Nuclear Magnetic Resonance Analysis of Tumor Necrosis Factor-induced Alterations of Phospholipid Metabolites and pH in Friend Leukemia Cell Tumors and Fibrosarcomas in Mice

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ABSTRACT

The alterations induced on the pool sizes of five phospholipid metabolites, glycerol 3-phosphorylcholine, glycerol 3-phosphorylethanolamine, phosphorylcholine, sn-glycerol 3-phosphate, and choline were studied by nuclear magnetic resonance (NMR) spectroscopy in murine tumors injected with recombinant murine tumor necrosis factor (TNF).

Solid tumors were obtained by s.c. injection of either Friend leukemia cells (clones 3Cl-1 and 745) in DBA/2 mice or murine fibrosarcoma cells (HeN4) in C3H/HeN mice. After tumor nodules had developed, TNF or bovine serum albumin was injected intratumorally. Treatment of both tumors with TNF resulted in a marked inhibition of tumor growth.

13P-NMR analyses of Friend leukemia cell tumors (and tissue extracts), 6 h after injection of TNF, showed: (a) a 1.5- to 3.5-fold decrease in the pool sizes of glycerol 3-phosphorylcholine and glycerol 3-phosphorylethanolamine; (b) a 7- to 8-fold increase of sn-glycerol 3-phosphate; (c) a 2- to 3.5-fold decrease of phosphorylcholine; (d) an alkaline shift (0.2 units) in intratumoral pH. Similar metabolic alterations occurred in TNF-treated HeN4 fibrosarcoma. 1H-NMR analyses of Friend leukemia cell tumor extracts also indicated, 6 h after tumor injection with TNF: (a) elevated choline levels (9x); (b) a 19-fold increase in the ratio [choline]/[phosphorylcholine]; (c) elevated (1.4x) levels of lactic acid; and (d) a 1.6-fold decrease in the [taurine]/[glycine] ratio.

The results are interpreted in the light of possible alterations in the activity of enzymes controlling the de novo biosynthesis and catabolism of phospholipids.

We conclude that NMR spectroscopy can be a useful means to monitor the level of some phospholipid precursors and/or derivatives as early markers of therapeutic efficacy in intact neoplastic tissues.

INTRODUCTION

Recent experimental evidence indicates that variations in the concentration of some phospholipid metabolites may represent biochemical responses of cells to factors regulating their differentiation and/or proliferative capacity in vitro and in vivo (1–6). Thus, studies of alterations in pool sizes of phospholipid metabolites during tumor growth or regression in vivo might not only contribute to the elucidation of the biochemical events controlling neoplastic growth, but also lead to the identification of “metabolic fingerprints” of therapeutic efficacy in both experimental and human tumors (5, 6).

Tumor-bearing mice injected with transplantable tumor cells represent useful preclinical experimental systems to study the mechanisms of action of different antitumor agents and the biochemical events underlying tumor regression. High-resolution NMR spectroscopy offers a powerful experimental approach to this problem, as it permits simultaneous and noninvasive detection of a number of intermediate metabolites in tissues, provided that their concentration and intracellular mobility are sufficiently high (5, 7, 8). In particular it has been shown that tumor 31P-NMR spectra generally exhibit conspicuous resonances from intermediates of the pathways of the de novo biosynthesis and catabolism of phospholipids such asGroPCho, GroPEtn in the spectral PDE region, and/or PCho and PETF in the PME region (2, 3, 6, 8–10). Phospholipid metabolites likePCho, GroPCho, and Cho can also be detected and monitored by 1H-NMR (8, 10–12).

Recent 31P-NMR studies carried out in our Laboratories showed that intratumoral injection with Mu-IFN-α/β (whose capacity to inhibit tumor growth had previously been demonstrated reviewed in Ref. 13), resulted in conspicuous alterations in the levels of some phospholipid metabolites in FLC tumors (5). In particular, significant decreases in the intratumoral concentrations ofGroPCho, GroPEtn, and PChowere observed in tumors as early as 2 days after injection of IFN. These effects, together with a marked increase in intratumoral pH, could be considered early biochemical “markers” of tumor regression (5) as they appeared prior to necrosis in IFN-treated tumors (14).

