Activity, as estimated tumor weight of tumors from rats treated with 0.00001. rTNF treatment of TB rats also demonstrated antineoplastic and lost less weight than TB rats that had been treated with saline (P < 0.00001). The cachectic effects of tumor, and prolongation of survival. Tachyphylaxis or tolerance to the cachectic effects of rTNF that results in tolerance to the cachectic effects of rTNF with continued treatment. Following treatment, TB rats that had been treated with rTNF ate significantly more and lost less weight than TB rats that had been treated with saline (P < 0.00001). rTNF treatment of TB rats also demonstrated antineoplastic activity, as estimated tumor weight of tumors from rats treated with rTNF were significantly less than controls (P = 0.003). The antineoplastic and antineoplastic effects of rTNF resulted in prolonged survival of TB rats treated with rTNF compared to control TB rats (P = 0.015). Survival of TB rats treated with rTNF was again significantly greater (P < 0.00001) and delayed weight loss (P = 0.0001) posttreatment that was further augmented by additional doses of rTNF. Antineoplastic activity of rTNF was less clear, and overall tumor growth curves were not affected by rTNF treatment. Survival of TB rats treated with rTNF was again significantly increased in a dose-dependent manner (P = 0.006). Repeated administration of low doses of rTNF to TB rats induces mild reduction in tumor growth, tolerance to the cachectic effects of rTNF that results in tolerance to the cachectic effects of tumor, and prolongation of survival.

INTRODUCTION

Cancer cachexia remains a syndrome for which no effective treatment has been developed. The development of cachexia in neoplastic and other disease states often interferes with effective therapy and is a poor prognostic sign (1, 2). Although the etiology of cancer cachexia is probably multifactorial, one presumptive mediator may be cachectin/tumor necrosis factor. This trimeric, M, 34,000 macrophage protein product has been suggested to be a mediator of cachexia from neoplastic, parasitic, and infectious diseases (3, 4). However, these three separate entities have separate, distinct metabolic features, making it unlikely that TNF or any single mediator causes all the changes associated with each.

Previous investigation has suggested that the cachexia in a rat model of cancer is due to an unidentified, stable humoral factor (5). Utilizing the L929 bioassay in the same animal model, levels of serum cachectin activity were found to correlate directly with tumor burden and inversely with declining food intake (6). Furthermore, tumor resection resulted in disappearance of previously detectable serum levels of cachectin activity and complete reversal of the weight loss and food intake decrement (6, 7). Finally, recent work suggests that anticachectin/TNF antibodies can attenuate the development of cachexia in other animal models (8). Although these studies suggest that TNF may be a mediator of experimental cachexia in laboratory animals, there is little evidence to support any role for TNF as a mediator of clinical cachexia in cancer patients. In three separate studies to measure levels of TNF or TNF activity in the serum of patients with cancer and cachexia (9-11), only one study was able to detect any levels of circulating TNF in cancer patients (11), and in that study levels did not correlate with clinical parameters of cachexia (11). Additional studies have suggested that tolerance to the anorectic effects of rTNF can occur with repetitive daily i.p. doses (12). Moreover, animals made tolerant to rTNF and subsequently challenged with a methylcholanthrene-induced sarcoma survived significantly longer than control animals previously unexposed to TNF (12). Thus, the precise cause of cancer cachexia is unknown, but some experimental evidence (6, 8, 12) suggests that TNF may have a role.

To elucidate further the role of TNF in cancer cachexia and to develop potential treatment strategies for this ubiquitous, morbid syndrome, non-tumor-bearing and tumor-bearing rats were made tolerant to rTNF by repetitive twice daily escalating i.p. low-dose bolus injections. rTNF treatment of both NTB and TB rats resulted in reduced food intake and weight loss. However, TB and NTB rats rapidly became tolerant to these negative effects and began to increase food intake and gain weight despite continued treatment. In the initial experiment, low-dose TNF treatment significantly decreased tumor growth and resulted in a delayed onset of the declining food intake and weight loss seen with progressive tumor growth. TB rats made tolerant to TNF lived significantly longer than controls. In a repeat experiment, the mild antitumor effects of TNF were not seen, but the anticachexia effects of TNF tolerance were again demonstrated in a dose-dependent manner.

