Recombinant Tumor Necrosis Factor-α Chronically Administered in Rats: Lack of Cachectic Effect (43042)

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Abstract. **Recombinant human tumor necrosis factor-α (rHuTNF) was injected into rats to test its reported cachectic effects. Rats were subcutaneously injected daily at 1730 hr with either saline or rHuTNF (0.25 mg/kg body wt) for either 5 or 14 days. Daily food intakes were significantly depressed only for the first day and first two days of rHuTNF injection in animals treated for 5 days and 14 days, respectively. There were no significant differences in daily body weights among the groups. Analysis of carcass composition revealed no significant differences in percentage of lipid or protein. Liver and inguinal pad weights were not significantly different. *In vitro* determination of lipogenesis showed it was enhanced in the inguinal pad and depressed in the liver only after 14 days of treatment. These results demonstrate that although *in vivo* rHuTNF may specifically alter tissue metabolism, it does not, by itself, result in a sustained cachectic effect.** [P.S.E.B.M. 1990, Vol 193]

Neoplasia and sepsis elicit a variety of metabolic adjustments in the host, often leading to generalized tissue wasting or cachexia (1, 2). Anorexia is also quite common, often contributing to the cachectic state (3, 4). It has been suggested that the depression of food intake is a natural protective response by the host to inhibit growth of the invading organism (or tumor) and to gain greater control over nutrient supply (4). How these metabolic alterations occur is unknown, but to further understand these changes, investigators have focused on identifying compounds unique to animals or patients with neoplasia or sepsis. Of the substances identified as having cachectic or anorectic effects, tumor necrosis factor-α (TNF) has received particular attention. Tumor necrosis factor, a product of macrophages (7, 8), initially sparked interest because of its ability to cause necrosis of certain tumor types (9–11). Further studies have demonstrated a variety of cytotoxic and metabolic effects (11–13), especially with respect to fat metabolism (12, 13). Direct evidence of TNF's *in vitro* effects on lipid metabolism comes from studies utilizing purified or recombinant TNF (rTNF). In cell culture conditions it has been shown that TNF can (i) depress lipoprotein lipase (LPL) (14–16) by inhibiting its synthesis (15) at the level of transcription (17); (ii) inhibit acetate incorporation into lipids, a measure of lipid synthesis (16); and (iii) stimulate free fatty acid release from adipocytes (16). However, Kern (18) reported that TNF did not affect LPL activity in primary cultures of human adipocytes. In addition, studies using human rTNF with isolated rat adipocytes, hepatocytes, and muscle cells indicate a minimal effect of the cytokine on cell metabolism (19).

Although TNF appears to alter lipid metabolism in some *in vitro* studies, relatively few investigations have been conducted to determine whether parallel effects occur *in vivo*. Feingold and Grunfeld (20) examined the effects of rTNF on lipogenesis and sterol synthesis in rats 1–17 hr after injection and showed that TNF stimulates lipid and sterol synthesis in liver but not adipose tissue. Semb *et al.* (21) also have shown that a single dose of purified TNF in animals can inhibit LPL in adipose tissue while stimulating it in the liver. The lipid-depleting effects of TNF have led to the hypothesis that TNF may be responsible for the cachexia observed in sepsis and neoplasia (22). Studies using a nonpurified source of TNF indicate that it does indeed have a cachectic effect (23). However, rTNF adminis-

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tered by continuous infusion or repeated injections in rats and mice suggests that, while rTNF initially induces a cachectic state, tolerance to the cytokine soon develops and food intake and body weight return to normal (24–26). Others (27) have shown that escalating doses of rTNF are necessary to maintain the cachectic state. Despite these findings, TNF is reported to be a cachectic agent by some investigators (28, 29). The present study confirms TNF's lack of sustained effect on food intake and body weight and further examines body composition and hepatic and inguinal lipid metabolism in rats chronically administered human rTNF for either 5 or 14 days.

Materials and Methods

^3H_2O (1 Ci/g) and Omnifluor were purchased from ICN Biochemicals, Inc., Costa Mesa, CA. Human tumor necrosis factor-α with a specific activity of 5×10^7 units/mg produced by recombinant DNA techniques was generously provided by Genentech Inc., South San Francisco, CA.