TNF has been reported to be toxic for tumor cells in vitro (15, 16 and references therein) and to induce necrosis of tumors in experimental animals (17–20). Moreover, it has been shown that although TNF was not cytotoxic for cell lines ofFLC in vitro, it did exert a very marked antitumor effect on s.c. FLC tumors, suggesting that the antitumor effects of TNF in this system, like those of Mu-IFN-α/β, were also host mediated (21).

In particular, preliminary 31P-NMR studies (21, 22) showed that injection of tumors with TNF also resulted in significant and rapid alterations in the spectral profiles ofphosphorylemonoesters and phosphoryldiesters, as well as in an increase of intratumoral pH in FLC tumors. These results emphasized the interest and feasibility of utilizing TNF as an antitumor agent for studying in more detail the metabolic response of tumors at early stages of tumor regression. This work reports the results of detailed NMR studies on two solid tumors injected with TNF.

MATERIALS AND METHODS

Mice. Five- to 8-week-old male and female DBA/2 and C3H/HeN mice were obtained from Charles River Italia S.p.A. (Milan, Italy).

3 The abbreviations used are: NMR, nuclear magnetic resonance; FLC, Friend erythro leukemia cells; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; GroPEtn, glycerol 3-phosphorylethanolamine; GroPCho, glycerol 3-phosphorylcholine; GroP, sn-glycerol 3-phosphate; PETn, phosphatidylethanolamine; PCho, phosphocholine; Cho, choline; PDE, phosphoryl diesters; PME, phosphorylemonoesters; BSA, bovine serum albumin; TNF, tumor necrosis factor; Mu-IFN-α/β, murine interferon α/β; NOE, Nuclear Overhauser effect.
C3H/HeN mice bearing a methylicholanthrene-induced fibrosarcoma in the hind leg were kindly provided by Dr. A. Mantovani, Istituto Mario Negri, Milan, Italy.

Tumor Cells. Mu-IFN-α/β-sensitive (745) and -resistant (3C1-8) Friend erythroleukemia cells (FLC) (23) were serially passaged by i.p. inoculation of DBA/2 mice (24). These cells have been shown to be resistant in vitro to the cytoxic effects of TNF (21). Mice were shaved on the dorsal area 1 to 2 days prior to tumor-cell inoculation and weekly thereafter, in order to evaluate the evolution of tumor growth.

Murine fibrosarcoma cells (HeN4) were isolated from a primary fibrosarcoma induced by methylicholanthrene in C3H/HeN mice by Dr. G. Taraboletti and Dr. A. Mantovani (Istituto Mario Negri, Milan), as previously described (25). Trypsinized tumor cells were passage s.c. in syngeneic C3H/HeN mice.

Histological Examination. Mice were killed by ether. The tumors were removed, cut in two pieces of approximately 0.5 cm2 and fixed in 10% formaldehyde for several days. Fixed tissues were subsequently processed for paraffin embedding, cut into 2- to 3-μm thick slices and stained with hematoxylin and eosin.

Tumor Necrosis Factor (TNF) and Control Preparations. Recombinant murine TNF (26) expressed in Escherichia coli, was purified to apparent homogeneity and had a specific activity of 8 × 107 U/mg protein (assayed on L929 cells in the presence of 1 μg/ml actinomycin D for 18 h). The endotoxin contamination of mouse TNF was estimated to be less than 0.5 ng/ml by Limulus amoebocyte lysate assay. In the experiments, TNF was diluted in buffered physiological solution (NaCl, 153 mM) containing BSA, purchased from Sigma, 100 μg/ml. As a control we used BSA dissolved in buffered physiological solution.

