Effect of Tumor Necrosis Factor on Enzymes of Gluconeogenesis in the Rat

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Abstract. **The effect of human recombinant tumor necrosis factor (TNF)-α on enzymes of gluconeogenesis in the rat was investigated by determining the activity of glucose 6-phosphatase, fructose 1,6-diphosphatase (FDP), and phosphoenolpyruvate carboxykinase in the liver and kidney of fed and fasted rats. The activity of transaldolase in the pentose phosphate pathway was also measured. Starvation of rats for 24 hr resulted in a 1.6- to 3.1-fold increase in liver and kidney glucose 6-phosphatase and phosphoenolpyruvate carboxykinase ($P \leq 0.05$), a decrease in liver and kidney FDP ($P < 0.002$), and an increase in liver and kidney transaldolase ($P = 0.0001$). Injection of 50 and 100 μg/kg/day of TNF for 5 days resulted in a significant ($P \leq 0.03$) decrease in kidney FDP only. Injection of 100 μg/kg/day of TNF for 5 days with a 24-hr fast on Day 5 resulted in a significant ($P = 0.04$) increase in liver transaldolase, and a significant decrease in kidney FDP and phosphoenolpyruvate carboxykinase. Comparison of the enzyme activities of rats injected with 100 μg/kg/day of TNF for 5 days with those of their pair-fed control partners revealed additionally a significant decrease in glucose 6-phosphatase in the liver ($P < 0.001$). It is concluded that TNF administration in the rat has different effects on the enzymes of gluconeogenesis in the liver and kidney, and these effects differ from those seen in starved or tumor-bearing rats.** [P.S.E.B.M. 1992, Vol 199]

Significant alterations in the metabolism of tumor-free tissues of the host can be induced by a distally growing cancer (1, 2). It has been proposed that the ensuing biochemical changes are caused by humoral factors that are released by the cancer or host tissues and act through as-yet undetermined mechanisms (2). The resultant biochemical derangement of the host, frequently accompanied by malnutrition, can lead to the syndrome of cancer cachexia (1).

Tumor necrosis factor (TNF)/cachectin, a cytokine produced by tumor and host cells, can induce metabolic changes in the host comparable to those seen in cancer cachexia (3–5). One of the central biochemical changes in cancer cachexia is a significant increase in gluconeogenesis (6–10).

The major objective of this investigation was to determine in the rat the effects of low, sublethal doses of TNF on the activity of gluconeogenic enzymes (glucose 6-phosphatase, fructose 1,6-diphosphatase, and phosphoenolpyruvate carboxykinase) in liver and kidney, the two major organs where gluconeogenesis operates (11). Another objective was to determine the effect of TNF on the *de novo* synthesis of ribose phosphate from glycolytic intermediates by measuring the activity of transaldolase, a key enzyme in the nonoxidative pentose phosphate pathway.

Materials and Methods

Animals. Male Sprague-Dawley rats (160 ± 14 g) were maintained on a 12:12-hr light:dark cycle and provided with water and chow *ad libitum*. When pair feeding was used, each control rat was given daily the same amount of food consumed on the previous day by his TNF-treated and weight-matched partner.

Experimental Animals. Rats were injected intraperitoneally with human recombinant TNF-α, generously donated by Asahi Chemical Industry of America. The preparation contained 2.3×10^6 units of TNF/mg of protein and a negligible amount of endotoxin as contaminant (0.35 pg of endotoxin/10^6 units of TNF, or the equivalent of 0.001 pg of endotoxin/μg (of TNF).

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TNF was injected at a concentration of 40 μg/ml in 10% glycerol (Experiments 1 and 2) or phosphate-buffered saline containing 0.1% bovine serum albumin (Experiments 3 to 5).

Five experiments were performed, each including six rats injected daily with TNF for 5 days (experimental) and four to six rats injected with the vehicle only (controls). Experiments 1 and 2 were designed to determine the dose at which a significant difference in the activity of one or more of the enzymes is observed. In Experiment 1, six rats were injected with 25 μg/kg of TNF twice daily at approximately 12-hr intervals, and four rats were injected with the vehicle only. Experiment 2 was similar to Experiment 1, except that the experimental rats received double the dose (50 μg/kg) twice daily.