MATERIALS AND METHODS

General. Eighty-nine male Fischer 344 rats, initially weighing between 130 and 160 g, were used in this study. Animals were individually housed in an environment that controlled light, ambient temperature, and humidity. Rats were allowed free access to water and were fed a casein-based, semisynthetic, nonscantometric diet (C 21; ICN, Cleveland, OH), which allowed accurate measurement of food intake. Food intake and body weight were determined every other day prior to tumor cell inoculation and were thereafter measured on a daily basis. All animals...
were allowed a 7-day accommodation period prior to experimentation.

The tumor utilized in this study was a transplantable methylcholanthrene sarcoma. Animals were tumorxed in the subcutaneous space just dorsal and cephalad to the right hind limb. Tumor inoculation was performed under light ketamine anesthesia (25 mg/kg i.m.), and each TB animal received a sterile, single-cell suspension of 5 x 10^6 tumor cells in 1 ml of Hanks’ balanced salt solution. NTB rats received the same anesthesia and an injection of 1 ml Hanks’ balanced salt solution alone.

From the time that the tumor was first palpable (usually 6–7 days after inoculation), the tumor was measured in three orthogonal linear dimensions daily (experiment 1) or every other day (experiment 2). Tumor weight was estimated from these measurements and the final excised tumor weight (13). Host weight is taken as total body weight in non-tumor-bearing animals and is total body weight minus the measured or estimated tumor weight in tumor-bearing animals. Animals underwent postmortem tumor resection for accurate measurement of tumor size and weight.

Materials. rTNF (Cetus Corp., Emeryville, CA) was reconstituted with 1.2 ml of sterile phosphate-buffered saline and brought to final concentration in phosphate-buffered saline with 0.5% bovine serum albumin. Bioactivity was 27 x 10^6 units/mg, and endotoxin contamination was 0.07 ng/ml by the Limulus amebocyte assay (information provided by Cetus Corp.). Bovine serum albumin (Calbiochem, San Diego, CA) was purchased free of nuclease and proteases.

Experiment 1. Forty-two rats were randomly assigned to two groups: NTB (n = 20) and TB (n = 22). When tumors became palpable in TB rats, NTB and TB rats were randomized to receive either control vehicle or rTNF i.p. twice daily for 9 days. The randomization resulted in four groups: NTB-control (n = 10), NTB-rTNF (n = 10), TB-control (n = 11), and TB-rTNF (n = 11). The solutions were coded so that the person who performed the experiment did not know what each animal received until the end of the study, when the code was broken. rTNF dosages were escalated according to the following schedule: 25 µg/kg twice daily for 3 days, 50 µg/kg twice daily for 3 days, and 100 µg/kg twice daily for 3 days. Following the 9-day treatment period, rats were carefully followed for body weight, food intake, tumor growth, and survival. Each time a TB animal died, an animal from the corresponding NTB group was sacrificed.

Experiment 2. Forty-seven tumor-bearing rats were randomly assigned to one of three groups when tumors became palpable and treatment with rTNF or control vehicle was instituted: TB-control (n = 15), TB-rTNF standard therapy (n = 16), or TB-rTNF additional therapy (n = 16). Control TB rats received saline vehicle i.p. twice daily for 15 days. rTNF standard therapy TB rats received escalating rTNF treatment according to the following schedule: 2.5 µg/kg i.p. twice daily for 3 days, 50 µg/kg i.p. twice daily for 3 days, 100 µg/kg i.p. twice daily for 3 days, and normal saline i.p. twice daily for 3 days. rTNF additional therapy TB rats received rTNF according to the same schedule except that they received 100 µg/kg i.p. twice daily for the last 6 days of the 15-day treatment period. The solutions were coded so that the person who performed the experiment did not know what each animal received until the end of the study, when the code was broken. Body weight, food intake, tumor growth, and survival were measured.

