Quantitation of Metabolic and Radiobiological Effects of 6-Aminonicotinamide in RIF-1 Tumor Cells in Vitro

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ABSTRACT

6-Aminonicotinamide (6AN) can be metabolized to 6-amino-NAD(P+), a competitive inhibitor of NAD(P+)-requiring processes, especially the pentose phosphate pathway (PPP) enzyme, 6-phosphogluconate dehydrogenase. The effect of 6AN on the flux of 1 and 6 13C-labeled glucose to lactate, via glycolysis and the PPP, was investigated using H-nuclear magnetic resonance. These studies showed that 6AN as a single agent caused a significant 89% (P < 0.0001) inhibition of glycolytic flux but had no detectable effect on the PPP. 31P-nuclear magnetic resonance studies of perfused RIF-1 cells indicated that 4 h of exposure to 6AN were sufficient to cause significant accumulation of 6-phosphogluconate, the substrate for this enzyme (P < 0.0001). A significant reduction in the phosphocreatine: inorganic phosphate ratio was observed under conditions that led to accumulation of 6-phosphogluconate (P < 0.006). Accumulation of 6-phosphogluconate and subsequent reduction in phosphocreatine correlated with significant potentiation of 6 Gy of irradiation by 6AN. These results suggest that the radiation enhancement effect of 6AN may be due to inhibition of glycolysis (mediated by 6-phosphogluconate) and the associated reduction in high-energy phosphates. Additional studies analyzing the metabolic effects of 6AN in combination with radiation are necessary to determine the role of inhibition of the PPP in 6AN enhancement of radiation.

INTRODUCTION

6AN, an analogue of niacin, is currently being evaluated in preclinical trials as part of an anticancer drug combination and as a radiosensitizer (1, 2). Although 6AN was found to have little therapeutic effect as a single agent (3), it has been shown to potentiate the effects of radiation in vitro (4), as well as a number of chemotherapeutic agents both in vitro and in vivo (5–7). Recently, we have shown that 6AN potentiates the response of tumors and cells to radiation both in vivo (8) and in vitro (9) and also enhances the effect of the drug combination 6-methyl mercaptopurine riboside plus N-(phosphonoacetyl)-L-aspartate in vivo (1). Indeed, when 6AN was given in combination with 6-methyl mercaptopurine riboside, N-(phosphonoacetyl)-L-aspartate, and radiation, 65% of murine mammary carcinoma CD8F1 tumors went into complete remission and 25% remained tumor free for greater than 1 year (1). This drug, therefore, may prove to be of clinical importance in combination therapy.

6AN acts by competition with niacin in pathways utilizing NAD(P+), being metabolized to 6ANAD(P+). 6ANAD(P+) in turn, can act as a competitive inhibitor of NAD(P+)-requiring processes. 6ANAD(P+) is a particularly potent inhibitor of the PPP enzyme, 6PG dehydrogenase, which is an important step in the synthesis of NADPH and ribose units required for biosynthesis and DNA repair. Inhibition of this enzyme by 6AN leads to accumulation of 6PG (9–11). In our previous studies using perfused RIF-1 cells, we were able to demonstrate not only an accumulation of 6PG following 6AN treatment but also a secondary inhibition of glycolysis and a decrease in PCr (9). 6AN has also been shown to inhibit poly(ADP-ribose) polymerase (5) and to induce the glucose-regulated stress protein GRP78 (12, 13). It is evident that 6AN has a number of effects on cellular metabolism. It is not clear, however, which of these are related to the chemosensitization induced by 6AN.

Here, we have undertaken to measure the flux of glucose carbon through glycolysis and the PPP prior to and following incubation with 6AN in vitro to quantitatively ascertain the effect of single-agent 6AN on glycolytic and PPP activity. In addition, we have investigated the effects of dose and time exposure on the radiation-enhancing effect of 6AN on RIF-1 cells in vitro.

