Cachectin/Tumor Necrosis Factor: A Possible Mediator of Cancer Anorexia in the Rat

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ABSTRACT

Cachectin/tumor necrosis factor (TNF) is a macrophage product which may have a role in cancer cachexia. Recombinant human cachectin/TNF (Cetus Corporation) was administered i.p. twice daily to male F344 rats at varying, nonlethal dosages for either 5 or 10 days, and daily rat food intake and body weight were measured. There was a dose-dependent cachectin/TNF-induced decline in food intake and body weight gain over the treatment period. However, after 1 day rats became tolerant to these effects and increased food intake and gained body weight despite receiving cachectin/TNF. Rats were subsequently inoculated with a transplantable methylcholanthrene-induced sarcoma, and survival was measured. Rats previously treated with high-dose (either 100 or 200 µg/kg/day) cachectin/TNF survived significantly longer following tumor inoculation than did control rats given saline or rats given 10 µg/kg/day of cachectin/TNF. Analysis of tumor growth curves and tumor weight indicated that high-dose cachectin pretreatment did not retard tumor growth. Analysis of food intake and tumor burden following tumor inoculation indicated that high-dose cachectin pretreatment decreased the reduction in food intake associated with progressive tumor growth and allowed rats to withstand a greater tumor burden at death. Rats immunized with low-dose human cachectin/TNF developed high IgG titers against human TNF, but failed to demonstrate the same protection against a methylcholanthrene-induced tumor challenge as rats made tolerant with repetitive twice daily high-dose cachectin/TNF. The observation of reduced cancer-associated anorexia and increased survival of tumor-bearing rats associated with previous tolerance to exogenous cachectin/TNF strengthens the contention that endogenously produced cachectin may be a factor in the pathogenesis of cancer anorexia in the tumor-bearing rat. The mechanism of this tolerance is unclear but does not appear to be a humoral immune response.

INTRODUCTION

Cancer cachexia includes anorexia and host tissue wasting. Its exact cause is unknown, but it is assumed to be caused by unidentified humoral factors (1). Previous work from our laboratory demonstrated that the anorexia and cachexia of rats bearing a methylcholanthrene-induced sarcoma were transferable across a parabiotic anastomosis (2). The results suggested that, in this rat tumor experimental model, cancer-associated cachexia is mediated via the circulation (2).

Cachexia may be caused by substances produced directly by tumors which enter the circulation and have distant effects on the host, substances like 1.2-toxohormone (3), bombesin (4), or a recently identified lipolytic and proteolytic factor produced by a murine colon cancer (5). Alternatively, cancer cachexia may be caused by a host response to a tumor which is an attempt to kill the tumor, but subsequently proves to be ineffective (6).

Cachectin/TNF has been suggested as a mediator of cachexia associated with infectious or neoplastic diseases (7, 8).

It appears that cachectin/TNF is clearly related to the pathophysiology of endotoxin and septic shock (9, 10); however, its role in cancer cachexia is less clear. Adakta et al. demonstrated cytotoxic activity consistent with TNF activity spontaneously produced in vitro by peripheral blood mononuclear cells from cancer patients (11), but Scuderi et al. failed to detect elevated serum levels of TNF in patients with neoplastic diseases (12). However, a recent study by Balkwill et al. measured circulating cachectin/TNF activity in 50% of cancer patients, but found no correlation between serum levels and clinical evidence of cachexia (13).

In these experiments, we examined the effects of repeated, nonlethal, i.p. doses of cachectin/TNF on the food intake and body weight of rats. Cachectin/TNF clearly had a dose-dependent negative impact on rat food intake and body weight, but rats became tolerant to the repeated i.p. administration of cachectin/TNF in the doses used and continued to eat food and gain weight despite cachectin/TNF administration. We then inoculated these rats with tumor to determine whether tolerance to cachectin/TNF's effects on food intake and body weight might protect against the anorectic effects of the tumor and prolong TB rat survival. Finally, we immunized rats against human cachectin/TNF to determine if antibodies against human cachectin/TNF might protect against TB anorexia. The prime interest for all these experiments was to examine whether the peptide cachectin/TNF might have a role in the pathophysiology of cancer anorexia in this experimental rat tumor model, in which we believe it is produced by an unidentified circulating factor (2).

MATERIALS AND METHODS

General. The experiments were performed on male Fischer 344 rats initially weighing between 150 and 200 g. In all experiments animals were individually housed in wire-bottomed cages within a temperature- and humidity-controlled room. A 12-h light/dark cycle was maintained. Rats were fed a casein-based, semisynthetic diet (C-21; ICN Nutritional Biochemicals, Cleveland, OH). C-21 is a nonscoratable paste diet which allows accurate measurement of daily food intake (14). It contains all the essential nutrients for normal rat growth and development. Rats in each experiment were allowed water ad libitum.