Experiments 3 and 4 were designed to determine whether any additional changes in enzyme activity would occur when the rats were also fasted for a 24-hr period on the fifth or last day of the injection schedule. In Experiment 3, six rats were injected with 50 μg/kg of TNF twice daily at approximately 12-hr intervals, and four rats were injected with the vehicle only. In Experiment 4, six rats were injected with 33 μg/kg of TNF at approximately 8-hr intervals, and six rats were similarly injected with the vehicle only.

Experiment 5 was designed to determine whether the known decrease in food intake induced by TNF administration would significantly alter the activity of gluconeogenic enzymes in liver and kidney. This experiment included four control rats fed *ad libitum*, six experimental rats injected with 50 μg/kg/day of TNF twice daily for 5 days, and six pair-fed control rats.

All rats were anesthetized with ether and sacrificed by guillotine decapitation on Day 6 of the experimental schedule (or Day 7 for the pair-fed rats). The liver and whole kidneys were excised, frozen immediately on dry ice, and kept frozen at −70°C until homogenized. In Experiment 5, blood was also collected from all the rats immediately following decapitation and the serum was separated and kept frozen until assayed. The organs were weighed and homogenized in a Potter-Elvehjem homogenizer in 10 vol of cold 0.2 *M* phosphate buffer (pH 7.4) containing 2 m*M* EGTA and 2 m*M* EDTA. The homogenates were centrifuged at 30,000*g* for 30 min and the supernatant fluids were divided into two aliquots, one for the determination of glucose 6-phosphatase activity and the other, to which concentrated mercaptoethanol was added to a concentration of 1 m*M*, for the determination of the other enzyme activities. It should be noted that although glucose 6-phosphatase is tightly bound to the endoplasmic reticulum of cells, it remains in the supernatant fluid following centrifugation of tissue homogenates at 30,000*g* (12). All supernatants fluids were frozen at −20°C until assayed.

Assay of Enzymatic Activities and Protein Concentration. Protein concentration in the supernatant fluids was determined by a biuret method adapted for use on the DACOS discrete analyzer (Coulter Electronics, Inc., Hialeah, FL). Serum glucose, lactate, and triglyceride concentrations were determined on an Ektachem discrete analyzer (Eastman Kodak Co., Rochester, NY). Fructose 1,6-diphosphatase (FDP; EC 3.1.3.11) activity was determined by the method of Latzko and Gibbs (13). Phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) and transaldolase (EC 2.2.1.2) activities were determined by the spectrophotometric methods described by Chang and Lane (14) and Brand (15), respectively.

Glucose-6-phosphatase (G6P; EC 3.1.3.9) activity was determined by an in-house procedure in which the rate of production of glucose is determined by enzymatic coupling to glucose oxidase and measurement of the hydrogen peroxide so formed by reacting it with 4-aminoantipyrine and *p*-hydroxybenzene sulfonate to form a quinoneimine dye that aborbs maximally at 505 nm (16). This homogeneous assay is much less tedious and time consuming than the method commonly used (17), which involves measurement of the inorganic phosphorus liberated following protein precipitation by acid. Briefly, our reaction mixture contained 10 μl of supernatant fluid, 25 μl of 50 m*M* glucose 6-phosphate, 150 μl of glucose reagent (pH 7.0), and 65 μl of water. The glucose reagent was purchased as the Glucose (Trinder) reagent from Sigma Chemical Co. (St. Louis, MO) and contains the following per liter of final incubation mixture: 0.5 mmol of 4-aminoantipyrine, 12 mmol of *p*-hydroxybenzene sulfonate, 9000 units of glucose oxidase and 6000 units of horseradish peroxidase. The reaction was started by addition of the sample and the optical density was monitored at 505 nm for 12 min. After a delay period of 7 min, to allow for any endogenous glucose in the sample to be exhausted, enzyme activity was calculated from the change in absorbance between 7 and 12 min using a molar extinction coefficient of 4900. One unit of activity is arbitrarily defined as the amount of G6P that catalyzes the formation of one μmol of hydrogen peroxide per minute at 30°C. Table I shows the correlation between the two methods using reaction mixtures containing increasing amounts of supernatant fluid from a rat liver homogenate whose activity was previously determined by the inorganic phosphorus method. The correlation was quite good ($r^2 = 0.99$) and the activity by the in-house method was 1/7 that by the inorganic phosphorus method. The reaction was linear up to 1.8 mU per reaction mixture by the in-house method and 12 mU by the inorganic phosphorus method.