MATERIALS AND METHODS

Cells. RIF-1 cells were grown in RPMI 1640 supplemented with 10% FCS (Intergen, Purchase, NY). Ninety-six h before the start of perfusion, approximately 5 × 10⁶ RIF-1 cells were seeded on 0.14 g of conditioned Cultispher-G collagen beads (Hyclone Laboratories, Inc., Logan, UT) in a spinner jar in 125 ml of medium. The medium for perfusion experiments was phosphate-free RPMI 1640 supplemented with 10% FCS and 1.2 μg/liter DNase I (Sigma Chemical Co., St Louis, MO). The total number of cells on the beads at the time of transfer from the spinner flask to the perfusion apparatus was approximately 1.7 × 10⁷ cells. Cells were counted by removing 1 ml of beads and medium from the spinner flask. Following sedimentation of the beads, 0.8 ml of the supernatant was removed and replaced by 0.8 ml of collagenase (10 mg/ml) in PBS. This mixture was incubated at 37°C for 30 min. With the collagen beads digested, it was possible for the released cells to be counted by erythrosin B dye exclusion. The total volume of the perfusion circuit, including the reservoir, was 125 ml. The head space of the medium reservoir was gassed with O₂/C₂O₂ (95%/5%). The design of the perfusion apparatus was as described previously for agarose threads (14). Cells were perfused on the collagen beads as a fluidized bed in a shortened, screw-cap NMR tube (Wilmad, Buena, NJ). The reduction in upward force, where the cross-sectional area of the tube increased at the neck, was sufficient to prevent flow of cells into this part of the tube.

Experimental Protocols. 13C-labeling experiments were carried out in spinner flasks. Cells were grown up as for the perfusion experiments. Cells were counted and split into two equal halves. Each half was incubated for 12 h in 125 ml of glucose-free and phosphate-free medium supplemented with 10% serum and 5 mM 13C-labeled glucose. One portion of the cells was incubated with [1-13C]glucose and the other with [6-13C]glucose (both 99%; Cambridge Isotopes, Andover, MA). Five-ml medium samples were removed at 2-h intervals and frozen for later acetone extraction. After 12 h, 13C-labeled medium in the spinner flask was replaced by unlabeled medium. Cells were then incubated for 4 h with 200 μM 6AN prior to a second incubation with 13C-labeled medium. Samples were again taken every 2 h up to 12 h after the end of the 6AN incubation. Incubating the cells with 13C-labeled glucose both before and after 6AN allowed each experiment to act as its own control.

The collagen beads were transferred to the perfusion apparatus, and the medium flow rate was adjusted to prevent the beads from settling at the bottom of the tube. 31P-NMR spectra were then acquired over the next 24 h. This period ensures that the cells are perfused properly and are growing, indicated by an increase in PCr and NTP. After 24 h, the perfusate medium was changed...
to one containing either 40 \( \mu \text{M} \) or 200 \( \mu \text{M} \) 6AN. The cells were then perfused for an additional 4 h, during which a \( ^{31} \text{P}-\text{NMR} \) spectrum was acquired. After 4 h, the 6AN was washed from the perfusion apparatus by perfusing with two lots of 6AN-free medium over a period of 30 min. Previous studies have shown that this procedure reduces the drug concentration by at least 99\% (9). Following the washout of 6AN, more \( ^{31} \text{P} \) spectra were acquired over the next 20 h.

**NMR.** A five-turn solenoid coil, wrapped around the bead-containing volume, was used to collect NMR spectra. \( ^{31} \text{P}-\text{NMR} \) spectra decoupled by WALTZ (Wonderful Alternating Phase Technique for Zero Residual Splittings) were obtained using a Bruker/GE 4.7 T Omega spectrometer (Bruker NMR, Fremont, CA) operating at 81.03 MHz. \( ^{31} \text{P}-\text{NMR} \) data were acquired with a spectral width of 10,000 Hz, a 60° pulse angle (20.3-\( \mu \)s pulse width), recycle time of 2 s, 4,096 data points, and 4,096 signal-averaged free induction decays. Under these conditions, spectra were partially saturated. One hundred thirty-five min of data acquisition were sufficient to give good signal:noise ratio. \( ^{31} \text{P} \)NMR spectra were processed with 5 Hz of exponential line broadening and referenced to PCr at 0 ppm. The NMR tube containing collagen beads was immersed in water to minimize inhomogeneities due to magnetic susceptibility gradients (15). A Helmholtz decoupling coil was built on the outside of the water bath.

