Hz or citrate/EDTA linewidths exceed 2.5 Hz, unreliable results will be obtained. The water-suppressed spectrum was obtained using pre-saturation. The power level of the pre-saturating pulse was such that a selective 180° pulse was 23.5 ms. Use of excess power to suppress the water resonance alters lipid resonances. Water was pre-saturated for 4 s at this power level prior to acquisition of each FID. The spectrum resulted from signal averaging 16 FID. Before Fourier transformation, the accumulated FID was multiplied by an exponential inducing 2 Hz of line-broadening. The sample temperature was 21°C while NMR observation was made.

## RESULTS

*NMR spectra of plasma from control and tumor-injected guinea pigs*

The aliphatic region of the plasma NMR spectra of guinea pigs is shown in Fig. 1. A clear difference in the linewidths of the methyl and methylene resonances is seen for plasma taken from animals in which line 10 ascites tumor was growing progressively as compared to control plasma taken from guinea pigs injected with heat-killed tumor cells. These spectra are similar to those from tumor-bearing patients and control patients [1]. These results suggest that the linewidth reduction of aliphatic lipid resonance induced by the presence of malignant tumors exists both in humans and guinea pigs. In other experiments with several other animal models, similar linewidth narrowing with malignant tumors was also observed (Fossel E.T., unpublished). We suggest that this observation is a reflection of a response of the host to the presence of malignant tumors, and may reflect a difference in the metabolism of lipoprotein lipids. The methyl and methylene resonances in the NMR spectra arise from the total pool of the plasma lipids. Thus, all types of lipids contribute to these resonances. The quantity of plasma lipid is substantial and has a half-life of 8–12 h. In an average 70 kg human, the turnover of lipid is approximately 100 g/day [19, 20]. Tumor could not produce such a quantity of lipoprotein lipid.

*Plasma NMR spectra of guinea pigs injected subcutaneously with line 1 tumor cells*

Figure 2 shows the time course of the average of the methyl and methylene linewidths for guinea pigs injected subcutaneously with line 1 tumor cells ($3 \times 10^6$) or heat-killed (70°C, 25 min) line 1 cells as control. Control values were also measured for each animal prior to innoculation with tumor cells. Pre-innoculation and control values (average = 40.5 Hz) are very similar to control values in humans [1]. As previously reported [17, 18], line 1 tumors became palpable on day 2 and grew rapidly to days 7–8, when they reached their greatest mass (12 × 17 mm). However, the absolute number of tumor cells could not be assessed in this model. After days 7–8, these tumors underwent rapid immunological rejection and had almost totally regressed by day 14 [17, 18]. Over this interval of growth (days 0–7), the average linewidth of the methyl and methylene groups decreased, progressively falling from 40.6 Hz to 27.1 Hz. These observations imply that the extent of tumor mass may be directly correlated to the change in the plasma NMR spectrum. After day 8, the linewidths of the

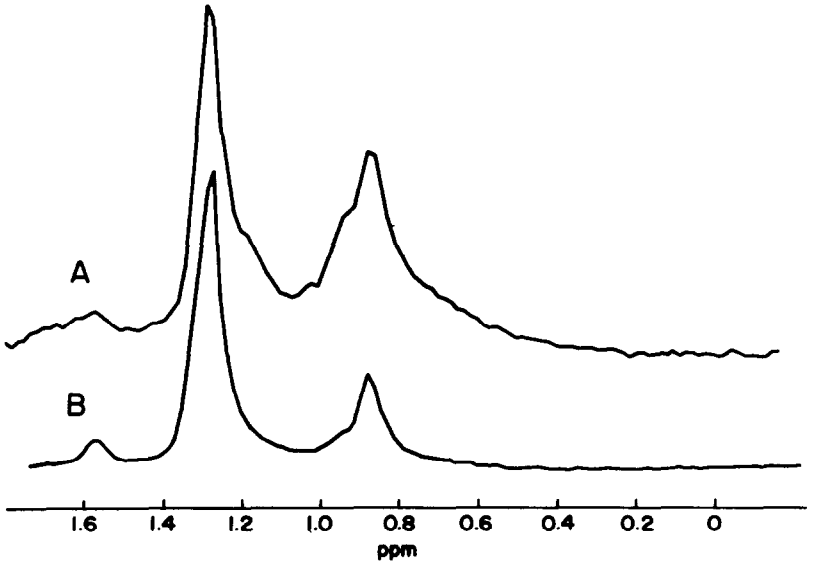


*Fig. 1. 360 MHz water-suppressed proton NMR spectra of plasma in guinea pigs. A is a day 7 spectrum of plasma from an animal which received heat-killed line 10 tumor cells, and B is from an animal that received live line 10 tumor cells as described in Fig. 3.*

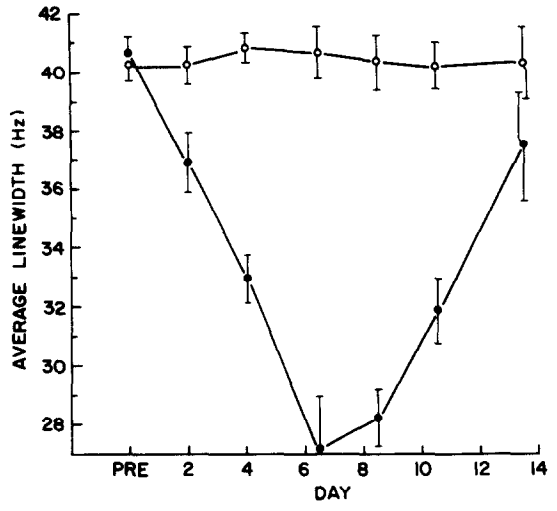


*Fig. 2. Average methyl and methylene linewidths measured from the citrated plasmas of strain 2 guinea pigs, injected subcutaneously with* $3 \times 10^7$ *viable line 1 tumor cells (●), or heat-killed tumor cells (○). Before injection, plasma was analyzed for both groups (pre-value) and then on days 2–3, 4, 6–7, 8–9, 10–11, and 13–14. For each data point (± S.D.),* n = 6.

methyl and methylene resonances became increasingly broader, again paralleling the loss of viable tumor cells in the animal. By day 14, the linewidths were essentially normal and tumor was no longer palpable in these experimental animals. Thus, it can be seen that the linewidths closely parallel the natural history of this tumor. Throughout this time there was no change in the plasma NMR spectra of the control, tumor-free animals.