The tumor was a transplantable MCA-induced sarcoma. The tumor was transplanted in the s.c. space by a sterile viable single cell suspension under i.p. pentobarbital (35 mg/kg) anesthesia. The sarcoma grows uniformly, and the natural history has been previously reported (15). It is locally invasive, does not metastasize, and kills its host 30 to 35 days following transplantation. Three orthogonal linear dimensions of the tumor were measured with calipers every other day from the time the tumor was first palpable. Tumor size was calculated by multiplying these three measurements together. At death, total body weight was recorded, and tumor was dissected free from the rat and weighed. Host weight was also recorded which equals total body weight minus excised tumor weight. Host weight equals total body weight in a NTB animal.

Cachectin/tumor necrosis factor was human recombinant cachectin kindly provided by Cetus Corporation (Emeryville, CA). Cachectin/TNF had a specific activity of 10³ units/mg as measured in the L929 assay, and the endotoxin level in the preparation was 0.18 pg/µg of TNF as measured in a standard Limulus amebocyte lysate assay.
Cachectin was administered i.p. to rats in the stated dosages, except in Experiment 4, it was given i.d. Control animals received similar volumes of saline. No anesthesia was used for these injections.

Experiment 1. Twenty-seven rats were acclimated to C-21 diet and individual cages. They were then randomized by a random number method equally into 3 groups (n = 9): cachectin/TNF at dosages of either 50 µg/kg (100 µg/kg daily) or 5 µg/kg (10 µg/kg daily) i.p. twice daily for 5 consecutive days; or a twice daily i.p. saline treatment. Food intake and body weight were measured daily during the treatment period. Seven days after the treatment period, the animals were each given 2.5 x 10^6 viable MCA sarcoma cells s.c., and subsequent survival was measured. Tumor size and food intake were also measured during tumor growth.

Experiment 2. This experiment was designed to answer a question raised by Experiment 1. Do rats become tolerant to the anorectic and cachetic effects of cachectin/TNF following repeated i.p. administration? Thirty-six male F344 rats were acclimated to C-21 diet and individual cages and randomized equally into 4 groups (n = 9): cachectin/TNF, 100 µg/kg i.p. every 12 h (200 µg/kg/day) for 10 days; cachectin/TNF, 50 µg/kg i.p. every 12 h (100 µg/kg/day) for 10 days; cachectin/TNF, 5 µg/kg i.p. every 12 h (10 µg/kg/day) for 10 days; and saline at similar volumes. Daily food intake and body weight were measured during the 10-day treatment period.Cumulative food intake and body weight change during the 10-day treatment period were calculated.

Experiment 3. This experiment was also designed to answer a question raised by Experiment 1, does cachectin/TNF pretreatment improve survival of rats subsequently inoculated with tumor? Fifty rats were acclimated to C-21 diet and individual cages and randomized equally into 2 groups (n = 25): saline i.p. every 12 h for 10 days or cachectin/TNF, 100 µg/kg i.p. every 12 h (200 µg/kg/day) for 10 days. Eight days later rats were each given 10^7 MCA sarcoma cells s.c. Two rats did not develop tumor (one in each group) and were excluded from the analysis. Survival was measured, and at death tumor weight, host weight, and tumor burden (tumor weight/total body weight) were recorded for each rat.

Experiment 4. This experiment asked whether the protective effects of cachectin/TNF pretreatment on TB rats were mediated via the humoral immune response. Thirty-five rats were again housed individually and acclimatized to diet and surroundings. One group of rats (n = 15) was immunized to human recombinant cachectin/TNF in a standard manner. On Day 0, rats under pentobarbital anesthesia were given 20 µg/kg of cachectin/TNF per rat in a 1:1 mixture with Freund's complete adjuvant (Difco) in 5 separate i.d. sites on the back of the rat. At four 1-wk intervals (Days 7, 14, 21, and 28), these rats were boosted with injections of 20 µg/kg of cachectin/TNF in incomplete Freund's adjuvant in a similar fashion. The control group (n = 20) received injections of saline in a similar fashion at the above time points. On Day 15, all rats from both groups were inoculated with 2.5 x 10^6 MCA sarcoma cells s.c. as described above. On Day 30, blood was obtained by retroorbital puncture and assayed for anti-human cachectin/TNF antibodies as described below. Food intake, tumor size, and survival were followed.