High-resolution \( ^{1} \text{H}-\text{NMR} \) spectra of medium extracts, at approximately pH 8, were acquired on a Bruker AMX 400-MHz magnet. Experimental parameters were 5000 Hz spectral width, 16,384 data points, a 90° flip angle of 12.1 \( \mu \)s, and a recycle time of 15 s. Thirty-two free induction decays were transformed without any filtering. Trimethylsilylpropionate-2,2,3,3-d4 was used as an internal concentration and chemical shift standard at 0 ppm. Under these conditions, spectra were fully relaxed, and metabolite concentrations could be calculated by comparison to the internal standard.

**Medium Extracts.** A crude extraction procedure similar to that described previously (16) was carried out to concentrate and remove serum protein from medium supernatants taken from cells incubated in spinner flasks in the presence of \( ^{13} \text{C} \)-labeled glucose. Briefly, an equal volume of cold acetone was added to the harvested medium sample and left on ice for 20–30 min. The precipitated protein was removed by centrifugation at 1500 \( \times \) \( g \) for 10 min. The supernatant was decanted from the protein fraction, and the acetone was removed from the sample under vacuum using a rotary evaporator. The temperature of the water bath used was not in excess of 37°C. Chelex-100 (Sigma) was added to the remaining water phase and shaken periodically over a period of approximately 2 h to chelate any paramagnetic ions present in the sample. Chelex-100 was removed by centrifugation at 3000 rpm for 10 min, and the resulting solution was lyophilized. The lyophilized powder was reconstituted in 0.5 ml of deuterated water.

**Surviving Fraction Experiments.** RIF-1 cells were grown in RPMI 1640 supplemented with 10% FCS, as described previously (9, 14), to establish log-phase growth. After 48 h, the medium was changed. Cells were harvested by trypsinization 3 h following the final radiation treatment. Two separate experiments were carried out: (a) cells were exposed to different concentrations of 6AN, from 0 to 200 \( \mu \text{M} \), for 15 h prior to 6 Gy \( \gamma \)-irradiation, as described previously (9), and (b) cells were exposed to 200 \( \mu \text{M} \) 6AN for different lengths of time, from 0 to 15 h, prior to radiation treatment. The trypsinized cells were counted and plated at various densities on a 5-cm Petri dish in 5 ml of RPMI 1640 supplemented with 10% FCS, 2 mm glutamine, and nonessential amino acids. After 9 days, the plates were stained with crystal violet and rinsed, and surviving colonies were counted.

**Statistics.** The change in metabolite ratios measured in \( ^{31} \text{P}-\text{NMR} \) spectra and the relative fluxes of carbon via the PPP and glycolysis were fitted to a straight line using the method of least squares analysis. The gradients of these lines were compared using analysis of covariance. Paired t-tests were used to measure differences at individual time points. Data from the surviving fraction experiments were compared by one-way ANOVA, with differences between individual time points or doses estimated by the Duncan-Waller multiple comparison test. Error limits are quoted in the text and on figures as \( \pm \text{SE} \).