Animal Procedures. Forty female Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing approximately 200 g were fed a semipurified diet *ad libitum*. After an initial adaptation period, animals were divided into four groups of approximately equal mean weight. Two groups of rats were subcutaneously injected daily at 1730 hr with TNF (0.25 mg/kg body wt) whereas controls received saline. One saline and one TNF group were sacrificed after 5 days and the others after 14 days. Food intake, corrected for spillage, and body weight were recorded daily.

Body Composition. Animals were sacrificed by decapitation between 0900 and 1100 hr and blood was collected. The liver and inguinal fat depots were rapidly removed and weighed. After removal of the gastrointestinal tract, the carcasses were weighed and frozen for subsequent analysis of body composition using a modification of the technique of Hartsook and Hershberger (30). Carcasses were autoclaved in sealed containers for 50 min at 120°C. Each carcass was mixed with three volumes of distilled water in a blender and then homogenized with a polytron tissue homogenizer (Brinkman Instruments, Westbury, NY). During homogenization, samples were withdrawn for water, ash, and fat analyses. Carcass fat was determined on samples by chloroform-methanol extraction. Carcass water was determined by drying samples at 85°C for 72 hr. Using these same samples, ash was measured by subsequent ashing at 600°C for 12 hr. Carcass protein was determined by difference.

Tissue Incubations. Eight slices of inguinal fat and two slices of liver, each weighing approximately 100 mg, were cut with a Stadie-Riggs tissue slicer. Each slice was incubated in a 25-ml Erlenmeyer flask with 2 ml of Krebs-Ringer bicarbonate buffer (½ calcium) containing 5 m*M* glucose and 2% (w/v) bovine serum albumin. Flasks were gassed with 95% O_2-5% CO_2, sealed with rubber stoppers, and incubated at 37°C in an oscillating water bath (90 cycles/min) for 2 hr.

Fatty Acid Synthesis. Radiolabel and hormones were added according to the parameter being measured. Duplicate slices of liver and inguinal fat were incubated in the presence of ^3H_2O (1mCi/ml) for measurement of basal fatty acid synthesis (FAS). Duplicate slices of fat were incubated in insulin (1000 microunits/ml) or norepinephrine (1 μg/ml) to measure stimulated and inhibited FAS, respectively. Reactions were stopped by the addition of 0.5 ml of 1 *N* H_2SO_4. Triglyceride fatty acids and total lipids were extracted from tissue slices by a modified Dole's procedure (31). Extracted fractions were dried in scintillation vials and reconstituted with scintillation fluid (15 g of Omniflour/3.75 liters of toluene). Radioactivity was counted in a Beckman LS 9800 liquid scintillation system. Rates of FAS were calculated for each organ based on total weight.

Statistics. Data were analyzed using SAS softwear package (32). Comparisons were carried out using General linear models procedure followed by a Duncan's multiple range test.

Results

Daily food intake of TNF- and saline-treated rats are shown in Figure 1. Treatment with TNF for 5 days depressed daily food intake for the initial day of injection; however, overall there were no significant differences in daily food intakes in this group (mean daily intakes were 15.33 ± 0.36 g and 15.38 ± 0.36 g for TNF and saline, respectively). Treatment with TNF for 14 days depressed daily food intake for the initial 2 days of injection, resulting in an overall depression of mean daily intake (14.20 ± 0.24 g and 14.95 ± 0.24 g for TNF and saline, respectively). However, this effect was transient and by the third day of injection, daily food intakes were no longer significantly different.

Daily body weights are shown in Figure 2. Mean daily body weight gain for 5-day TNF- and saline-injected rats was 3.77 ± 0.34 g and 3.27 ± 0.35 g, whereas 14-day body weight gains averaged 2.13 ± 0.23 g and 2.43 ± 0.23 g, respectively. Tumor necrosis factor treatment had no significant effect on body weight gain when given for either 5 or 14 days.