All enzyme activities were determined at 30°C on a Cobas centrifugal analyzer (Roche Diagnostics, Nutley, NJ), except for transaldolase activity, which was determined at 37°C on a Beckman DU-7 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

Table I. Comparison of the Inorganic Phosphorus and In-House Methods for the Determination of G6P Activity

G6P added[a] (mU)	Inorganic phosphorus method[b] (mU)	In-house[c] (mU)	Inorganic phosphorus/ in-house ratio
3	3.0	0.39	7.7
6	6.5	0.96	6.8
9	9.1	1.40	6.5
12	12.3	1.83	6.7
15	13.1	2.16	6.1

[a] The G6P added to each reaction mixture was in the form of the supernatant fluid of a liver homogenate whose activity was measured previously by the inorganic phosphorus method. All activities are in mU/incubation mixture.
[b] G6P activity was determined as described in Ref. 17.
[c] G6P activity was determined as described in Materials and Methods.

Statistical Analysis. All data were analyzed by the two-tailed, unpaired Student's *t* test.

Results

The mean enzyme activities in units/g wet wt in the livers and kidneys of the control rats used in Experiments 1–4 are shown in Table II. The values for the fed rats in Experiments 1 and 2 represent the activities in normal rats and are comparable to those reported by other investigators (18–20). For G6P, where the unit of activity by the in-house method is equal to approximately 1/7 units by the traditional inorganic phosphorus method, multiplication of the G6P activities in Table II by seven yields values that are also similar to those reported by other investigators (18–20).

A comparison of these normal activities with those of the 24-hr starved control rats showed the changes normally associated with starvation in rat liver and kidney (19). Most noticeably, the mean G6P and PEPCK in the liver of the fasted control rats in Experiments 3 plus 4 (n = 10) were 3.1- and 1.6-fold greater, respectively, than those in the liver of the fed control rats in Experiments 1 plus 2 ($n = 8$; $P = 0.0001$ for both enzymes). Similar results were obtained in the kidney, where the mean G6P and PEPCK in the fasted control rats were 2.5-fold greater than those in the fed ones ($P = 0.05$ and 0.0001, respectively). Transaldolase was also 30–40% greater in the liver and kidney of the fasted control rats ($P = 0.0001$ for both tissues). FDP, on the other hand, showed a significant decrease in fasted liver (28%) and, to a lesser extent (12%), in fasted kidney ($P < 0.002$ for both tissues).

Essentially similar results were obtained when the enzyme activities were calculated as units/g of protein in the tissue supernatant fluids. However, the activities in this case were about 7-fold greater.

Comparisons of the mean liver and kidney enzyme activities of the TNF-treated and control rats are shown in Figure 1 in units/g tissue wet wt. For simplicity, the mean (± SE) activities of the TNF-treated rats in each of the four experiments are shown relative to the mean activities of the corresponding control rats arbitrarily set at 1. Mean activities significantly different from the control means at a *P*-level of 0.05 or less are indicated by asterisks.

Interestingly, TNF treatment of rats resulted in more changes in the kidney than in the liver, whether the rats were fed or fasted. When 50 μg/kg/day of TNF was injected (Experiment 1), none of the liver enzymes showed a statistically significant difference, but kidney FDP was significantly decreased (11%; $P = 0.03$). Injection of 100 μg/kg/day of TNF (Experiment 2) resulted again in a 10% decrease in kidney FDP ($P = 0.02$). Injection of 100 μg/kg/day of TNF with a 24-hr fast on Day 5 (Experiments 3 and 4) resulted in some additional changes. Liver showed a significant 18% increase in transaldolase in both Experiments 3 and 4 ($P = 0.04$ and 0.003, respectively), while kidney showed

Table II. Enzyme Activities (Mean ± SD Units/g Wet Tissue Wt) in Liver and Kidney of Control Rats