**RESULTS**

**\( ^{1} \text{H}-\text{NMR} \) Experiments.** Fig. 1 shows the lactate methyl region of a \( ^{1} \text{H}-\text{NMR} \) spectrum of incubation medium taken from RIF-1 cells after 12 h in the presence of [\( ^{1-13} \text{C} \)]glucose. The central doublet is due to the \( ^{1} \text{H} \) spin spin coupling of the methyl moiety of [\( ^{3-13} \text{C} \)]lactate. \( ^{1-13} \text{C} \) J coupling of 125 Hz causes further splitting of the methyl protons of \( ^{13} \text{C} \)-labeled lactate, giving rise to the outer satellite peaks. [\( ^{3-13} \text{C} \)]Lactate can be formed from the metabolism of both [\( ^{1-13} \text{C} \)]Glucose and [\( ^{6-13} \text{C} \)]glucose in glycolysis. Lactate labeled at the methyl position can also be formed from the metabolism of [\( ^{6-13} \text{C} \)]glucose in the PPP. The \( ^{13} \text{C} \) label of [\( ^{1-13} \text{C} \)]glucose, entering the oxidative branch of the PPP, however, is lost as carbon dioxide at the 6PG dehydrogenase step. Comparison of lactate labeling from metabolism of [\( ^{1-13} \text{C} \)]glucose and [\( ^{6-13} \text{C} \)]glucose, therefore, allows estimation of the relative fluxes of glucose carbon through glycolysis and the PPP. This technique for estimation of the relative direct fluxes from glucose to lactate via glycolysis and the PPP has been described in some detail previously (17, 18). Briefly, the fractional enrichment of lactate for cells incubated with [\( ^{1-13} \text{C} \)]glucose and [\( ^{6-13} \text{C} \)]glucose is calculated using equations A and B, respectively:

\[
R_1 = \frac{[\text{[\( ^{3-13} \text{C} \)]Lactate}]}{([\text{[\( ^{3-13} \text{C} \)]Lactate} + [\text{[\( ^{3-13} \text{C} \)]lactate}])L_{C1}} \quad (A)
\]

\[
R_6 = \frac{[\text{[\( ^{3-13} \text{C} \)]Lactate}]}{([\text{[\( ^{3-13} \text{C} \)]Lactate} + [\text{[\( ^{3-13} \text{C} \)]lactate}])L_{C6}} \quad (B)
\]

where \( R_1 \) and \( R_6 \) represent the fractional labeling of lactate when [\( ^{1-13} \text{C} \)]glucose and [\( ^{6-13} \text{C} \)]glucose are used as substrates, respectively. This fractional enrichment is analogous to the specific activity of a radiolabeled compound. \( L_{C1} \) and \( L_{C6} \) are the fractional enrichments of the substrates [\( ^{1-13} \text{C} \)]glucose and [\( ^{6-13} \text{C} \)]glucose, respectively. The relative flux of glucose carbon via glycolysis (\( G \)) is given by the relation:

\[
G = \frac{R_1}{R_6} \quad (C)
\]

and the relative flux of glucose carbon via the PPP (\( P \)) is given by the relation:

\[
P = 1 - \frac{R_1}{R_6} \quad (D)
\]
It can be seen from equations C and D that the amount of unlabelled lactate produced during the experiment is irrelevant, because the denominator of equations A and B, representing total lactate, is the same regardless of substrate. This means that lactate produced endogenously from other sources, or present in the medium or serum prior to the start of the experiment, does not affect the estimation of relative glycolytic and PPP fluxes, as could occur with a single labeling experiment. Recycling of carbon in the PPP, i.e., activity of the nonoxidative branch of the pathway, followed by re-entry of carbon into the oxidative branch of the pathway, cannot be measured using this technique; the C1 label of glucose is always lost at the first pass, and the C6 label does not get scrambled to another position. This means that the appearance of labeled carbon in lactate is independent of its number of passes through the PPP. This experiment, therefore, only measures the flux of carbon directly from glucose to lactate via glycolysis and the PPP. It is not clear, however, how important recycling of carbon in the PPP is metabolically, i.e., what contribution it makes to ribose and NADPH synthesis in RIF-1 cells in vitro.

Table 1 shows the relative fluxes of labeled carbon, from glucose to lactate, via glycolysis and the PPP at time intervals both in the presence and absence of 6AN, calculated using equations A–D. Data are the mean (±SE) of four separate experiments. In untreated cells, the contribution of glycolysis and the PPP to lactate were approximately 95 and 5%, respectively over the 12 h of the study. After the 4-h exposure to 6AN, however, this profile changed with a decrease in the proportion of glucose metabolism via glycolysis and an increase via the PPP. This change was significant at 8 h (P < 0.05), 10 h, and 12 h (both P < 0.02) after the 4-h exposure to 200 μM 6AN. It is not clear whether any significant change in the relative fluxes of glucose to lactate occurred during the first 6 h after 6AN treatment. Although no significant changes were measured, very little lactate accumulated, and the 1H-13C satellites were barely detectable, indicating that inhibition of lactate production had occurred during this period.