Tumor Preparation for NMR Analysis. FLC solid tumors were induced in DBA/2 mice injected s.c. with 5 × 106 3C1-8 cells in the dorsal area. Fibrosarcoma HeN4 cells were implanted s.c. in the dorsal area of C3H/HeN. After tumor nodules had developed, either TNF (4 μg in 0.2 ml) or BSA was injected intratumorally. Histological examinations of FLC and fibrosarcoma tumors revealed that the tumor mass was less compact in TNF- than in BSA-injected tumors, with the appearance in the former of some degenerating cells 6–12 h after injection. More extensive areas of necrosis were observed 24 h after TNF treatment.

For NMR analyses tumors were rapidly dissected, washed with 0.15 M NaCl solution containing 20% deuterium oxide, and inserted into NMR tubes. Several tumors were packed at the bottom of the NMR tube, up to a height of at least 2.5 cm, in order to optimize the filling factor of the radio frequency coil in the probe. The high level of tissue homogeneity of these preparations was reflected by the 31P-NMR spectra, which typically exhibited fairly narrow and symmetric signals.

Ascitic tumors were obtained by i.p. injection of DBA/2 mice with 5 × 106 in vivo passaged FLC. After 10 days of in vivo growth the peritoneal cavity was washed with 3 ml of 0.15 M NaCl and tumor cells were washed twice with the same solution at 4°C, before preparing the tumors extracts.

Tumor Extracts. Tissue extracts for 31P-NMR analyses were prepared (unless otherwise stated) from tumors frozen immediately after dissection at liquid nitrogen temperature and converted to powder by mechanical action. The use of a Spex freezer mill (Spex Industries Inc., Metuchen, NJ) was found effective to minimize loss of material. The powdered tissues were then homogenized in 60% (by volume) ethanol solution and centrifuged at 27,000 × g for 30 min (at 4°C). The supernatant was evaporated to dryness by rotovapor, the residue was resuspended in deuterium oxide-EDTA (100 mM), and the pH was measured (assayed on L929 cells in the presence of 1 μg/ml actinomycin D for 18 h). The endotoxin contamination of mouse TNF was estimated to be less than 0.5 ng/ml by Limulus amoebocyte lysate assay. In the experiments, TNF was diluted in buffered physiological solution (NaCl, 153 mM) containing BSA, purchased from Sigma, 100 μg/ml. As a control we used BSA dissolved in buffered physiological solution.

NMR OF TNF-TREATED TUMORS

31P-NMR analyses carried out on tumors maintained in the NMR tube at 4°C showed a maximum variation of 0.02 pH units in 40 min. The reproducibility of pH determinations in dissected tumors, already demonstrated in previous work (5), was assessed in this study in five separate experiments involving a total of 35 BSA-treated FLC tumors, dissected from DBA/2 mice on days 8 or 9 of tumor growth. All these examinations gave pH values comprised between 6.98 and 7.02, with an average pH of 7.00 and SD of ± 0.02. The same results were obtained from a group of six untreated FLC tumors and from two groups of BSA-treated HeN4 fibrosarcomas (11 days of growth, 5 tumors per group).

No significant spectral alterations were observed in the linewidths or in the relative peak areas of the signals in either the phosphoryl-containing or the phosphoryldiester spectral regions, during time intervals as long as 2 h after tumor dissection. Chemical shifts were measured in ppm with respect to orthophosphoric acid (85 g/100 ml) as external reference. Under these conditions the resonance of glycerol 3-phosphorylcholine was at +0.49 ppm (p). Tissue extracts were analyzed at 4°C, with 60° angle pulses, acquisition time of 0.82 s and interpulse delay of 2.5 s (unless stated otherwise).

Peak assignments were made for phospholipid metabolites in the 31P-NMR spectra both on the basis of previous experience (5) as well as by addition to the extract preparations of the corresponding standard compounds, under various conditions of pH.

31P-NMR signal areas of tumor extracts were corrected for the differential magnetic saturation and NOE enhancement (27) factors of the various compounds. The correction factors were determined by measuring each signal under two different conditions of pulse sequence and gated broad band hetero-decoupling: (a) the usual experimental conditions described above for analysis of tissue extracts; (b) a sequence of 90° pulses, separated by an interpulse delay of 30 s (to allow full recovery of the magnetization before the next pulse); (c) decoupling power "on" during acquisition and "off" during the interpulse delay in order to prevent the building up of NOE enhancements.