Comparison of organ weights and body composition (Table I) reflects TNF's lack of effect on food intake and body weight. Neither 5-day nor 14-day treatments had a significant effect on carcass lipid, carcass protein, liver, or inguinal pad weight.

Values for inguinal triglyceride fatty acid and total lipid synthesis after 5 days are shown in Figure 3. Values for inguinal triglyceride fatty acid and total lipid synthesis after 14-day TNF treatment are shown in Figure 4. The effect of TNF was not apparent until 14 days.

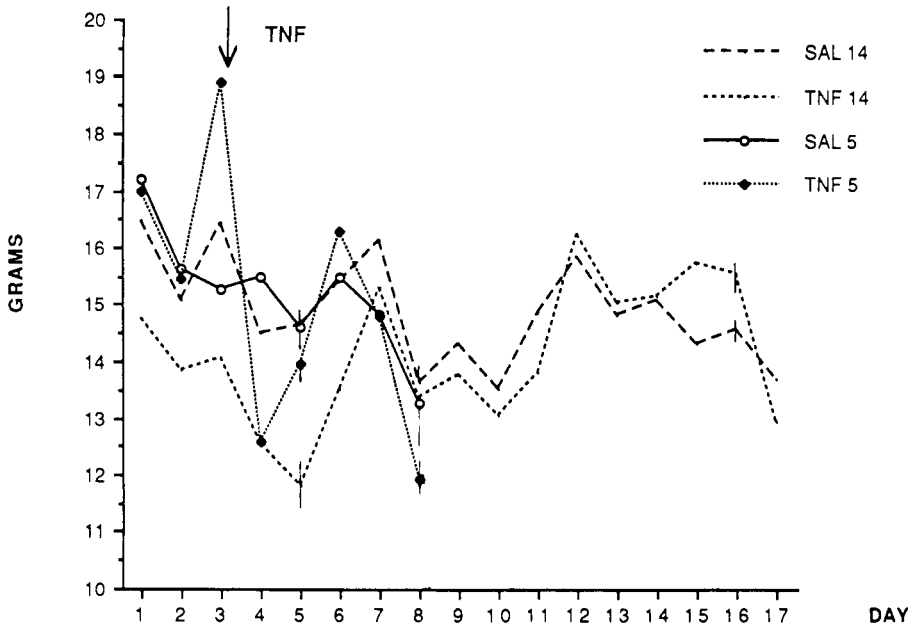

Figure 1. Daily food intakes of 5-day and 14-day treated rats. Animals were injected subcutaneously daily at 5:30 PM with either rTNF (n = 10) or saline (n = 10). Values are mean ± SE.

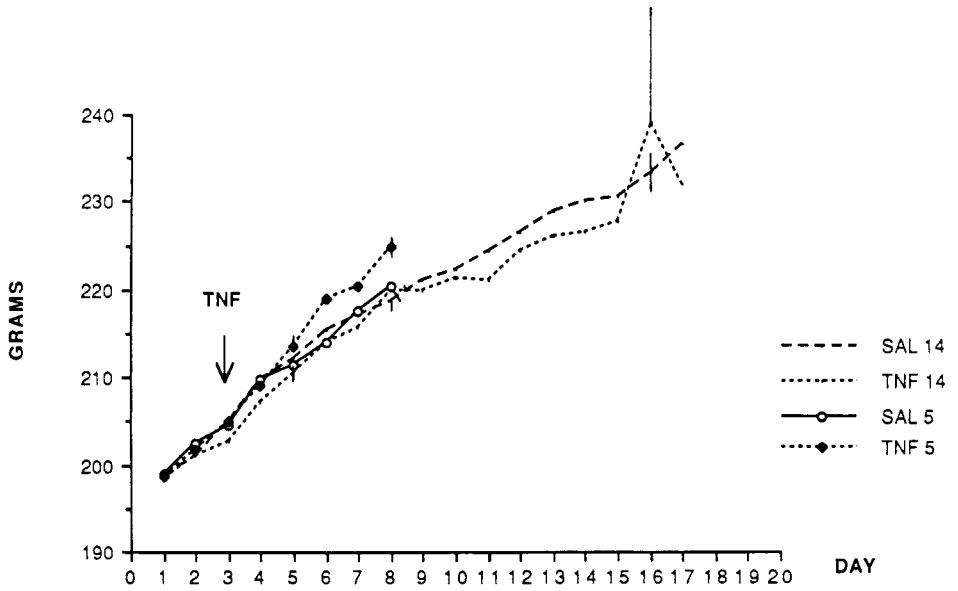

Figure 2. Daily body weights of 5-day and 14-day treated rats. See Figure 1 for protocol. n = 10 for each group. Values are mean ± SE.