Statistics. Survival analysis was performed utilizing the Breslow modification of the Kruskal-Wallis test (14). Analysis of body weight, food intake, and extrapolated tumor weight was performed using a multivariate repeated measures analysis of variance applied to those measurements within three study intervals: before treatment, during treatment, and following treatment. Univariate comparisons were made using Student’s t test.

RESULTS

Experiment 1

Food Intake. Food intake (Fig. 1a and b) was not significantly different in any group of rats during the accommodation period preceding tumor inoculation on day 0. NTB and TB rats each ate between 11 and 13 g/day. Inoculation of sarcoma or sham injection transiently decreased food intake, but values returned to baseline levels within 2 days. In NTB rats (Fig. 1a), rTNF treatment resulted in a sustained, significant decrease in food intake compared to saline-treated controls (P < 0.00001). Additionally, by the fourth day of rTNF treatment (day 10), NTB rats were eating significantly greater amounts than on days 1–3 of treatment (P < 0.001), even though the administered dosage of rTNF was increasing at this time. After treatment (day 16), food intake in NTB rats treated with rTNF rapidly returned to control levels. Food intake in TB rats (Fig. 1b) initially followed a pattern similar to that in NTB animals (Fig. 1a). A transient, minor decline in food intake was seen with tumor cell inoculation on day 0 followed by a marked decrease in food intake during rTNF treatment (day 6) (P < 0.00001) (Fig. 1b). Again, by day 4 of rTNF injections (day 10), TB rats were eating significantly greater amounts of food than on day 1 of rTNF treatment at a time when the rTNF dose was increasing (P < 0.001). On day 10 post tumor inoculation, both control TB rats and rTNF-treated TB rats began to decrease food intake in a parallel fashion secondary to the anorectic effects of the tumor. During the treatment period, rTNF-treated TB rats always ate significantly less than control TB rats (P < 0.0001). However, in the posttreatment period (beginning on day 16), rTNF-treated TB rats returned to normal food intake levels (11 g/day) at a time when control TB rats

Fig. 1. Food intake of non-tumor-bearing (a) and tumor-bearing (b) rats treated with rTNF in experiment 1. NTB rats were sham injected and TB rats were inoculated with tumor on day 0. rTNF or saline (control) was given i.p. twice daily between days 6 and 15. Data are mean ± SEM.
had demonstrated a clear reduction in food intake (7 g/day). rTNF-treated TB rats continued to have a significantly greater food intake throughout the posttreatment period until postinoculation day 27 (P < 0.00001) (Fig. 1b).

Body Weight. In both TB and NTB rats, daily weight gain was between 5 and 8 g/day prior to tumor or sham inoculation (day 0) and was not significantly different (Fig. 2, a and b). Tumor or sham inoculation caused a transient, minor decrease in weight gain that lasted 2 days. In both NTB and TB rats (Fig. 2, a and b), rTNF treatment resulted in a marked weight loss compared to saline-treated control animals after the first dose of rTNF (P = 0.00003) (day 6) that rapidly returned to levels similar to those in control animals (day 7–10) and then fell significantly again during the highest doses of rTNF (day 13 and 14, P < 0.00003). In NTB rats, the amount of weight gain was not significantly different during the posttreatment period (Fig. 2a). However, TB rats treated with rTNF gained weight in the posttreatment period, whereas control TB rats were losing weight (days 19–23; Fig. 2b). During the posttreatment period, rTNF-treated TB rats demonstrated a significant reduction in weight loss (P < 0.00001) until day 28, when the groups began to overlap (Fig. 2b).

Tumor Growth and Survival. Following the inoculation of tumor on day 0, estimated tumor weight progressively increased in rats treated with either saline or rTNF (Fig. 3a). However, rTNF treatment had an antitumor effect, as rats treated with it had a significant reduction in estimated tumor weight until postinoculation day 25 (P = 0.003) (Fig. 3a). In addition, TB rats treated with rTNF lived significantly longer than saline-treated controls (P = 0.003) (Fig. 4a).

