CACHETIN/TUMOR NECROSIS FACTOR INDUCES
CACHEXIA, ANEMIA, AND INFLAMMATION

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Severe weight loss and debilitative wasting of lean body mass frequently
complicate the treatment of patients suffering from malignancy or chronic
infection. Termed cachexia, this syndrome of anorexia, anemia, and weakness
further increases cancer mortality; some data indicate that as many as 30% of
cancer patients die from cachexia, rather than tumor burden (1-3). The severity
of cachexia may be unrelated to tumor size or parasite load, and profound
wasting has been observed in patients with tumor burdens of only 0.01-5.0%
body mass (4). If not reversed, cachexia-associated derangements of homeostasis
lead to immunological deficiencies, organ failure, and multiple metabolic abnor-
malities. While it is clear that a variety of mechanisms participate in the patho-
genesis of cachexia, and that cachexia adversely affects prognosis, the etiology
of this syndrome is not known.

For a number of years we have been searching for endogenous, humoral
mediators of cachexia, beginning with the characterization of metabolic changes
in trypanosome-infected rabbits that develop profound cachexia and lose up to
50% of lean body mass within weeks. In later stages of disease a paradoxical
increase in circulating triglycerides occurs, attributable to systemic suppression
of lipoprotein lipase (LPL) (5). A bacterial LPS-inducible serum factor which
suppresses LPL in mice, and several other key lipogenic enzymes in the adipocyte
cell line 3T3-L1, was isolated and named cachectin (6, 7). Cachectin evokes a
state of cellular cachexia by suppressing the expression of several mRNAs
encoding essential lipogenic enzymes (8, 9). Myocytes also show changes in
cellular metabolism after exposure to cachectin in vitro, including a prompt
decrease in resting transmembrane potential difference and depletion of intra-
cellular glycogen stores with increased lactate efflux, and a later increase in
hexose transporters (10, 11). It has been suggested that cachectin may play a
Central role in cachexia, and the administration of conditioned medium containing cachectin from LPS-induced macrophages induces anorexia and weight loss in mice (12).

Independent genetic sequence analysis subsequently proved the identity of cachectin and tumor necrosis factor (TNF) (13-15). The availability of recombinant human cachectin allows the spectrum of biological responses attributable to this hormone to be characterized in detail. High doses of cachectin are extremely toxic, and acute intravenous administration induces catabolic hormone release, tissue injury, and fatal shock (16, 17). Moreover, anticachectin antibodies can passively protect animals against shock and death during endotoxemia (18) or Escherichia coli bacteremia (19), suggesting that cachectin is the principal mediator of acute responses to Gram-negative bacterial invasion. Recent evidence suggests that chronic low-dose treatment with cachectin is also toxic, causing anorexia and weight loss (20). Cachectin-induced cachexia was elegantly demonstrated by Oliff et al. (21), who report severe weight loss and increased mortality in mice bearing transgenic tumors that persistently secrete human cachectin.

The present report gives evidence that chronic administration of sublethal doses of recombinant human cachectin to rats induces a syndrome strikingly similar to disease-associated cachexia. Anorexia, weight loss, depletion of whole-body protein and lipid stores, reduction of red blood cell mass, leukocytosis, and tissue inflammation were induced by twice daily injections given for 7-10 d. Sham-injected pair-fed controls lost weight but did not develop the specific manifestations of cachectin toxicity and cachexia. These data suggest that chronic exposure to cachectin, as likely occurs during cancer or chronic disease, participates in the metabolic and hematologic responses of cachexia.

Materials and Methods

Cachectin. Recombinant human cachectin was expressed in a yeast system, prepared as previously described, and was the generous gift of the Chiron Corporation, Emeryville, CA (16). The purified protein was diluted in 5 mM PBS (pH 7.4) to a final concentration of 2 mg/ml and was filter sterilized. The endotoxin/LPS content was <25 ng LPS/mg cachectin, as assayed by the Limulus amebocyte lysate test (M. A. Bioproducts, Walkersville, MD).