Table I. Carcass Composition and Organ Weights[a]

Treatment	Carcass lipid (%)	Carcass protein (%)	Liver weight (g)	Inguinal weight (g)
Saline, 5 days	11.30 ± 0.39	20.85 ± 0.25	7.77 ± 0.19	2.48 ± 0.16
TNF, 5 days	10.55 ± 0.22	20.52 ± 0.23	7.97 ± 0.11	2.41 ± 0.12
Saline, 14 days	11.52 ± 0.60	21.13 ± 0.25	8.37 ± 0.22	2.64 ± 0.16
TNF, 14 days	10.79 ± 0.36	21.09 ± 0.36	7.97 ± 0.27	2.56 ± 0.10

[a] Carcass composition and organ weights of rats treated with TNF or saline for 5 or 14 days. See Figure 1 for protocol. n = 10 in each group. Values are mean ± SE. No significant differences.

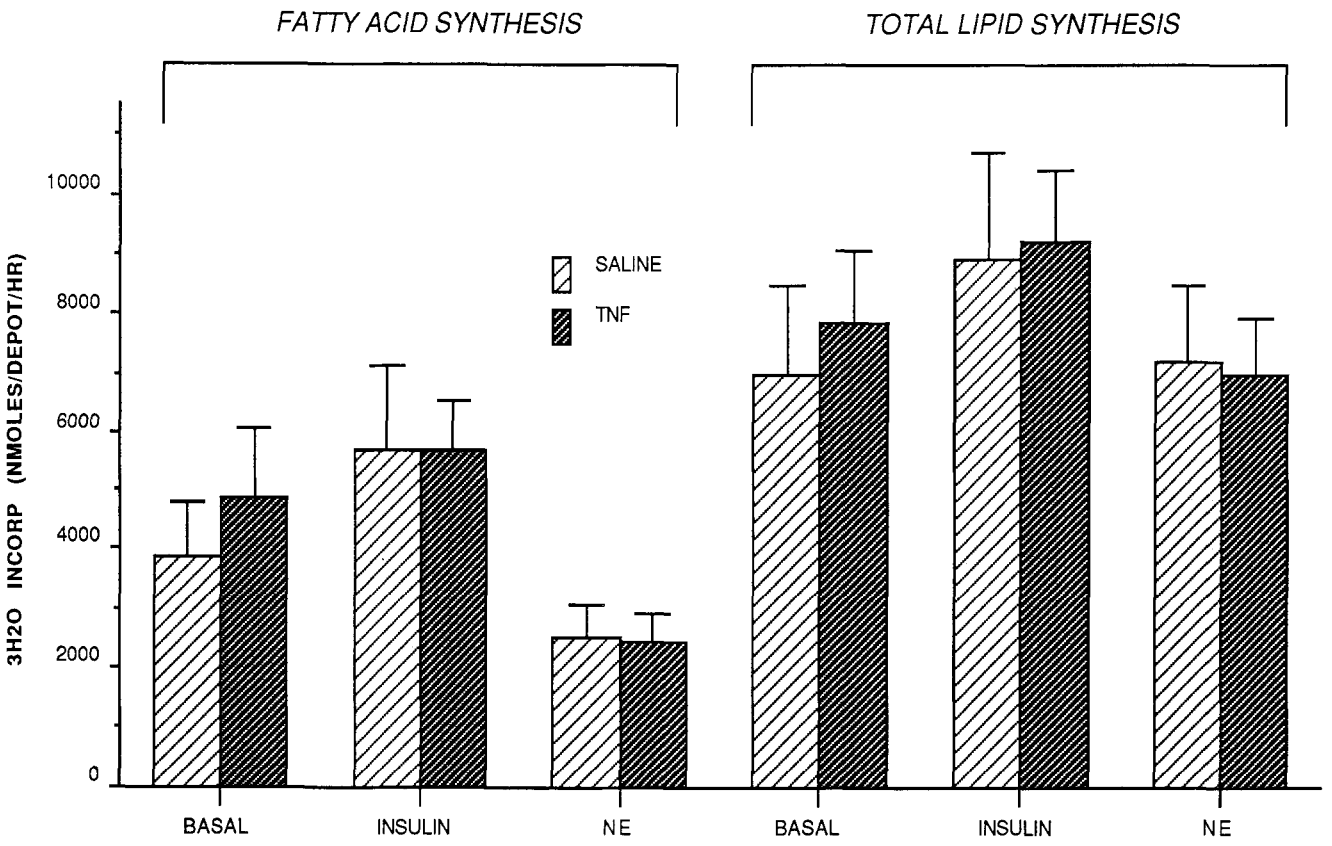

Figure 3. Triglyceride fatty acid and total lipid synthesis in inguinal pad of 5-day treated rats. Animals were injected subcutaneously daily at 5:30 PM with rTNF or saline. Fatty acid synthesis was measured in slices of adipose tissue using ^3H_2O as a tracer. n = 8 for saline-basal, n = 9 for saline-insulin, n = 9 for saline-norepinephrine (NE), n = 10 for TNF groups. Values are mean ± SE. There was no significant difference between treatments for any condition.

Overall, TNF appeared to increase FAS when given for 14 days but not for 5 days. In basal and insulin-stimulated states, TNF treatment enhanced triglyceride fatty acid synthesis. Synthesis of total lipids was elevated only in the insulin-stimulated state. With norepinephrine-inhibited lipogenesis, 14-day TNF treatment did not stimulate lipogenesis.

Hepatic synthesis of fatty acids and total lipids are presented in Figure 5. Five-day treatment had no significant effect on either fatty acid or total lipid synthesis. However, 14-day treatment depressed synthesis of total lipids with no effect on fatty acids.

Discussion