Measurement of these relative fluxes does not give any indication as to the degree of inhibition of glycolysis and the PPP by 6AN. The inhibitory effect of 6AN can better be assessed by calculating the concentration of lactate produced via glycolysis and via the PPP at each time point. Because the proportion of glucose metabolized to lactate by the two pathways and the concentration of lactate accumulated in the medium at each time point are known, both in the presence and absence of 6AN, the inhibition of glycolysis and the PPP can be calculated. Fig. 2 shows the accumulation of lactate via each of the two pathways, in which total lactate after 12 h in the control case is 100%. The amount of lactate accumulated, in both the presence and absence of 6AN, via the two pathways at each time point is calculated relative to this time point. These data have been corrected for serum lactate, and the data also assume that 6AN has no effect on other pathways that may lead to accumulation of lactate. No significant change in the concentration of lactate produced via the PPP was observed at any time point. The concentration of lactate produced via glycolysis, however, was reduced significantly at all time points following incubation with 200 μM 6AN for 4 h (P < 0.05 after 2 h, P < 0.01 from 4 h to 10 h, and P < 0.0001 after 12 h).

The data in Fig. 2 have also been fitted by linear regression to give a rate of accumulation of lactate via glycolysis and the PPP in both the presence and absence of 6AN. Accumulation of lactate via glycolysis in untreated RIF-1 cells proceeded at a rate of 8.4%/h (R² = 0.996; P < 0.0001), and following 6AN at a rate of 0.9%/h (R² = 0.749; P < 0.0001). Analysis of these data by analysis of covariance indicates that a significant 89% inhibition of glycolysis occurred following incubation with 6AN (P < 0.0001). Accumulation of lactate via the PPP occurred at a rate of 0.3%/h (R² = 0.558; P < 0.007) and 0.4%/h (R² = 0.918; P < 0.0001) in controls and following 4 h of exposure to 6AN, respectively. It is evident, therefore, that there was no significant change in PPP flux during incubation with 6AN.

Surviving Fraction Experiments. Fig. 3a shows a dose-response curve for cells incubated with 6AN for 15 h prior to 6 Gy irradiation.
It can be seen that 40 μM 6AN did not significantly enhance the cell kill achieved by 6 Gy of γ-irradiation alone. Increasing the concentration of 6AN to 80 μM, however, caused a significant reduction in the surviving fraction of RIF-1 (P < 0.05). 6AN in concentrations of 120, 160, and 200 μM, prior to 6 Gy of irradiation, similarly increased the efficacy of radiation. Although the latter concentration of 6AN gave the highest level of radiation sensitization and was most similar to our previous studies using 40 μM 6AN in a different lot of serum (9), it was not significantly greater than 80 μM 6AN.

Fig. 3b plots the decrease in surviving fraction of RIF-1 cells following exposure to 200 μM 6AN. Exposure to 6AN for periods varying from 1 to 15 h contributed to an increased level of cell killing by radiation that was significant for all durations of exposure (P < 0.05).

This result is contrary to our previous studies, using a different lot of serum, in which 40 μM 6AN was sufficient to potentiate the effect of 6 Gy of irradiation (P < 0.001; Ref. 9). Absolute levels of nicotinamide, with which 6AN competes in the synthesis of NAD(P)⁺, and of glutathione, which is involved in recycling of NADP⁺, were found to be quantitatively comparable in both lots of serum (data not shown). Other serum factors, therefore, must be involved in counteracting the effects of 6AN. These serum differences were also noted in NMR experiments in which accumulation of 6PG and decreases in PCr were measured (data not shown). Apart from the minimum 6AN concentration requirements of the different lots of serum, the metabolic responses of RIF-1 to 6AN, including the time exposure effect, were identical in both lots of serum provided the minimum concentration had been reached.