Experiment 2

Food Intake and Body Weight. Food intake and body weight change followed trends similar to those seen in experiment 1 (Fig. 5, a and b). No significant differences were demonstrated during the accommodation period; that is, days −2 and −3 prior to tumor inoculation. Tumor inoculation (day 0) again produced a brief decline in both parameters which rapidly returned to baseline levels. During the treatment period (days 6–20), TB rats treated with both standard and additional therapy rTNF registered an initial significant decline in both parameters (P < 0.00001) when compared to controls (Fig. 5, a and b). In both rTNF treatment groups, food intake was significantly greater and weight loss significantly less by day 3 of the treatment period (day 9) than it was on treatment day 1 (day 6) (P < 0.00001; Fig. 5, a and b). Additionally, when analyzing the entire treatment period, TB rats that received the additional 3 days of rTNF therapy had significantly less food intake (P = 0.03) and significantly greater weight loss (P = 0.0004) than TB rats that received standard therapy.

Toward the end of treatment and following treatment period, food intake stabilized or increased for both groups of rTNF-treated TB rats, whereas control TB rats demonstrated a severe progressive decline in food intake (Fig. 5a). Both treatment
groups had significantly greater food intake than controls during the last 4 days of the treatment period and following the treatment period until experiment day 28 \( (P < 0.0001) \). The group of TB rats receiving additional therapy with rTNF had significantly greater food intake during this period than rats receiving standard rTNF therapy \( (P < 0.00001) \) (Fig. 5a). In addition, the weight loss of both treatment groups in the post-treatment period was significantly less than that of controls from experimental day 20 until day 28 \( (P < 0.00001) \) (Fig. 5b). Moreover, TB rats receiving additional therapy with rTNF lost significantly less weight than rats receiving standard therapy (days 21–30; Fig. 5b) \( (P = 0.005) \).

Tumor Growth and Survival. Unlike results in the previous experiment, tumor growth rate was not apparently affected by rTNF treatment (Fig. 3b). There were some significant decreases in estimated tumor weight in the rTNF additional therapy group at selected days \( (P = 0.009) \) but not in the standard rTNF-treated rats. In the posttreatment period, there were no apparent differences in estimated tumor weight in any group (Fig. 3b).

Despite the apparent lack of effect on tumor growth, survival was significantly increased by rTNF treatment of TB rats (Fig. 4b). Mean survival for control TB rats was 29 days, standard rTNF therapy increased mean survival to 32 days \( (P = 0.012 \) versus control TB rats), and additional rTNF therapy increased mean survival to 36 days \( (P = 0.0001 \) versus control TB rats). TB rats receiving additional rTNF therapy also had significantly prolonged survival compared to TB rats treated with standard therapy \( (P = 0.006) \) (Fig. 4b).

**DISCUSSION**

Cancer cachexia is a multifactorial syndrome that essentially represents the failure of the tumor-bearing host to respond to net energy loss by increasing food intake. Although usually thought of as a late manifestation of malignancy, it may be present prior to clinical detection of malignancy (15, 16). Since cachexia has negative impact on cancer therapy and it is estimated that two-thirds of cancer patients die solely from progressive wasting of host tissue (1, 17), delay or reversal of this syndrome may have clinical significance. Evidence for hormonal mediation of cachexia is largely indirect; however, TNF has been implicated as a candidate (18). The precise role of TNF in the genesis and maintenance of cancer cachexia is not totally defined. Nevertheless, TNF has been shown to induce a wasting syndrome which parallels cachexia (19, 20), produce hyperlipidemia and prevent storage and synthesis of lipids, and cause pronounced weight loss and anorexia in animal models (20–23) which can be prevented by anti-TNF antibodies (8, 24).