Tumor necrosis factor treatment for either 5 or 14 days did not affect body weight. Although the TNF treatment produced an initial depression of food intake, this effect was transient and did not affect body weight. These findings are in agreement with those reported by Patton *et al.* (24), who demonstrated that repeated daily injections suppressed feeding but tolerance to TNF developed in 3–5 days, normalizing food intakes and body weights. However, others (27, 33) report data demonstrating that TNF has a marked cachectic effect. These conflicting findings have been credited to differences in route of administration and dosage of the drug (34). It has been suggested that single daily administration of TNF may not be physiologic and that continuous infusion or at least two daily injections, preferably intraperitoneally, may be necessary to test its cachectic effects. However, Socher *et al.* (25) found only an initial cachectic effect in mice when TNF was given either twice daily (ip) or continuously infused. Others (26) found only a transient depression of body weight with TNF given intravenously. Cachectic effects have been demonstrated when the drug was tested either in the presence of a tumor (33) or with escalating doses above the LD_{50} of the drug (27). It is clear that, regardless of route of administration, TNF, in constant doses, does not induce a sustained cachexia or anorexia in healthy animals.

The dosage used in the present study was based on the work of Patton *et al.* (24) who examined rTNF's effects on the gastrointestinal tract in rats over a dose range of 0–0.70 mg/kg body wt. A maximal response, measured in increased stomach content, was reported at 0.25 mg/kg body wt, shown to be due to inhibition of gastric emptying. Severe gastrointestinal distress was also observed at this dose. Despite these initial physiologic effects of TNF, tolerance developed quickly. In-