**31P-NMR Experiments.** The surviving fraction data indicated that much shorter exposures to 6AN than had been used previously were sufficient to cause sensitization to radiation. Shorter exposures to 6AN could be important clinically, because infusions of 6AN could be used prior to radiotherapy. We therefore undertook to investigate the effect of a 4-h incubation with 6AN on the metabolism of perifused RIF-1 cells. Fig. 4 shows 1H-decoupled 31P-NMR spectra of perifused RIF-1 cells 12 h after incubation for 4 h with 6AN. Fig. 4, a and b, shows spectra of cells perfused in the presence of 40 and 200 μM 6AN, respectively. It can be seen that 6PG accumulation was observed in the presence of 200 μM 6AN, a dose at which maximal radiosensitization occurred, but not in the presence of 40 μM 6AN, a dose that also had no radiosensitizing effect. Quantitation of peaks in 31P-NMR spectra was achieved by dividing the integral of a peak by that of P₁. The P₁ observed in the NMR spectrum was due almost entirely to P₁ in the perifusate, which, therefore, remained constant over the course of the experiment. Also, the extracellular volume was much larger than that of the intracellular volume, so changes in cell number had a negligible effect on measured P₁.

Changes in the ratio of 6PG:P₁ over time, for each of these condi-
Dubrova suggested that control of the PPP is based upon deinhibition of G6P dehydrogenase, the rate-controlling enzyme. This hypothesis, i.e., that the PPP is not very active under basal conditions, is supported by other studies, indicating that PPP activity is not essential for growth (22, 23) but is necessary to reverse the oxidation of NADPH induced by physical or chemical oxidative stress. Previous studies have noted an increase in PPP activity after radiation (4, 24) and exposure to oxidizing agents (22, 23). Tuttle et al. (22) studied a G6P dehydrogenase null variant Chinese hamster ovary cell line and found that growth was feasible but that these cells were more sensitive to radiation or oxidizing agents. Similarly, in the study of Pandolfi et al. (23), cells with a rearranged G6P dehydrogenase gene without measurable G6P dehydrogenase activity were viable, although they had decreased cloning capacity. However, if these cells were exposed to hydrogen peroxide or diamide, there was a further, dose-dependent decrease in their cloning capacity. Pandolfi et al. (23) concluded that G6P dehydrogenase activity is not necessary for pentose biosynthesis but is essential as a protective agent against oxidative stress. These data are compatible with our previous results (9), which showed that exposure of RIF-1 tumor cells to 6AN alone did not alter the surviving fraction, but 6AN followed by the oxidizing stress of radiation significantly decreased the surviving fraction. Additional studies investigating the effect of sequential 6AN + radiation are necessary to determine whether radiation enhancement by 6AN is related to inhibition of the PPP, glycolysis, or both.

A proposed mechanism for potentiation of alkylating agent activity (including radiation) by 6AN is the inhibition of poly(ADP-ribose)-polymerase. A number of studies, however, have indicated that inhibition of this enzyme has only marginal effects on 1,3-bis(2-chloroethyl)-1-nitrosourea activity (12, 25). Belfi et al. (7) concluded recently that 6AN activity was related to induction of GRP78 rather than poly(ADP-ribose)polymerase (7). Reduction in NAD+ levels due to competitive synthesis of 6ANAD+, in the presence of 6AN, has been proposed as an alternative mechanism for potentiation of alkylating agents and induction of GRP78 (12, 25). Although NAD+ concentrations have not been measured in this study, accumulation of 6ANAD(P+) was not sufficient to cause a reduction in the flux of glucose through the PPP.