	Expt. No.	*n*/State[a]	TNF dose[b]	G6P	FDP	PEPCK	Transaldolase
Liver	1	4/Fed	50	1.42 ± 0.53	11.5 ± 0.83	2.20 ± 0.19	6.1 ± 0.30
	2	4/Fed	100	1.27 ± 0.48	9.1 ± 1.09	3.54 ± 0.24	6.4 ± 0.10
	3	4/Fasted	100	4.51 ± 1.70	7.2 ± 0.55	3.33 ± 0.47	7.6 ± 0.79
	4	6/Fasted	100	3.86 ± 1.08	7.6 ± 0.90	5.78 ± 0.22	8.8 ± 0.60
Kidney	1	4/Fed	50	0.77 ± 0.41	5.9 ± 0.31	3.6 ± 0.60	7.6 ± 0.80
	2	4/Fed	100	1.22 ± 0.14	5.5 ± 0.28	5.1 ± 1.23	6.8 ± 0.71
	3	4/Fasted	100	2.26 ± 0.72	4.8 ± 0.23	11.7 ± 1.70	8.8 ± 0.60
	4	6/Fasted	100	2.64 ± 1.25	5.2 ± 0.26	10.3 ± 0.54	11.2 ± 0.90

[a] Rats were fed *ad libitum* in Experiments 1 and 2 and fasted on Day 5 in Experiments 3 and 4.
[b] Total daily dose injected in μg/kg body wt.