*Plasma NMR spectra of guinea pigs injected intraperitoneally with line 10 tumor cells*

Similar data are shown in Fig. 3 for the ascites growth of line 10 tumor cells. The pre-innoculation and control average methyl and methylene values were 39.7 Hz. On the second day after injection of $3 \times 10^6$ viable cells intraperitoneally, the average linewidth value became smaller (35.3 Hz) and continued to decrease as the tumor grew, reaching a value of 21.3 Hz on days 7–8. In this model, the number of tumor cells can be accurately assessed, as indicated in Fig. 3. In humans, a plasma NMR value of 33 Hz or lower was empirically defined as indicating malignancy [1]. This animal model crossed that value on days 2–3 when there were approximately $1.5 \times 10^7$ tumor cells growing in the peritoneum. After days 7–8, it was not possible to maintain the animals with tumor cells continuing to grow. Again, the progressive narrowing of the linewidth of the tumor growth implies a reciprocal relationship between the number of viable tumor cells and the linewidth values for methyl and methylene resonances.

*NMR spectra of plasma of guinea pigs injected intraperitoneally with sterile oil*

As a further control of these experiments, six guinea pigs were injected with sterile oil (Marcol)

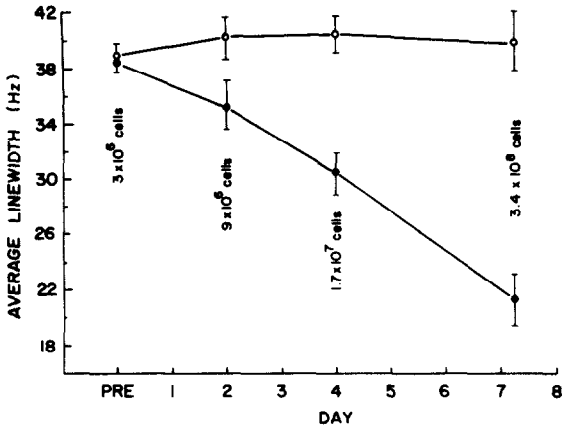


*Fig. 3. Water-suppressed proton NMR data for average methyl and methylene resonances from citrated plasma from guinea pigs which were injected intraperitoneally with* $3 \times 10^7$ *viable line 10 tumor cells (●), or heat-killed tumor cells (○). Samples were taken before injection of tumor cells and on days 2, 4 and 7–8. Numbers indicate the number of viable tumor cells at the various times.*

intraperitoneally. Citrated plasma was taken for NMR analysis before injection and on days 2, 4, and 8. The methyl and methylene resonances of plasma from these animals did not differ significantly from those that received heat-killed tumor cells. This was true even though intraperitoneal injection of sterile oil induces ascites fluid accumulation with recruitment of neutrophils, lymphocytes, and macrophages. The ascites fluid from these animals had broad (normal) methyl and methylene resonances. However, the ascites fluid from animals which received intraperitoneal injections of viable line 10 tumor cells had narrow methyl and methylene resonances. There were close similarities between the NMR spectra of plasma and ascites fluid under the various experimental conditions.

*Plasma NMR spectra of guinea pigs during a delayed hypersensitivity reaction*

In additional experiments, guinea pigs previously immunized with human serum albumin and complete Freund's adjuvant were rechallenged with antigen to produce a delayed hypersensitivity response. Citrated plasma was taken for NMR analysis before and 3 days later. A typical delayed hypersensitivity response was documented. This had no effect on the NMR spectra of these animals as the methyl and methylene resonances of these animals were not different from those of control animals.

## DISCUSSION

Our experiments allow us to conclude also, that the narrowing of lipoprotein lipid methyl and methylene linewidths is a specific response of the host to growing tumor cells. In the mammalian hosts tested and with all types of tumors tested, the plasma lipoprotein average aliphatic linewidths change as a response to viable tumor cells. In these experiments, inbred guinea pigs were identically treated except

for the tumor cells or sterile oil they received. Thus, the response observed in NMR linewidth values was directly related to the tumor cells the guinea pigs received. From these experiments, one can conclude that an inflammatory response *per se* is probably not responsible for the NMR changes in plasma observed in tumor-bearing animals.

In addition, under these carefully controlled conditions, there was a reciprocal relationship between the number of viable tumor cells and the average methyl and methylene proton NMR resonance linewidths. Such a relationship is more difficult to establish in patients with cancer, since a good estimate of the total number of viable tumor cells is not available in patients. However, in two patients previously described with acute myelomonocytic leukemia who were followed over a period of chemotherapy, both patients' plasma NMR values accurately reflected responses to chemotherapy [1]. Both patients responded initially; one had a relapse and the other remained in remission. The average methyl and methylene values reflected these events in the same way as the guinea pigs injected with line 1 or line 10 tumors.

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