It is noted that both 6AN and 2-deoxyglucose induce GRP78, inhibit glycolysis, and enhance radiation treatment in vivo (26) and in vitro (27, 28). Jain et al. (27) showed that the degree of inhibition of glycolysis by 2-deoxyglucose correlated with the ability of cells to repair radiation damage to DNA. These workers suggested that reduction in cellular ATP, due to inhibition of glycolysis by 2-deoxyglucose, inhibited cellular repair processes postradiation. Both 6AN and 2-deoxyglucose have been shown to be more effective radiosensitizers under hypoxic conditions. Purohit and Pohlit (28) suggested that hypoxic cells were more susceptible to radiation damage in the

**DISCUSSION**

The 1H-NMR experiments, in which RIF-1 cells were incubated with either [1-13C]glucose or [6-13C]glucose in the presence or absence of 6AN, confirmed our previous data in which a reduction in glucose utilization and lactate production was observed (9). This earlier result was in agreement with Kolbe et al. (19), who also observed a more than 50% reduction in metabolism of glucose to lactate. In this study, we have demonstrated an 89% reduction in glycolytic flux in the presence of 6AN. Inhibition of glycolysis is secondary to the accumulation of 6PG, induced by 6AN-mediated inhibition of 6PG dehydrogenase in the oxidative branch of PPP. 6PG inhibits glycolysis at the phosphoglucone isomerase step (20), which catalyzes the conversion of 6PG to fructose-6-phosphate.

It is clear from this study that exposure to 6AN alone induced greater inhibition of glycolysis than of the PPP, the primary site of action of 6AN. Although 6AN may not have caused a significant change in the flux of carbon through the PPP, it can be seen from the 31P-NMR experiments that it did induce a significant accumulation of 6PG. The flux of glucose through the PPP, in the context of competitive inhibition by 6ANAD(P+), was maintained by a shift in the equilibrium of 6PG dehydrogenase, which led to an accumulation of the enzyme substrate 6PG. Thus, despite the inhibition of 6PG dehydrogenase, the rate of ribose-5-phosphate and NADPH synthesis required for DNA synthesis and repair was maintained. It is noted that minimal accumulation of lactate occurred over the first 6 h in the presence of 200 μM 6AN, and therefore there is some uncertainty with regard to PPP inhibition during this period. This is the likely cause for the wide variation in PPP activity noted during the first 6 h of the study (Table 1).

Krebs and Eggleston have noted previously that PPP activity is determined by the NADPH:NADP+ ratio, which strongly favors inhibition of the PPP under typical metabolic conditions (21). They suggested that control of the PPP is based upon deinhibition of G6P dehydrogenase, the rate-controlling enzyme. This hypothesis, i.e., that the PPP is not very active under basal conditions, is supported by other studies, indicating that PPP activity is not essential for growth (22, 23) but is necessary to reverse the oxidation of NADPH induced by physical or chemical oxidative stress. Previous studies have noted an increase in PPP activity after radiation (4, 24) and exposure to oxidizing agents (22, 23). Tuttle et al. (22) studied a G6P dehydrogenase null variant Chinese hamster ovary cell line and found that growth was feasible but that these cells were more sensitive to radiation or oxidizing agents. Similarly, in the study of Pandolfi et al. (23), cells with a rearranged G6P dehydrogenase gene without measurable G6P dehydrogenase activity were viable, although they had decreased cloning capacity. However, if these cells were exposed to hydrogen peroxide or diamide, there was a further, dose-dependent decrease in their cloning capacity. Pandolfi et al. (23) concluded that G6P dehydrogenase activity is not necessary for pentose biosynthesis but is essential as a protective agent against oxidative stress. These data are compatible with our previous results (9), which showed that exposure of RIF-1 tumor cells to 6AN alone did not alter the surviving fraction, but 6AN followed by the oxidizing stress of radiation significantly decreased the surviving fraction. Additional studies investigating the effect of sequential 6AN + radiation are necessary to determine whether radiation enhancement by 6AN is related to inhibition of the PPP, glycolysis, or both.
presence of 2-deoxyglucose, because they are reliant on anaerobic glycolysis as an energy source. It can be seen that a number of similarities between 6AN and 2-deoxyglucose exist, which suggests that they may have similar mechanisms of action. Both 6AN and 2-deoxyglucose cause inhibition of glycolysis, reducing the amount of cellular energy available for DNA repair following radiation.