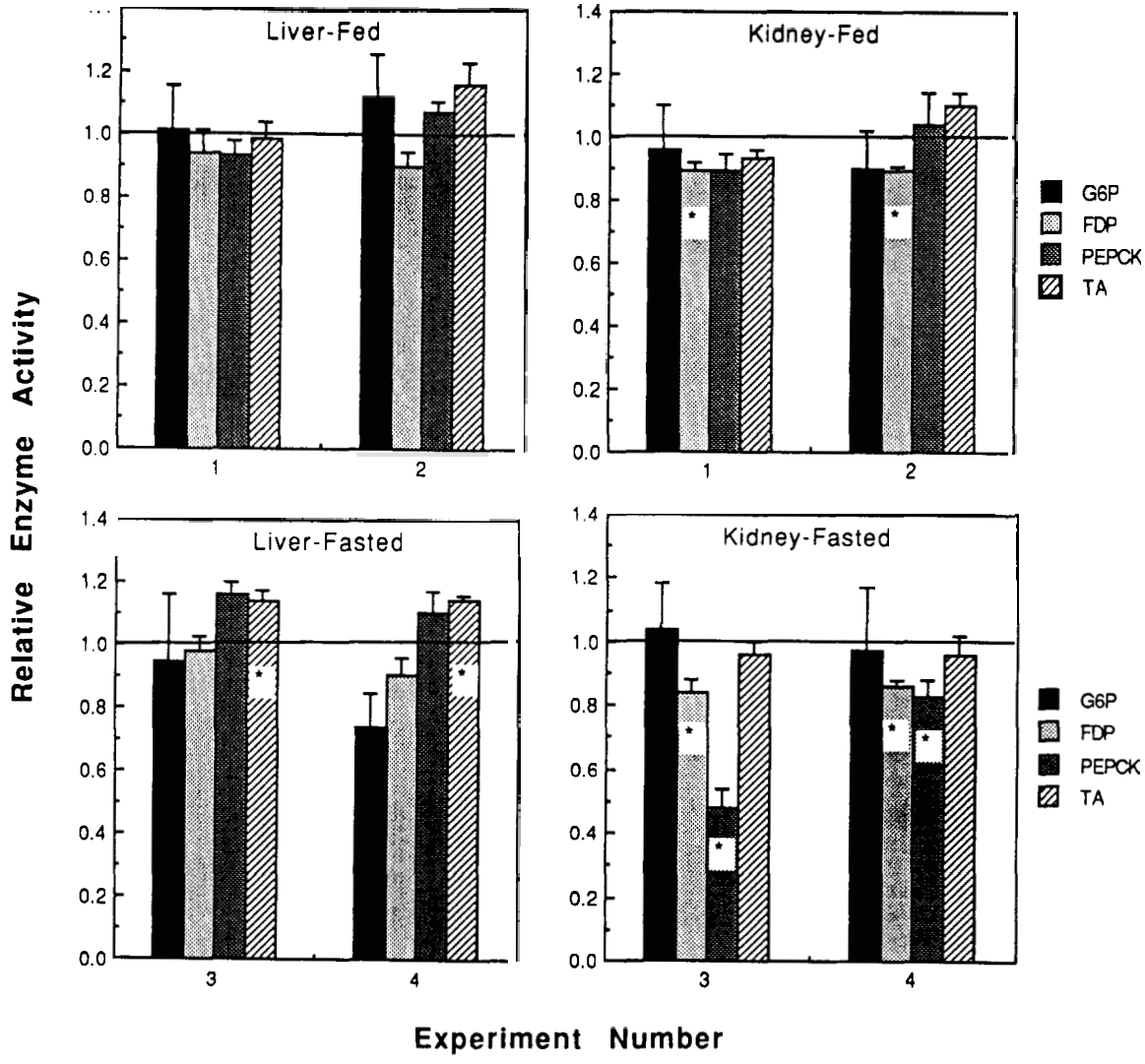

Figure 1. Effect of TNF on enzyme activity in the liver and kidney of rats fed *ad libitum* (Experiments 1 and 2) and rats fasted for 24 hr on Day 5 of the experimental schedule (Experiments 3 and 4). Each experiment consisted of six rats injected intraperitoneally with 50 μg/kg/day (Experiment 1) or 100 μg/kg/day (Experiments 2–4) of human recombinant TNF-α for 5 days, and 4–6 control rats injected with the vehicle only. The mean (± SE) enzyme activities in units/g tissue wet wt are shown as fractions/multiples of those of the control rats. Mean activities significantly different from the control means at a *P*-level of 0.05 or less are indicated by an asterisk. TA, transaldolase.

a more pronounced decrease in FDP (14–16%; $P = 0.0001$) and a significant decrease in PEPCK (52%, $P = 0.0005$ for Experiment 3; 17%, $P = 0.02$ for Experiment 4).

It should be noted again that the control rats in Experiments 3 and 4 were fasted for 24 h, as were the experimental rats, so that the changes observed after TNF administration were above and beyond those observed with fasting alone.

Mean daily body weight of the TNF-injected rats and their pair-fed control partners in Experiment 5 were not statistically different from those of control rats fed *ad libitum*. On the other hand, food intake of the TNF-injected rats was 20% lower than that of the control rats fed *ad libitum* on Day 1 after TNF injection (19.5 ± 3.8 vs 24.5 ± 2.65 g/day, mean ± SD; $P = 0.06$), but subsequently rose to the control level by Day 2. A significant transient decrease in food intake upon TNF administration has been shown by other investigators (21, 22).

Figure 2 shows the enzyme activities (mean ± SE) of the TNF-treated and pair-fed control rats relative to the activities of the control rats fed *ad libitum* arbitrarily set at 1. Mean activities of TNF-treated rats significantly different from those of pair-fed control rats ($P \leq 0.05$) are indicated by asterisks. Comparison of the activities of TNF-treated and pair-fed control rats showed a decrease in G6P in the liver (44%; $P = 0.0002$) and, to a lesser extent, in the kidney (31%; $P = 0.07$). However, G6P activity was significantly lower than that of the control rats fed *ad libitum* in both liver and kidney G6P (37–44%; $P \leq 0.003$). There was also a decrease in liver and kidney FDP (10–15%; $P = 0.01$) and in kidney transaldolase (14%; $P = 0.03$).