RESULTS

Effects of Intratumoral Injection of TNF on the Growth of Friend Leukemia Cell Tumors and HeN4 Fibrosarcoma. Fig. 1 shows the effects of intratumoral injection of TNF on the growth of established 3C1-8 FLC tumors (A) and HeN4 fibrosarcoma (B) implanted s.c. in syngeneic mice. Daily treatment of 3C1-8 FLC tumors with TNF for 7 days resulted in a clear-cut inhibition of tumor growth (Fig. 1A). Mean day of death was: 26.3 ± 0.8 days for control BSA-treated mice and 31.5 ± 1.5 days for TNF-treated mice. All mice (8/8 in each group) died with extensive spleen and liver metastases.

As shown in (Fig. 1B) two intratumoral injections of TNF in C3H/HeN mice with established fibrosarcoma also resulted in a marked inhibition of tumor growth, as compared to control BSA-treated tumors. On day 33 of tumor growth mice were sacrificed. The mean weights of tumors were as follows: 4.05 ± 0.18 g for TNF-treated mice and 15.86 ± 2.8 g for BSA-treated mice.

Phospholipid Metabolites Detected in 31P-NMR Spectra of FLC Tumor Extracts. Fig. 2 shows typical 31P-NMR spectra of ethanolic extracts obtained from either ascitic or s.c. solid FLC.
NMR OF TNF-TREATED TUMORS

Fig. 1. Effect of TNF on the growth of established 3C1-8 FLC tumors (A) and HeN4 fibrosarcoma (B) implanted s.c. in syngeneic mice. Arrows, days of intratumoral injection. In A, 6-week-old male DBA/2 mice were injected s.c. with \(5 \times 10^6\) in vivo passaged 3C1-8 FLC. On day 9 of tumor growth, either BSA (100 \(\mu\)g/ml in NaCl 153 mM buffered saline) or TNF (20 \(\mu\)g/ml) was injected intratumorally (0.2 ml) for 7 days. There were eight mice per group. In B, 7-week-old male C3H/HeN mice were injected s.c. with \(10^6\) HeN4 fibrosarcoma cells. On day 11 of tumor growth, mice were injected twice with 0.2 ml of either BSA (100 \(\mu\)g/ml in NaCl 153 mM buffered saline) or TNF (20 \(\mu\)g/ml), intratumorally. There were eight mice per group. O, BSA-treated mice; •, TNF-treated mice.

Fig. 2. \(^{31}\)P-NMR spectra (161.9 MHz, 4°C) of ethanolic extracts (reading pH 7.01 ± 0.02) of FLC tumors from DBA/2 mice. A, i.p. ascitic tumor, clone 745 (observe pulse angle 90°, number of scans (NS), 12,608), day 10 of growth; B, i.p. ascitic tumor, clone 3C1-8 (pulse angle 90°; NS, 15,600), day 10 of growth; C, s.c. solid tumor, clone 745, day 22 of growth (pulse angle 60°; NS, 3,740); D, s.c. solid tumor, clone 745, day 8 of growth (pulse angle 90°; NS, 17,420). Peak 1, phosphorylethanolamine; peak 2, phosphorylcholine; peak 3, inorganic phosphate; peak 4, glycerol 3-phosphorylethanolamine; peak 5, glycerol 3-phosphorylcholine; peak 6, \(\gamma\)-ATP; peak 7, \(\beta\)-ADP; peak 8, \(\alpha\)-ADP; peak 9, \(\alpha\)-ATP; peak 10, nicotinamide adenine dinucleotide; peak 11, diphosphodiester; peak 12, \(\beta\)-ATP.