ELISA for Rat Anti-Human Cachectin/TNF Antibodies. Recombinant human cachectin/TNF was diluted to 10 µg/ml in 0.05 M sodium carbonate buffer, pH 9.6, and 100 µl per well were applied to 96-well Immulon Microelisa plates (Dynatech, Alexandria, VA) and kept at 4°C for 16 h. The plates were washed 3 times with phosphate-buffered saline/0.05% Tween-20 and blocked for 60 min with phosphate-buffered saline/1% bovine serum albumin/0.05% Tween-20 blocking solution. The plates were washed, and 200 µl of serial dilutions of rat sera from Experiment 4 were added in triplicate for 120 min. The plates were washed, and 200 µl of a 1/1000 dilution of goat anti-rat IgG alkaline phosphatase (Sigma) were added to each well for 120 min. The plates were washed, and 175 µl of 1 mg/ml of p-nitrophenyl phosphate (Sigma) were added. After 45 min the reaction was stopped with 50 µl of 3 M NaOH, and the plates were counted at 405 nm in a Microelisa reader (Dynatech). Results are expressed as the dilution of serum that gave 50% maximal absorbance.

Statistics. Data are presented as the mean ± SEM. Parametric results are analyzed by Student's independent t tests, and nonparametric results are analyzed by the Wilcoxon rank sum test. Survival curves are plotted as Kaplan-Meier curves and are compared by Breslow analysis.

RESULTS

Experiment 1. Prior to the experimental period, all rats were consuming similar amounts of food daily (12 to 15 g; Fig. 1A). Cachectin/TNF injection significantly lowered rat food intake only on Day 1 in the 10-µg/kg/day group and on Days 1, 2, 3, and 4 in the 100-µg/kg/day group. Saline administration only affected food intake on Day 1 (Fig. 1A). Despite the repeated daily administration of 100 µg/kg i.p. of cachectin/TNF, rats ate near normal food amounts on Day 5 (Fig. 1A). Following the experimental period, all rats rapidly returned to normal food intake (12 to 15 g); there was no compensatory hyperphagia seen in cachectin/TNF-treated rats.
Prior to the experimental period, all rats were gaining weight at an amount of 5 g/day (Fig. 1B). Saline injections initially dropped daily weight change to 0, and subsequently rats gained 2 to 4 g/day despite saline administration. Animals given low-dose cachectin/TNF (10 µg/kg/day) lost weight (7 g) on Day 1, but subsequently gained similar weight to saline controls (Fig. 1B). Animals given high-dose cachectin/TNF (100 µg/kg/day) lost the most weight on Day 1, but were able to gain similar amounts of weight to saline-treated control rats on Days 3, 4, and 5 despite the continued administration of cachectin/TNF (Fig. 1B).

These animals were given tumor 7 days later, and subsequent survival was measured. Rats pretreated with either saline or 10 µg/kg daily of TNF/cachectin had similar survival, but rats pretreated with high-dose (100 µg/kg/day) cachectin/TNF lived significantly longer than both other groups (Fig. 2A). When one compared tumor growth in the rats pretreated with 100 µg/kg/day of cachectin/TNF to rats pretreated with saline, there was no significant difference in tumor growth (Fig. 2B). But when one compared the post-tumor inoculation food intake in these two groups, one detected significant differences. From post-tumor inoculation Day 20 onward, rats pretreated with 100 µg/kg/day of cachectin/TNF ate significantly more food than rats pretreated with saline (Fig. 2C).

Experiment 2. Saline treatment again had little effect on food intake of NTB rats. Cachectin/TNF appeared to have a dose-dependent effect on food intake of NTB rats (Fig. 3). Tolerance to the cachectin/TNF effects on food intake was clearly demonstrated. At each daily dose (10 µg/kg, 100 µg/kg, and 200 µg/kg), food intake significantly increased and returned toward normal levels despite the continued administration of cachectin/TNF. Despite a demonstration of tolerance to the repeated administration of i.p. cachectin/TNF (Fig. 3), cumulative food intake and body weight change during the 10-day treatment period significantly decrease as the daily dose of cachectin increased (Table 1). These results clearly demonstrate that cachectin/TNF is capable of reducing food intake and body weight in a dose-dependent manner.