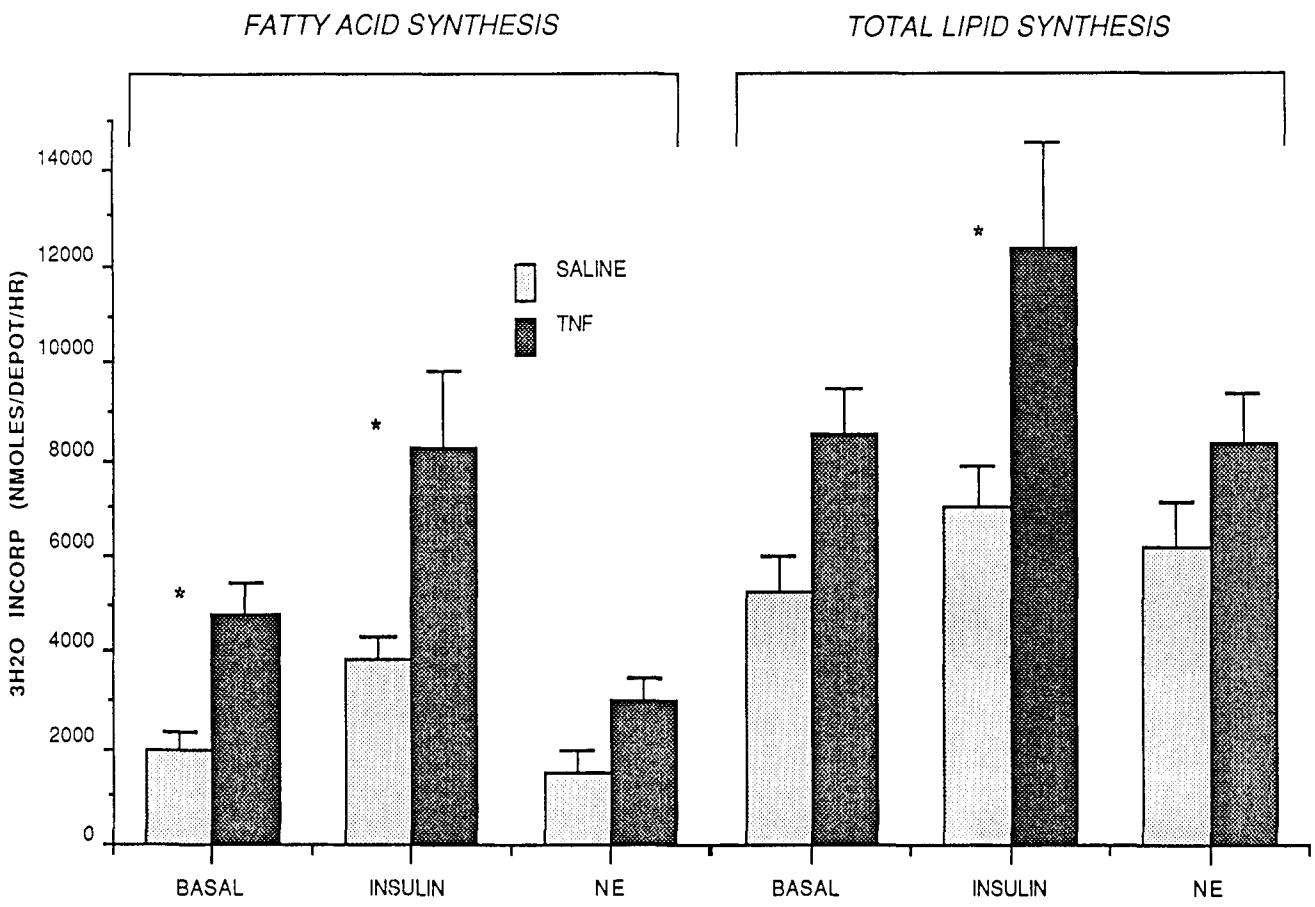

Figure 4. Triglyceride fatty acid and total lipid synthesis in inguinal pad of 14-day treated rats. See Figure 3 for protocol. $n = 10$ for all groups. Values are mean ± SE, $^*P < 0.05$.

jections given every 5th or 10th day produced a decrease in food intake with each injection, indicating tolerance develops within 24 hr and persists for 5–10 days.