Fig. 3. \(^{31}\)P-NMR spectra (161.9 MHz, 4°C) of intact 3C1-8 FLC tumors, dissected 8 days after s.c. inoculation (5 \(\times 10^6\) cells) and 6 h after intratumoral treatment with 0.2 ml of A, BSA, 100 \(\mu\)g/ml in NaCl 153 mM buffered saline (eight tumors) or B, TNF, 20 \(\mu\)g/ml in saline, containing BSA (eight tumors). Spectrum (C) was obtained from the ethanolic extract of the tumor masses analyzed in B. The peak at 4.55 ppm was assigned to GroP. Assignment of peaks 1–5 in the spectrum of the extract (C) is the same as in Fig. 2. Assignment of peaks in the tissues: peak a, mainly arising from phosphorylethanolamine, with contribution from \(\alpha\)-glycerol 3-phosphate; peak b, mainly arising from phosphorylcholine and another compound of still unidentified nature; peak c, inorganic phosphate; peak d, glycerol 3-phosphorylethanolamine; peak e, glycerol 3-phosphorylcholine.

Effects of Intratumoral Injections of TNF on Phosphorylmonooester and Phosphoryldiester Signals of FLC Tumors. DBA/2 mice were injected s.c. with 3C1-8 cells. When tumor nodules were visible (8 days), they were injected with TNF or BSA. Six h after injection the tumors were dissected as intact tumor masses and \(^{31}\)P-NMR analyses immediately performed at 4°C. Phosphorylethanolamine and phosphorylcholine signals were the major constituents of two rather broad resonances, a and b, centered at 4.1 and 3.8 ppm, respectively, in the phosphorylmonooester spectral region of BSA-treated tumors (Fig. 3.4). Glycerol 3-phosphorylethanolamine and glycerol 3-phosphorylcholine gave rise in these spectra to two rather narrow resonances (d and e) in the phosphoryldiester regions.

Intratumoral treatment with TNF induced conspicuous reductions in the relative signal areas of glycerol 3-phosphorylethanolamine and glycerol 3-phosphorylcholine, both resonances showing 1.5-fold reductions with respect to those of tissues treated with BSA (Fig. 3, A and B; Table 1).

Another effect of TNF on the spectra of intact tissues was a
NMR OF TNF-TREATED TUMORS

Table 1  Effects of intratumoral injection of TNF on the $^{31}$P-NMR relative signal areas of phosphorylmonoesters [$A_{(PME)}$], phosphoryldiesters [$A_{(PDE)}$], and on pH in intact FLC tumors and HeN4 fibrosarcoma

<table>
<thead>
<tr>
<th>Tumor (day of growth)</th>
<th>Number of tumors*</th>
<th>Treatment/time (h)</th>
<th>pH</th>
<th>$A_{(PME)}$</th>
<th>$A_{(total P)}$</th>
<th>$A_{(PME)}$</th>
<th>$A_{(total P)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLC-3Cl-8 (8)</td>
<td>8</td>
<td>TNF/6</td>
<td>7.22</td>
<td>0.13</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLC-745 (8)</td>
<td>8</td>
<td>BSA/6</td>
<td>7.02</td>
<td>0.19</td>
<td>0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeN4 (11)</td>
<td>6</td>
<td>TNF/6</td>
<td>7.28</td>
<td>0.20</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>BSA/6</td>
<td>6.98</td>
<td>0.21</td>
<td>0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeN4 (11)</td>
<td>5</td>
<td>TNF/6</td>
<td>7.14</td>
<td>0.15</td>
<td>&lt;0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>BSA/6</td>
<td>7.02</td>
<td>0.18</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>BSA/12</td>
<td>7.22</td>
<td>0.20</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>BSA/12</td>
<td>6.98</td>
<td>0.19</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Number of tumors analyzed in each NMR experiment, in order to optimize the filling factor of the radiofrequency coil in the probe (see "Materials and Methods").

Effects of Mouse TNF on Phosphorylmonoesters' and Phosphoryldiesters' Signals of HeN4 Fibrosarcoma. $^{31}$P-NMR studies showed that, 6-12 h after TNF treatment, HeN4 fibrosarcoma exhibited spectral alterations (Fig. 4) similar to those already observed in FLC tumors, i.e., (a) marked reductions in the glycerol 3-phosphorylcholine and glycerol 3-phosphorylethanolamine relative peak areas in the phosphoryldiester region; (b) significant increase and/or appearance of a peak due to sn-glycerol 3-phosphate in the phosphorylmonoester region.