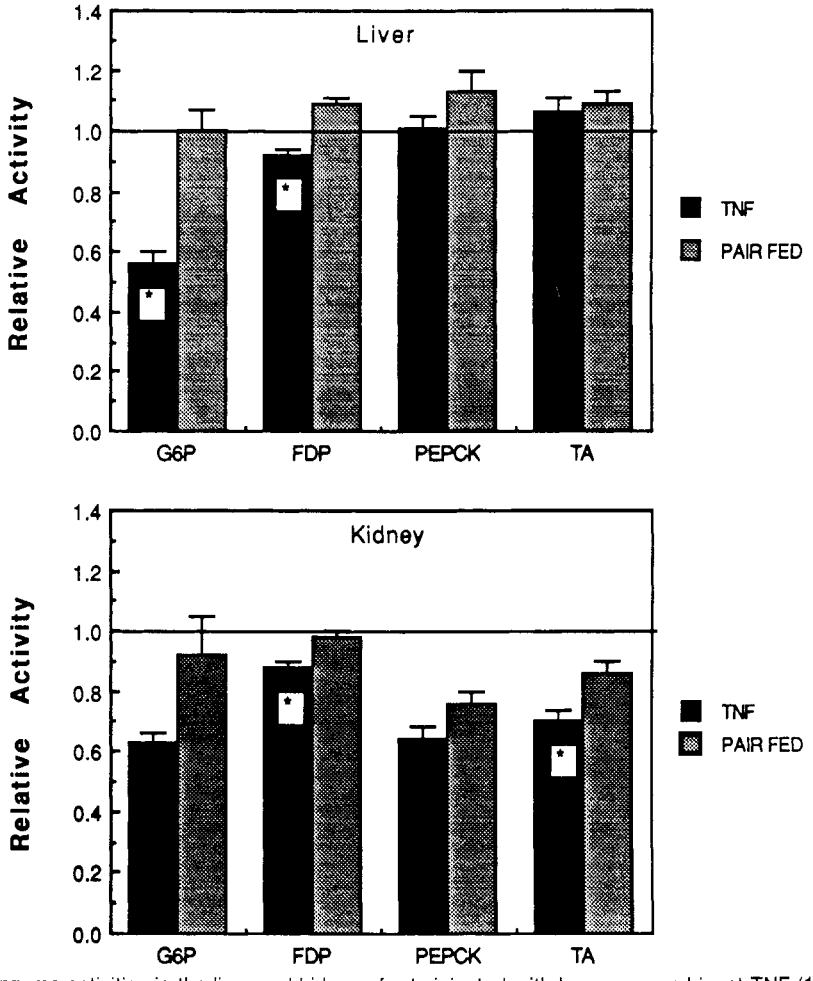

Figure 2. Comparison of enzyme activities in the liver and kidney of rats injected with human recombinant TNF (100 μg/kg/day for 5 days; $n = 6$) with those of pair-fed control rats ($n = 6$) and control rats fed *ad libitum* ($n = 4$). The mean (± SE) enzyme activities in units/g tissue wet wt are shown relative those of the control rats fed *ad libitum*, arbitrarily set at 1. An asterisk within the TNF column indicates a significant difference from the mean of the pair-fed control rats at a *P*-level of 0.05 or less.

Mean serum triglyceride concentration in the TNF-treated rats (108 ± 27 mg/dl) was 92% greater than that of the control, pair-fed rats (56 ± 12 mg/dl; $P = 0.002$) and 36% greater than that of the control rats fed *ad libitum* (73 ± 13 mg/dl; $P = 0.08$). In contrast, mean serum glucose (167 ± 5 mg/dl) and lactate (3.45 ± 0.40 mmol/liter) concentrations in the TNF-treated rats did not differ significantly from those of the pair-fed control rats (164 ± 7 mg/dl and 3.15 ± 0.54 mmol/liter, respectively) or the control rats fed *ad libitum* (166 ± 7 mg/dl and 3.60 ± 0.87 mmol/liter).

Discussion

In the present study, starvation of rats for 24 hr resulted in a 60–200% increase in liver and kidney G6P and PEPCK, a 30–40% increase in liver and kidney transaldolase, and a 39% and 12% decrease in liver and kidney FDP, respectively (Table II). Somewhat similar results were obtained by Katz (19), who showed doubling of liver G6P and PEPCK after a 24-hr period of starvation in the rat, but FDP was unchanged. In the kidney cortex, there was also a 140% and 40% increase in PEPCK and G6P, respectively (19).

The administration of TNF to rats resulted in changes in G6P, PEPCK, and transaldolase that were quite different from those seen in starvation (Figs. 1 and 2; Table II). Of particular interest is the observation that PEPCK, which approximately doubled in the liver and kidney following starvation, was relatively unchanged in the liver of TNF-treated rats and decreased appreciably in the kidney of TNF-treated and fasted rats. Also transaldolase, which increased significantly in the liver and kidney of fasted rats, showed an increase in the liver that was significant only when the rats were also fasted (Fig. 1), while it was relatively unchanged (Fig. 1) or decreased in the kidney (Fig. 2). The increase in liver transaldolase is probably a reflection of increased DNA synthesis, which has been observed in rats following administration of TNF doses comparable to those used in this study (23). On the other hand, FDP is the only enzyme that showed changes/decreases following TNF administration that were analogous to those seen in starvation (Figs. 1 and 2).