Monoclonal Antibody. Murine monoclonal anti-recombinant human cachectin antibody purified from ascites fluid from tumors of American Type Culture Collection (Rockville, MD) cell line HB 9228 was a generous gift of the Chiron Corporation. F(ab')2 fragments were prepared by incubation with pepsin (25 μg/ml) in citrate buffer (pH 3.5) for 8 h at 37°C according to previously described methods (22). The appearance of F(ab')2 was monitored by pHAST gel electrophoresis; the final product was purified using mono-Q column chromatography and dialyzed exhaustively against normal saline. Purified F(ab')2 completely neutralized at least 50 ng/ml of recombinant human cachectin (10^4 U/mg) at an antibody concentration of 10 μg/ml as assayed in the standard L929 cell cytotoxicity assay (23).

Endotoxin. LPS (E. coli strain 0127:B8, Difco Laboratories Inc., Detroit, MI) was diluted in PBS (pH 7.4) to a final concentration of 0.1 ng/ml.

Experimental Protocol. Female Sprague-Dawley rats weighing 250–300 g were housed in individual metabolic cages with 12-h light-dark cycles, and provided water and Purina Rat Chow ad libitum for at least 7 d before use. All animals were weighed daily at 8:00 a.m. when total 24 h food and water intake were recorded. Animals were studied in groups of 5-10, and during the study periods (7–10 d) were given twice daily (9:00 a.m.
and 8:00 p.m.) intraperitoneal injections of recombinant human cachectin diluted in 0.5 ml of 5 mM PBS (pH 7.4). Previous studies estimated the LD₅₀ for recombinant human cachectin in Sprague-Dawley rats at 700 μg/kg (16); we used a sublethal dose (250 μg/kg) for the present experiments. To generate an appropriate control population, control and pair-fed control animals were handled similarly except that human albumin, instead of cachectin, was injected at similar doses. Pair-fed control animals were allowed access to only the average quantity of food consumed by rats of the cachectin-treated group (gram of food/kg body weight) during the previous 24 h.

Hematocrits were determined from 10-μl samples of freely flowing blood from a tail vein puncture. At the completion of any study period complete blood count was determined from intracardiac blood samples; animals were killed by pentobarbital overdose administered via the inferior vena cava. Necropsy was performed, and tissue sections from vital organs were obtained for histopathological analysis.

**Body Composition Analysis.** Whole-body content of water, fat, and protein were determined by previously described methods (24). Whole carcasses were cut into 2.5 × 2.5 cm pieces, autoclaved to soften the pelts (210°C for 20 min), then homogenized in a blender with 500 ml of distilled water. Aliquots of the homogenized solution were weighed, frozen, and lyophilized (200 millitorr) for 1 wk. Total carcass water was calculated by subtracting total dry weight from total carcass weight. Total carcass fat content was determined gravimetrically by exhaustive extraction of a 2 g lyophilized sample with 2:1 chloroform/methanol (24). Total nitrogen in the lyophilized sample was determined by Dumas methodology with an automated elemental analyzer (model 240; Perkin-Elmer Corp., Norwalk, CT); a factor of 6.25 was used to convert total body nitrogen to total body protein.

**Estimation of Blood Volume.** Total circulating blood volume was estimated at the completion of a 10-d experimental period by dilution of ⁵¹Cr-labeled RBCs according to a modification of previously described methods (25). Heparinized syngeneic donor blood (18 ml) was incubated with ⁵¹Cr (2 mCi) for 45 min at 25°C. The mixture was centrifuged (1,000 g, 20 min) and the cell pellets were rinsed with two volumes of 0.9% saline. The cells were then resuspended to the original blood volume in 0.9% saline. Recipient rats were anesthetized with pentobarbital and the left carotid artery was cannulated with polyethylene tubing (PE-50, outside diameter 0.965 mm; Becton Dickinson, Inc., Parsippany, NJ). ⁵¹Cr-labeled donor blood (1 ml) was injected into the arterial cannula at time 0; at t = 30 min, 500 μl of blood was withdrawn through the cannula and discarded; then, 20 μl of blood was sampled in a graduated capillary tube and sample radioactivity was quantitated in a gamma counter (counting error <2%). Blood volume was estimated by dividing the counts per minute injected by counts per minute per milliliter of arterial blood sampled at t = 30 (25). RBC mass was then calculated by multiplying the estimated blood volume by percent packed cells (hematocrit) and by 1.056 (specific gravity of rat blood) (25), and RBC mass was expressed as a percentage of body weight.