Experiment 3. Rats pretreated with 200 µg/kg/day of cachectin/TNF in a similar manner to Experiment 2 lived significantly longer following tumor inoculation (P < 0.001) than saline-pretreated controls. Median survival for saline control rats was 29 days versus 36 days for cachectin-pretreated rats (Fig. 4). Similar to Experiment 1, retarded tumor growth did not appear to be the reason why cachectin/TNF-pretreated rats lived longer. In fact, at death, tumors weighed significantly more in cachectin/TNF-pretreated rats (Table 2). Host weight at death was not significantly changed by cachectin/TNF pretreatment. However, tumor burden at death was significantly greater in rats pretreated with cachectin, indicating that these rats tolerated a larger tumor burden.

Experiment 4. Rats immunized with low-dose cachectin/TNF in Freund’s complete adjuvant, and then boosted weekly for 4 wk with cachectin/TNF in incomplete Freund’s adjuvant developed a marked IgG response as determined by ELISA assay (Table 3). The mean titer which gave one-half maximal absorbance in immune animals was approximately 1:12,800. Control animals demonstrated no antibody response at 1:25 serum dilution, the lowest titer tested.

However, immunizing the rats with low-dose human cachectin/TNF at weekly intervals did not alter the response to a tumor challenge. Rats immunized with cachectin/TNF showed no difference in tumor growth compared to nonimmune control rats (Fig. 5A). Also these immune rats declined their food intake in response to increasing tumor burden at the same rate as control animals (Fig. 5B). The mean overall survival following tumor inoculation in the control group was 38.1 ± 1.0 days which was not statistically different from 39.9 ± 1.2 days in the immune group. The median survival in the control group was 39 days compared to 40 days in the immune group.

DISCUSSION

Cachectin/TNF is a macrophage-produced peptide originally isolated in the course of studies attempting to delineate basic mechanisms of cachexia in chronic diseases (16). It is called a hormone because it binds by high-affinity receptors to adipocytes and myoblasts (17) as well as malignant cells (18).
CACHETIN AND CANCER ANOREXIA

Fig. 3. Effect of cachectin on food intake in NTB rats. Cachectin administration began on Day 8 and continued through Day 17. Initially, all cachectin-treated groups showed a significant decline in food intake compared to saline-treated controls (P < 0.001). Despite continued cachectin treatment, all groups approached food intake similar to saline-treated controls in a dose-dependent manner. mcg/kg/d, µg/kg/day. Points, mean; bars, SEM.

Table 1 Cumulative food intake and body weight change during 10-day treatment period

Comparisons are made by the nonparametric Wilcoxon rank sum test. Numbers for food intake and body weight change of cachectin-treated rats are all significantly different from each other and saline-treated controls (P < 0.005).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Food intake (g)</th>
<th>Body wt change (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>126 ± 5</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>Cachectin/TNF, 10 µg/kg/day</td>
<td>111 ± 7</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Cachectin/TNF, 100 µg/kg/day</td>
<td>90 ± 5</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Cachectin/TNF, 200 µg/kg/day</td>
<td>75 ± 5</td>
<td>-7 ± 2</td>
</tr>
</tbody>
</table>

* Mean ± SEM.

This current study examines the effects of recombinant human cachectin/TNF on food intake. Cachectin/TNF is a pre-served molecule across species: it has been noted that the aminoterminal sequence of mouse cachectin is strongly homologous to that reported for human cachectin/TNF (19). In initial work by Cerami et al., nonlethal i.p. bolus doses of crude purified native cachectin obtained from endotoxin-treated macrophages depressed food intake, but after 3 days of administration mice began to return to normal food intake (20). Similar tolerance to repeated administration of recombinant human Genentech TNF has also been shown in rats by Patton et al. (21). These earlier observations closely parallel our current observation with recombinant human cachectin/TNF in rats (Figs. 1A and 3); that is, after a clear dose-dependent depression in food intake (Table 1), rats become resistant (or tolerant) to the negative effect.
effects of the peptide on food intake. This tolerance begins to occur early after only 1 day of administration.