It is possible that the tolerance developed may be due to formation of antibodies against rTNF, especially if species-specific TNF is not used. However, it has been shown that when human rTNF was injected (ip) into rats, antibody formation did not begin until the 10th day of injection (24). Thus, the resistance to cachexia observed in the present study for the 5-day treatment group is not likely due to antibody formation against the TNF. Others (35) confirm this effect of tolerance to TNF within the first days of injection. A more parsimonious explanation for the lack of sustained cachectic effect is that other factors are necessary to maintain the cachexia. Cachectic effects have been ascribed to TNF when the source of TNF was not pure, such as conditioned medium (23), or when other physiologic processes had been altered as well (33). Oliff *et al.* (33) observed a decrease in body fat, lean body mass, and food consumption in animals that are chronically infected with TNF-producing tumors. However, the presence of an invading cell line can induce numerous effects, only one of which is production of TNF. In point of fact, Oliff *et al.* (33) state that with daily injections of TNF they "were unable to document persistent weight changes" (unpublished data).

The necessity of other factors for full TNF activity may also extend to its cytotoxic effects. Recently, it has been shown that *in vivo* necrosis of solid Meth A tumors can be achieved either with high doses of rTNF (which result in extensive side effects) or by low doses of rTNF in combination with detoxified endotoxin and nontoxic poly(A:U), or submicrogram doses of toxic endotoxin (36). These low doses of TNF or the latter components are not cytotoxic if administered independently. *In vitro* tumor growth is not affected by toxic endotoxin, rTNF, or the combination, although it is depressed by murine tumor necrosis serum. In other tests of TNF's toxicity, Rothstein and Schreiber (37) reported that hemorrhagic necrosis and lethal shock in normal mice can be produced only in the presence of rTNF and bacterial products but not with TNF alone (except for pharmacologic doses). These effects are not additive, but rather suggest a synergism between TNF and other factors.