**Histopathology.** Tissue specimens obtained from vital organs at necropsy were fixed with 10% formaldehyde in PBS for 5 d. Tissue sections were stained with hematoxylin and eosin (H&E) for histopathologic analysis.

**Statistics.** All data are presented as mean ± SE. Comparison between groups was made using Student's t test for unpaired observations or by one-way analysis of variance where appropriate. p < 0.05 was considered statistically significant.

**Results**

**Food Intake and Weight Changes During Cachectin Administration.** No animals died during the course of chronic administration of recombinant human cachectin at a dose of 250 μg/kg given intraperitoneally twice daily. This cachectin dosage induced a significant reduction of food intake as compared with controls receiving albumin injections (Fig. 1). Cachectin-induced anorexia was abolished if the animals were injected daily with anticachectin antibodies (10 mg/kg administered intraperitoneally, once daily).
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Figure 1. Anorexia induced by the chronic administration of cachectin. Rats were randomly divided into separate groups and received twice daily injections of either albumin (controls, 250 μg/kg, n = 6) (circles), or cachectin (250 μg/kg, n = 5) (squares) for 8 d. A separate group (n = 6) receiving cachectin twice daily also was injected with anticachectin antibodies (10 mg/kg) each morning (triangles). Food intake for each group is shown as the percentage of food intake in albumin-injected controls; all studies carried out in metabolic cages. *p < 0.05 as compared with albumin-injected controls.

Figure 2. Weight loss associated with cachectin-induced anorexia. The mean change in body weight over the 8-d study is depicted for the following experimental groups: albumin-injected controls (250 μg/kg, twice daily, n = 6) (stippled bars); cachectin (250 μg/kg, twice daily, n = 5) (angled bars); antibody-treated cachectin-injected rats (n = 6) (crosshatched bars); and pair-fed, albumin-injected controls (250 μg/kg, twice daily, n = 6) (horizontal bars).

At the end of the study period, the cachectin-treated animals, which had consumed only 60% of the albumin-injected controls' food intake, had statistically significant weight loss (p < 0.05 as compared with albumin-injected controls) (Fig. 2). Weight losses by cachectin-injected rats did not differ significantly from those of pair-fed controls. Daily administration of anticachectin antibodies to cachectin-treated animals protected against weight loss as well as anorexia. A separate group of animals were injected with endotoxin/LPS (10 ng/kg, twice daily); neither anorexia nor weight loss was observed with this dose of endotoxin given for 10 d (data not shown).

In some experiments, we observed a loss of the anorectic effect of chronic cachectin administration after 3–5 d. Anorexia was reinstituted when the dose of cachectin was increased to 500 μg/kg. By the eighth or ninth day, a repeat tolerance to cachectin was similarly counteracted by redoubling the dose of administered cachectin (to 1,000 μg/kg). The nature of this tachyphylaxis is not understood at present. To adjust for this response in subsequent experiments, we routinely titrated increasing cachectin doses to maintain food intake at ~60% of the albumin control group intake. No differences were observed between the animals that did or did not develop tachyphylaxis for any of the parameters described below.

Body Composition Analysis. The body compositions of normal and pair-fed control rats (Table I) agree closely with previous reports from other laboratories.
Anemia and Decreased RBC Mass. A significant anemia developed in cachectin-treated animals, but not in the albumin-treated controls or pair-fed controls (Fig. 3). Hematocrits fell within 3 d and continued to decline throughout the study period. There was no evidence of gross hemorrhage or external blood loss either during the experiment or at necropsy, and no microscopic blood loss was detected in urine or stool.