Tolerance to cachectin's toxic effects was observed in these studies in which the cachectin was administered intermittently. However, other work using a constant i.v. infusion of a different brand of human recombinant cachectin in rats did not demonstrate tolerance to its effect on food intake. In fact, the continuous i.v. infusion of Asahi cachectin/TNF resulted in a progressive decline in food intake and death of the animal (22). Oliff et al. recently inserted the human cachectin/TNF gene into a mammalian expression vector and transfected hamster ovary cells to produce a tumor which continuously secreted cachectin/TNF. Nude mice bearing this cachectin/TNF-secreting tumor progressively declined food intake and developed severe body wasting consistent with cancer cachexia (23). Differences between intermittent bolus i.p. administration of cachectin/TNF and continuous secretion or infusion of the molecule might include absorption of TNF. Perhaps repeated i.p. injections of cachectin/TNF have decreased activity on food intake because the administered amount of the peptide is not absorbed into the circulation. To refute this hypothesis, we studied whether repeated i.p. administration of cachectin/TNF and tolerance to its effects on food intake altered uptake or clearance of a subsequent i.p. dose of cachectin/TNF versus control animals. Serum levels of cachectin/TNF activity following i.p. administration were the same between control rats and rats made tolerant by repetitive i.p. injections indicating that absorption into the circulation was not different (24). Tolerance to intermittent bolus administration of cachectin/TNF is also reflected by body weight. Although body weight gain is reduced by repeated i.p. administration of cachectin/TNF (Table 1), after 1 day rats are able to gain weight despite repeated i.p. bolus administration of cachectin (Fig. 1B). This is markedly different from chronic secretion of escalating doses of cachectin by a transplanted tumor which produces extremely high blood levels of TNF and causes progressive reduction in food intake, loss of body weight, and death (23).

The surprising observation made here is that, when rats were made tolerant to cachectin/TNF's effects on food intake and body weight and were subsequently inoculated with a tumor, they lived significantly longer than controls (Figs. 2A and 4). The dose of i.p. cachectin/TNF pretreatment required to observe this effect was either 100 µg/kg/day or 200 µg/kg/day for 5 to 10 days. There was no apparent difference between 10-day pretreatment with cachectin/TNF compared to 5-day pretreatment, nor was there a difference between 200 µg/kg/day compared to 100 µg/kg/day. However, it was apparent that 10-µg/kg/day pretreatment did not affect survival following tumor challenge. Pretreatment with cachectin/TNF did not reduce subsequent tumor growth, which might account for improved survival (Fig. 2B: Table 2). Instead it reduced and postponed the anorectic effects of the tumor on the host. At 20 days following tumor inoculation, when control rats were declining food intake, cachectin/TNF-pretreated rats were eating significantly more food (Fig. 2C). This ability to eat despite a growing tumor mass was also reflected by a greater ratio of tumor weight to total body weight or tumor burden. Rats pretreated with high-dose cachectin/TNF had a greater tumor burden at death (Table 2), which is another indication that they tolerated their tumor better.

The mechanism of this tolerance to cachectin/TNF is unclear. Several observations demonstrate that the mechanism is not a humoral immune response. (a) Rats immunized against human recombinant cachectin/TNF are not protected from a challenge with tumor despite high titers of anti-human cachectin/TNF IgG antibodies. (b) Rats made tolerant by 100 to 200 µg/kg/day of cachectin/TNF i.p. for 5 to 10 days in a manner that improves survival to tumor challenge have a minimal antibody response to cachectin/TNF with titers less than 1:100 (data not shown). (c) The kinetics of the tolerance response in terms of the rate of recovery of food intake and body weight change in only 2 days during cachectin administration does not follow the time course expected with a primary humoral immune response.

The inability of high titers of anti-human cachectin/TNF antibodies to protect against cancer anorexia does not imply that endogenous rat cachectin has no role in the etiology of cancer anorexia. Antibodies may be ineffective across species barriers. Investigators have demonstrated that cachectin/TNF from the human and the mouse has highly variable effects on target cells from different species (25). Others have shown that neutralizing antibodies against human cachectin were ineffective against murine cachectin and vice versa (26). We have observed that a sensitive radioimmunoassay for human cachectin does not detect rat cachectin in serum which has high levels of cachectin bioactivity.

This study suggests that cachectin/TNF plays a role in the pathophysiologic effect of cancer anorexia in the TB rat. The idea of tolerance or resistance to the detrimental effects of intermittent cachectin/TNF on food intake and body weight was observed in this study and by others recently (21). Tolerance to the toxic effects of cachectin/TNF is reproducible, but its mechanism is unknown. The mechanism may be mediated via down regulation of cellular receptors which are known to be present on most tissues (16), or by modulation of a related immune or cytokine response (like interleukin-1 or interferon) by repeated treatment with cachectin/TNF, or by a TNF-induced increased metabolic state which increases animal resistance. Tolerance to the effects of cachectin/TNF on food intake and body weight translated to tolerance to the anorectic effects of tumor and prolonged survival. This observation implies that cachectin/TNF is a mediator of the anorexia seen in the sarcoma-bearing rat. Future studies to detect circulating cachectin/TNF levels and using antibodies to rat cachectin/TNF to reverse cachexia in the TB rat will be necessary to clearly document its presence and activity.

REFERENCES

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