Analysis of the respective extracts showed that the ratio $A_{(GroP)}/A_{(GroPC)}$ reached the value of 2.18 12 h after tumor treatment with TNF, while it was maintained at the much lower value of 0.14 in the extracts of BSA-treated tumors. The larger contribution given by the glycerol 3-phosphate signal to the PME spectral profile of TNF- versus BSA-treated tumors, together with the values of the relative signal areas of the PME band profile in the spectra of the corresponding tissues, indicate a net decrease in the relative signal area of phosphorylcholine in TNF-treated tumors.

Effects of Intratumoral Injections of TNF on pH of FLC Tumors and HeN4 Fibrosarcoma. The inorganic phosphate signal exhibited in TNF-treated tumors a downfield chemical shift of 0.23 ppm versus the respective controls, indicative of an alkaline shift of 0.2 units in intratumoral pH (Table 1). An even higher pH variation was found in a group of 10 tumors (pH 7.38), analyzed 20 h after TNF treatment versus a group of nine BSA-treated controls (pH 6.98) (data not shown). Analogous results were exhibited by FLC 745 solid tumors 6 h after TNF treatment (Table 1).

Significant increases in the average intratumoral pH value...
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Fig. 5. $^1$H-NMR spectra (400 MHz, 4°C) of ethanolic extracts of 3C1-8 FLC tumors. DBA/2 mice were given injections s.c. of $5\times 10^5$ FLC and treated with either TNF or BSA as described in Fig. 3, legend. Top, extracts of five tumor samples (day 14 of growth) 6 h after treatment with TNF; bottom, extracts of five control tumor samples, treated with BSA. Peaks: Leu, leucine; Val, valine; Lac, lactic acid; Ala, alanine; Cr, creatine; Cho, choline; PCho, phosphorylcholine; GroPCho, glycerol 3-phosphorylcholine; Tau, taurine; Gly, glycine. Chemical shifts were referred to 4, 4-dimethyl 4-silapentane sodium sulfonate.

(Fig. 4; Table 1) were also observed in TNF-treated fibrosarcoma.

All these results (together with those obtained on control tumors, see also “Materials and Methods”), indicated that only TNF-treated tumors exhibited consistently higher pH values than those of BSA-treated tumors, with alkaline shifts ranging between 0.12 and 0.40 pH units (depending upon the tumor and time of treatment), as determined from four independent experiments.

Combined $^3$P- and $^1$H-NMR Analyses of Variations in the Concentration Levels of Phospholipid Metabolites in Extracts of TNF-treated FLC Tumors. High resolution $^1$H-NMR spectra permitted the detection of several metabolites in the extracts of 3C1-8 FLC freeze-clamped tumors dissected from mice on day 14 after implantation and 6 h after TNF treatment (Fig. 5). The identification of peaks due to alanine, valine, leucine, glycine, taurine, and creatine was made on the basis of previous assignments in tumor cell extracts (8, 10, 12). Careful assignment of the signals respectively arising from N$^+$ (CH$_3$)$_3$ of choline (3.180 ± 0.002 ppm), phosphorylcholine (3.200 ± 0.002 ppm), and glycerol 3-phosphorylcholine (3.208 ± 0.002 ppm) was made in this study with the use of standard solutions at different pH values, as well as by addition to the extracts of the respective standard compounds. Phosphorylcholine and glycero 3-phosphorylcholine peaks in the spectra of tumor extracts were slightly separated and not completely overlapping, as suggested by Agris and Campbell in their analysis of Friend leukemia cell extracts (11); the different finding might be the consequence of the influence exerted by different ionic strength conditions on the chemical shifts of these peaks (especially on that of glycerol 3-phosphorylcholine). Moreover, carnitine, whose presence was identified on FLC extracts by Agris and Campbell (11) as a distinct $^3$P-NMR signal, gave, when added to our extract, a resonance completely overlapping with that of glycerol 3-phosphorylcholine. The detection of phosphorylcholine and glycerol 3-phosphorylcholine N$^+$ (CH$_3$)$_3$ groups as distinct although partially overlapping peaks in $^1$H-NMR spectra provided the possibility of making two independent determinations of the ratio of the concentration levels of these two compounds, [GroPCho]/[PCho], from either $^3$P- or $^1$H-NMR spectra of tumor extracts (Fig. 6). In the BSA-treated tumors this ratio was 2.3 ± 0.2 when computed from $^1$H-NMR signals and 2.2 ± 0.2 from $^3$P-NMR spectra [after introducing, in the latter case, the correction factors (“Materials and Methods”) arising from different relaxation times and NOE enhancements of the two compounds]. The coincidence, within experimental errors, of these two values suggests that free carnitine, if even present, would possess a concentration below the detection levels allowed by the sensitivity of this approach.