Varnes (4) suggested that under hypoxic conditions, cells treated with 6AN were unable to produce NADPH from alternative pathways, leading to increased radiosensitivity. The current study shows that under oxygenated conditions, 6AN does not inhibit the PPP. However, because in vivo many tumors are hypoxic, NADPH depletion due to PPP inhibition may have a significant role in radiation enhancement by 6AN. In this study, we have not addressed the question of PPP flux under conditions of oxidative stress such as would be expected after radiation, wherein it would be expected that flux through the PPP would be increased (4, 24). Thus, additional experiments to determine whether 6AN can block the increase in PPP activity induced by X-ray therapy are necessary.

We have shown previously that perfusion of RIF-l cells with 6AN for 15 h led to a significant accumulation of 6PG and a reduction in PCr:P, (9). In this study, we have shown that a similar accumulation of 6PG can be obtained with only 4 h of exposure to 6AN. Similarly, no significant difference in the reduction of PCr:P was observed between RIF-1 cells perfused for 4 and 15 h in the presence of 6AN. Although no significant change in ATP was observed in these studies with RIF-1 cells, a reduction in the ATP:ADP ratio has been observed following 6AN treatment in a number of previous studies by other investigators (24, 29, 30). This reduction in high-energy phosphates is attributed to a secondary inhibition of glycolysis through competitive inhibition by 6PG on the glycolytic enzyme, phosphoglucose isomerase (20). The similarity in the response of RIF-1 cells to 6AN over different durations of exposure was substantiated further by the surviving fraction data, in which there was no significant increase in the 6-Gy irradiation response when the period of preincubation with 6AN was increased from 2.5 to 15 h. Significant enhancement of radiotherapy by 6AN correlated with conditions in which an accumulation of 6PG and a reduction in PCr was observed, suggesting that these metabolic effects are required for the potentiation of radiation by 6AN. The data presented here indicated that exposure to 6AN for 4 h prior to radiation, and perhaps as acute as 1 h, would be sufficient to enhance the effect of radiation in vitro. This could prove to be important clinically, because it may be possible to administer 6AN to patients, as a radio- and chemosensitizer, either by infusion or by bolus injection, prior to treatment.

In our previous studies, using only [1-13C]glucose, the accumulation of glucose carbon in the oxidative branch of the PPP was found to be 3.9% of that accumulating in lactate via glycolysis following 6AN (9). The work presented here, using both [1-13C]glucose and [6-13C]glucose, indicates that this represents only a small fraction of the carbon passing through the PPP, given that approximately 30% of all glucose carbon was metabolized to lactate via the PPP following exposure to 6AN. In the previous study, we observed a significant reduction in the amount of label lost as carbon dioxide after perfusion with 6AN. The data presented here indicate that this reduction was not due to a decrease in the amount of C1 label lost as carbon dioxide in the oxidative branch of the PPP, but rather, this change may be due to a reduction in oxidative metabolism via the citric acid cycle. It is likely that such a reduction in citric acid cycle flux is linked to the reduced glycolytic rate and the related decrease in the availability of substrate.

These data indicate that when the concentration of 6AN is increased above a certain threshold determined partly by some serum factors, a near-maximal activity is reached rapidly with only a few hours of
exposure. The $K_i$ of 6ANAD(P$^+$) for 6PG dehydrogenase has been shown to be very low, suggesting that above a certain concentration of 6AN, accumulation of 6ANAD(P$^+$) becomes rate limiting in the equilibrium of 6PG dehydrogenase, such that it is sufficient to cause a significant accumulation of 6PG. This accumulation of 6PG, in turn, leads to inhibition of glycolysis and a reduction in cellular energy. It is hypothesized that 6AN may potentiate the activity of radiation by inhibition of energy-dependent repair processes, as has been suggested as a mechanism for the activity of 2-deoxyglucose (27). The data presented in this study suggest that in RIF-l cells coincubated with 6AN, this may occur by reduction of available energy, in the form of high-energy phosphates.

ACKNOWLEDGMENTS

We thank Elizabeth M. Obee for help with the statistics. In addition, we gratefully acknowledge the excellent advice and suggestions of an anonymous reviewer.

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Cancer Res 1997;57:3956-3962.

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