Tolerance to the anorectic effects of rTNF has been demonstrated in several laboratory models in which intermittent bolus doses have been administered (12, 23, 25–28). In the current
study, tolerance to the anorectic effects of TNF developed in both NTB and TB animals at approximately the same time in therapy and with similar magnitude (Fig. 1, a and b). Several points should be noted. In NTB rats, tolerance to TNF does not appear to have an adverse effect, since there were no treatment deaths and treated animals recover food intake and weight gain after treatment (Figs. 1a and 2a). Tolerance may be beneficial to the TB host because it appears to enable treated animals to survive longer than controls (Fig. 4). However, tolerance to the anorectic and toxic effects of TNF is transient, and that may explain why the development of cachexia could only be delayed and not prevented. It is interesting to note that the beneficial effect of rTNF in TB rats appears to be dose related. TB rats treated with 15 days of rTNF therapy had significantly greater survival and food intake as well as less weight loss than rats treated with only 12 days of therapy. It may be possible that different treatment regimens with more prolonged therapy may further delay the onset of cachexia or prevent this syndrome entirely. In this initial study, the doses were chosen to avoid the hypersensitivity and increased lethality of rTNF in TB animals reported by others (29). Since in these experiments TB rats were not hypersensitive to rTNF, future studies may safely use greater dosages and more prolonged treatment to demonstrate a more significant benefit.

The mechanism of rTNF tolerance is not well understood. Tolerance to the anorectic effects of rTNF also protects rodents from the lethal effects of rTNF, endotoxin, and a septic challenge (25, 30–32). The detrimental effects of TNF in some disease states may be related to hydroxyl radical generation and direct membrane damage (33, 34). Recent investigation has suggested that resistance to the cytolytic effect of rTNF may be due to augmented production of glutathione and other free radical scavengers (35–37). Thus, intermittent rTNF exposure may result in increased ability to buffer oxidative challenges, not only by rTNF, but by other cytokines and inflammatory mediators as well. An additional, perhaps related, possible mechanism is down-regulation of TNF receptors or changes in receptor affinity such that effector cell toxicity is reduced (38, 39). Interestingly, tachyphylaxis or tolerance to TNF does not appear to occur during continuous exposure to this cytokine (20, 40).

In the current study, rTNF treatment not only produced tolerance to the anorectic effects of rTNF, but in experiment 1, it also appeared to diminish tumor growth. Estimated tumor weight in experiment 1 was significantly reduced in rats treated with rTNF (Fig. 3a). A similar finding did not occur in experiment 2, in which tumor growth rate did not appear to be affected by rTNF treatment (Fig. 3b). However, there was a clear dose-related survival benefit associated with rTNF treatment of TB rats in both experiments (Fig. 4, a and b). What is not clear is the mechanism of the prolongation of survival by rTNF. Is it antitumor effects or is it anticachexia effects of rTNF treatment that induced tolerance to endogenous host or tumor-produced TNF? We suggest that the prolongation of survival induced by repetitive exposure of TB rats to rTNF is not due to antitumor effects of rTNF but rather due to anti-
cachexia effects. Tumor growth was only affected in one experiment. The onset of cachexia (both weight loss and anorexia) was significantly postponed in both experiments in a dose-dependent manner. We have previously treated rats with this sarcoma with even higher doses of rTNF in different regimens and have had minimal antitumor effects (data not shown). We have previously correlated the activity levels of TNF in the serum of TB rats with parameters of cachexia and have demonstrated the disappearance of these levels with resection of the tumor and the disappearance of cachexia (6). In addition, tolerance to the anorectic effects of rTNF induced in NTB rats by repetitive exposure to i.p. boluses of rTNF in a regimen similar to the regimen used here allowed the delayed onset of anorexia and the prolongation of survival when these rats were transplanted with this same sarcoma (12). The current experiment extends this observation to TB rats; that is, TB rats can be made tolerant to rTNF, and this tolerance prolongs survival and delays the onset of cachexia with some minor antitumor effects. Thus, rTNF treatment may be beneficial by its potential antineoplastic and anticachectic effects.

Future investigation for anticachexia therapy should involve combination therapy with rTNF and insulin. Several reports from our laboratory, using the same tumor model, have shown that insulin can partially reverse the anorexia, weight loss, and compositional changes of cancer cachexia (41, 42). In addition, recent work documents that it reverses many of the toxicities induced by cachectin/TNF (43). Since rTNF treatment is able to induce a delay in the cachectic effects of the tumor, it may be that insulin therapy combined with rTNF will have synergistic anticachexia activity and further ameliorate cachexia and prolong survival.

REFERENCES


Prolonged Survival of Tumor-bearing Rats with Repetitive Low-Dose Recombinant Tumor Necrosis Factor


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