The most important feature of $^1$H-NMR spectra was the presence in the extracts of TNF-treated tumors of a conspicuous signal arising from Cho, which was, instead, barely detectable in the spectra of the controls (Fig. 6). The ratio A(Cho)/A(PCho) was lower than 0.1 in the latter and 2.5 ± 0.3 in TNF-treated tumors extracts.

The combination of $^3$P- and $^1$H-NMR analyses of these extracts permitted the quantitative determination of the concentrations of six phospholipid metabolites in TNF and in BSA-treated tumors (Table 2).

It was noted that, consistently with the observations reported above for the $^3$P-NMR analyses of FLC and HeN4 tumors and
Table 2  Effect of TNF on the concentrations (µmol/g wet weight of tissue) of water-soluble phospholipid metabolites in tissue extracts of 3CI-8 FLC tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GroP</th>
<th>PCho</th>
<th>GroPCho</th>
<th>Cho</th>
</tr>
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<tbody>
<tr>
<td>TNF</td>
<td>0.155</td>
<td>0.172</td>
<td>0.081</td>
<td>0.055</td>
</tr>
<tr>
<td>BSA</td>
<td>0.020</td>
<td>0.190</td>
<td>0.286</td>
<td>0.114</td>
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</tbody>
</table>

DISCUSSION

Injection of TNF in either FLC tumors or HeN4 fibrosarcoma induced a marked inhibition of tumor growth (Fig. 1) and clear-cut alterations in the concentration of some metabolites, as early as 6 h after treatment. As one of the FLC clones used in this study (3CI-8) has been shown to be resistant to the cytotoxic effect of TNF (21), it seems possible that the metabolic changes observed represent host-mediated effects. At the present time, however, we cannot exclude the possibility that FLC (3CI-8) are rendered sensitive to the cytotoxic effect of TNF under in vivo conditions and that the biochemical changes detected reflect direct effects of TNF on tumor metabolism.

Effects of TNF on the Levels of Phospholipid Metabolites. 31P-NMR analyses (Fig. 2) showed that high concentrations of PCho, GroP, GroPEt and GroPCho were detected both in viable ascitic cells, as well as in solid FLC tumors at early stages of tumor growth. It seemed therefore unlikely that the presence of these phospholipid metabolites was merely the result of tumor degeneration and necrosis.

Injection of FLC solid tumors with TNF (Figs. 3 and 6; Tables 1 and 2) resulted (6 h after treatment) in a 1.5- to 3.5-fold decrease in the pool sizes of PDE (GroPEt and GroPCho). On the other hand, the most significant variations in the PME spectral region were an increase (up to 8-fold) of GroP and decreases of the other components, especially PCho (up to 3.5-fold decrease). Injection of HeN4 fibrosarcoma with TNF resulted in analogous variations in the pool sizes of both PDE and PME compounds.

These alterations cannot be simply considered as biochemical events associated with TNF-induced tumor necrosis. In fact, similar effects on the levels of PCho, GroPEt, and GroPCho were also observed in FLC tumors 2 days after treatment with Mu-IFN-α/β, i.e., well before the appearance of necrotic areas in the tumor (5, 14). Furthermore, no effect on the pools of these metabolites was detected in FLC tumors in which large areas of necrosis had been induced by X-ray treatment (5).