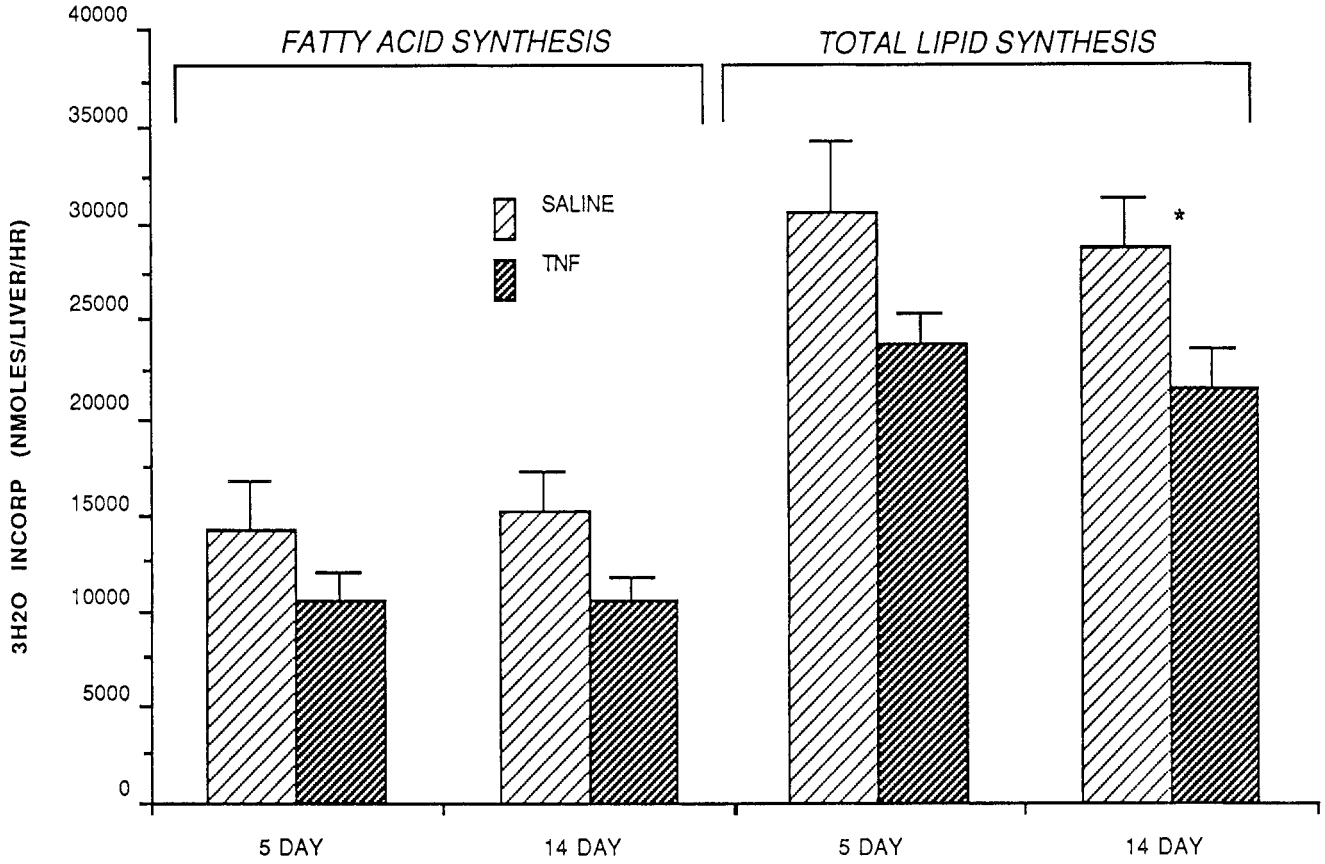

Figure 5. Triglyceride fatty acid and total lipid synthesis in liver of 5-day and 14-day treated rats. See Figure 3 for protocol. $n = 8$ for saline-5 day, $n = 9$ for saline-14 day, $n = 10$ for TNF groups. Values are mean ± SE, $^*P < 0.05$.

In the present experiment, TNF had no sustained effect on food intake when administered for either 5 or 14 days. Analysis of organ weights and carcass composition reflects the data on food intake. There were no differences due to TNF given for either 5 or 14 days in liver or inguinal tissue weights and a slight but nonsignificant depression of total body fat.

Our data from tissue incubations indicate that 14-day TNF treatment enhanced lipogenesis in the inguinal pad but depressed it in the liver. Furthermore, it appears that the TNF did not cause an insulin resistance as the insulin-induced lipogenesis was roughly the same in animals treated with TNF or saline. Within each treatment for adipose tissue, insulin generally stimulated and norepinephrine depressed lipid synthesis, indicating viability and responsiveness of the tissue. Although these alterations provide evidence of tissue-specific effects of TNF, they appear to balance each other and, hence, do not affect overall body weight or lipid stores. However, Feingold and Grunfeld (20) showed that a single injection of rTNF at a dosage approximately one half of ours into rats resulted in elevated hepatic sterol and lipid synthesis with no change in adipose tissue lipogenesis. Major differences in design between these studies may explain our disparate findings. Feingold and Grunfeld evaluated acute effects (1–17 hr after injection) while we examined chronic effects. In addition, their animals were fasted after injection, ours were not. The extended length of our study may have allowed for metabolic adjustments to TNF which would not be apparent in a short-term study.

The biologic half-life of TNF has been reported to be approximately 6 min (38). Thus, it may be argued that the single injections used in the present study were quickly inactivated. However, we did observe anorexia for the first 1–2 days of injection, hence the TNF was initially effective. This depression of food intake is possibly due to the severe gastrointestinal stress induced by TNF (24), an effect which would persist beyond the half-life of TNF. Since continuous infusions resulting in sustained TNF levels do not produce wasting (25), mechanisms other than inactivation of TNF must be responsible for the lack of cachexia.

It appears that in normal rats, chronic daily injections (up to 14 days) or recombinant human TNF at 0.25 mg/kg body wt does not result in a sustained cachectic response. It does not appear that higher doses

or more chronic administration of TNF may have produced more striking differences, as other studies (25) indicate that tolerance to TNF occurs with continuous infusion or elevated doses. The tolerance to TNF is not likely due to antibody formation (24), at least for the 5-day treatment group. We propose that other factors are necessary to mediate the lipid-depleting effects that have been observed in infection or with less purified preparations of TNF.

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