Tumor-bearing animals show changes in the activity of gluconeogenic enzymes that are similar to those seen in starvation and, therefore, are also different from those seen following TNF treatment. Gutman *et al.* (20) reported that liver G6P and PEPCK increased by 60–150% over the control values of rats bearing a benzopyrene tumor, while FDP did not change significantly. These changes could not be attributed to a decrease in food intake by the tumor-bearing rats, because their food intake was similar to that of the control rats. Singh *et al.* (24) also determined the activity of these enzymes in 19-hr fasted rats and in rats bearing a transplantable sarcoma. In the fasted rats, PEPCK and G6P increased significantly, as in the present study, but FDP was unchanged. In the tumor-bearing rats, liver PEPCK increased gradually, with an increase in the tumor to body ratio until it attained a level equal to that of the fasted rats. In contrast, the activities of G6P and FDP remained at the level of the fed control rats until the tumor to body weight ratio was about 0.35, after which they increased rapidly. The authors suggested that these changes could be caused by hormones or humoral factors released by the tumor.

Our observations suggest that TNF is not responsible for the increase in gluconeogenesis seen in the tumor-bearing host. TNF may, however, have a different effect when acting in the presence of other cytokines. Tredget *et al.* (25) have shown that significant increases in plasma lactate, glucose, and triglycerides occurred following the combined infusion of TNF and interleukin 1 in 48-hr fasted rabbits, but did not occur when the cytokines were infused independently.

TNF may also have a different effect on gluconeogenesis at doses higher than those used in this study. We have chosen to use low, sublethal doses of TNF to avoid the excessive tissue damage and ensuing biochemical changes that may prevent an accurate assessment of its biochemical effects. For example, Tracey *et al.* (26) observed that intravenous infusion of human recombinant TNF in the rat at doses close to the LD_{50} (700 μg/kg) results, within 12 hr in appreciable tissue necrosis accompanied by severe metabolic acidosis with hyperglycemia and hyperlactacidemia. In our study, serum glucose and lactate concentrations were not affected by the administration of 100 μg/kg/day of TNF for 5 days.

More recently, Tracey *et al.* (3) also showed that intraperitoneal injection of sublethal doses of TNF (500 μg/kg/day for 7 to 10 days), which are still 5-fold greater than those injected intraperitoneally per day in the present study, results in cachexia, anemia, and histologic abnormalities in several tissues, including the liver, but not the kidney. In a previous study, we did not observe any damage upon electron microscopic examination of liver sections from rats injected intraperitoneally with 100 μg/kg/day of TNF for 5 days (27).

Our results suggest that intraperitoneal administration of low doses of TNF to rats inhibits, rather than stimulates, gluconeogenesis in the liver, and more so in the kidney. The inhibition occurs mainly at the level of G6P and FDP in the liver, and at the level of G6P, FDP, and PEPCK in the kidney. Since the serum glucose concentration was normal, inhibition of G6P and FDP may have resulted in the accumulation of glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-diphosphate. This is consistent with our previous observation (27) that liver and, to a much lesser extent, kidney glucose 6-phosphate dehydrogenase activity increases significantly at low doses of TNF. This enzyme acts in conjunction with the glutathione peroxidase-reductase redox cycle and is activated by the decrease in the $NADPH/NADP^+$ ratio that occurs whenever hydrogen peroxide is generated (28). Since it is known that TNF and other cytokines can stimulate the formation of free radicals by leukocytes (29), it is possible that the major initial response of the body to TNF treatment is prevention of the oxidative damage caused by free radicals. In the liver, this is accomplished by channeling more glucose 6-phosphate via the oxidative portion of the pentose phosphate pathway.

Furthermore, since PEPCK and transaldolase in the liver are normal or elevated, it is also possible that there is a demand for DNA synthesis, leading to enhanced channeling of accumulated hexose and triose phosphates via the nonoxidative portion of the pentose phosphate pathway. The situation may be different in the kidney, which histologically appears to be relatively unaffected by TNF (3). In this organ, TNF treatment results in a decrease in PEPCK, as well as G6P and FDP, activity, and only a slight increase in glucose 6-phosphate dehydrogenase activity, suggesting that the main response to TNF is enhancement of the activity of the oxidative portion of the pentose phosphate pathway. It should be noted that the synthesis of PEPCK in the liver and kidney is apparently regulated by different means (30), which may explain the differential effect of TNF on the two tissues. The mechanisms by which TNF effects these changes are not yet known.

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