1H-NMR analyses of FLC tumor extracts showed that the concentration of choline, another phospholipid metabolite, was also markedly increased (9×), 6 h after injection with TNF into the tumor. This effect, combined with the observed decrease of [PCho] resulted in a striking increase of about 19× in the ratio [Cho]/[PCho], in TNF-treated tumors.

The results clearly indicate the occurrence of important metabolic alterations, induced by TNF on the phospholipid metabolism of these tumors. Alterations in some enzymatic activities of the major metabolic pathway (29 and references therein) of the de novo biosynthesis and catabolism of PtdCho (Fig. 7), and the analogous pathway of PtdEt might explain these phenomena, and/or offer interesting working hypotheses for further investigations.

High concentrations of GroPCho, first reported by Burt et al. in 31P-NMR spectra of muscles (30), were subsequently detected in a variety of developing or differentiating tissues (31). The disappearance of this compound in mature cells (2, 4, 31) suggested that GroPCho might be a marker of differentiation. As regards the metabolic role of this compound, Burt and Ribolow (31) suggested that it might regulate phospholipid metabolism and therefore membrane fluidity and function of membrane-bound proteins, by inhibiting the activity of lyso-phosphatidylcholine-phospholipase (EC 3.1.1.5). The decreased levels of GroPCho observed in TNF-treated tumors might actually result not only from altered activities of lyso-phospholipases and/or phospholipases (EC 3.1.1.32 and EC 3.1.1.4) but also from the activation of sn-glycerol 3-phosphorylcholine phosphodiesterase (EC 3.1.4.2), as suggested by the conspicuous increases observed in the levels of glycerol 3-phosphate and choline (Table 2). This hydrolyase has already been reported to be active in animal liver, brain, and kidney (32–34), all tissues which also contain rather high concentrations of GroPCho. It was also suggested that alterations in the activity of this enzyme occur in human brain in some degenerative disorders (35), while a high activity has been reported in human muscle homogenates (36). Moreover the activity of GroPCho phosphodiesterase has been shown to be sensitive to...
The decrease observed in the [taurine]/[glycine] ratio in TNF-versus BSA-treated tumors, seems therefore to indicate a partial deprivation of tumor cells of their “detoxifying defences,” which allowed them to maintain, before treatment, high proliferative rates. This decrease might in particular arise from a higher production in TNF-treated tumor cells of oxidative and peroxidative compounds, with a consequent larger formation and disposal of taurine complexes.

Effects of TNF on pH and on the Pool Size of Lactate. Intratumoral pH was shifted towards more alkaline values by injection of TNF in either FLC tumors or fibrosarcoma. Analogous results were also induced in FLC tumors by treatment with Mu-IFN-α/β (5).

This effect might result from partial inhibition, induced by these biological response modifiers, on the glycolytic capacity of tumor cells, with a consequent decrease in lactic acid production. However, the higher value (×1.4) of lactate in TNF-versus BSA-treated FLC tumors indicated that this is not the case. The elevation in lactic acid produced by the TNF-treated tumor is also in agreement with the finding by Kettelhut, Fiers, and Goldberg (40) of acidosis in TNF-treated rats. The alkaline shift of pH in TNF-treated tumors, therefore, suggests the occurrence of additional mechanisms of pH regulation, whose effects are opposite to and overcome enhanced lactic acid production.

Alkaline shifts of up to 0.7 pH units were already reported by Glickson et al. (8) for RIF-1 tumors 4 days after treatment with cyclophosphamide. This result was interpreted as originating from the growing fraction of the tumor, since the maximum shift correlated with a return of the tumor to the plateau level of DNA synthesis. The appearance of an alkaline shift in TNF-treated FLC tumors and fibrosarcoma is too early to suggest the existence of an analogous correlation in these tumors.
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Nuclear Magnetic Resonance Analysis of Tumor Necrosis Factor-induced Alterations of Phospholipid Metabolites and pH in Friend Leukemia Cell Tumors and Fibrosarcomas in Mice

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