To determine whether the decreased hematocrit was due to relative expansion of the plasma volume or to diminished RBC mass, blood volume was estimated at the end of the experimental period by dilution of 51Cr-labeled RBCs (Table II). Blood volumes in the cachectin group were not significantly different than in sham-injected or pair-fed control groups. Thus, when corrected for the diminished packed cell volume, RBC mass was significantly decreased in the anemic cachectin-treated group. Cachectin-induced anemia was not associated with an increased number of circulating reticulocytes (1.2 ± 0.3%) compared with the control group (2.1 ± 0.3%). A marked leukocytosis was observed after cachectin administration but not in controls or pair-fed animals (Table II).
Pathologic Changes. Cachectin-treated animals exhibited gross pathology not observed in control or pair-fed rats. The mesenteric adipose of the cachectin-treated animals was invariably darkened and thickened, changes rarely observed in the controls injected with albumin. Chronic exposure to cachectin also led to organ-specific liver and heart weight increases (Table 1). Although spleen weights were increased somewhat, the changes were not statistically significant as compared with albumin-controls or pair-fed controls (data not shown).

Microscopic examination revealed a number of pathological alterations in tissues from rats injected with cachectin. The most prominent changes were in omentum, spleen, liver, and heart.

Omentum. While a mild degree of omental inflammation was present in the controls, there was extensive evidence of a more severe inflammatory reaction in omental tissue from the cachectin-injected animals (Fig. 4). The histologic changes consisted of infiltration by chronic inflammatory cells, proliferation of small blood vessels, and reactive mesothelial hyperplasia. The cellular reaction was pleomorphic, but consisted largely of lymphocytes and macrophages with occasional clusters of neutrophils. When the inflammatory reaction was scored semiquantitatively (from 0–4), the severity of the reaction was scored as 3.2+ in five cachectin-treated animals, 0.8+ in three normal controls, and 1+ in four control pair-fed animals.

Spleen. Cachectin-treated animals exhibited depletion of lymphocytes from the marginal zone of the lymphoid follicles (Fig. 4). Although the severity of such depletion varied, it was obvious in all animals as compared with albumin-treated or pair-fed controls.

Liver. The livers of animals receiving cachectin exhibited proliferation of small bile ducts in the portal tracts, surrounded by a pleomorphic mononuclear infiltration (Fig. 4). In some sections focal hepatic parenchymal cell necrosis was observed. Small foci of extramedullary hematopoiesis were also present.

Heart. The hearts of cachectin-treated animals exhibited a distinct endocardia and subendocardial lesion not present in controls. There was focal infiltration of subendocardium by chronic inflammatory cells (Fig. 4), associated with pronounced hypertrophy and hyperplasia of intimal endothelial cells and subintimal fibroblasts. There was also focal myocardial cell hypereosinophilia and necrosis.
Figure 4. (A and B) Representative histological sections of omentum, stained with H&E from control albumin-injected (A) and cachectin-injected rat (B). There is focal inflammatory infiltrate in the control (scored as +), and diffuse, more extensive infiltrate in the cachectin-treated animal (scored as 4+).
Figures 4A, 4C, and 4D: H&E stained sections of spleens from control (C) and cachectin-treated animals (D). The marginal zone (M in C, control) is absent in the cachectin-treated animal.
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Figure 4. (G and H) Subendocardial region of the myocardium of control (G) and cachectin-treated animals (H). Note subintimal inflammation and fibroblast proliferation in the cachectin-treated heart.
The areas around capillary muscles were most obviously affected. There was no evidence of adherent thrombi.

Other Organs. The bone marrow from the controls was normocellular. The marrow of the anemic, cachectin-treated group was not significantly different from controls and there was no evidence of increased cellularity. The kidneys were not appreciably different in cachectin-treated animals as compared with controls. The lungs of the cachectin-exposed animals showed foci of interstitial inflammation, but such foci were also present in control animals. It was not possible to regularly differentiate tissues of animals from each group by examination of the lung alone.

Discussion

The present data indicate that chronic exposure to cachectin is capable of inducing cachexia. Anorexia and weight loss after cachectin administration were associated with the depletion of whole-body protein and lipid stores. Moreover, these studies have identified other cachectin-specific responses, such as anemia, leukocytosis, and tissue inflammatory changes, which were independent of reduced food intake (as determined by comparison to pair-fed controls). These deleterious changes are similar to derangements occurring in malignancy- or infection-associated cachexia, suggesting that cachectin may well contribute to cachexia in some disease states.

Persistent exposure to cachectin prevented the adaptive protein conservation response usually observed during nonstressed partial starvation. As would be expected from previous animal and human studies (reviewed in reference 26), food-restricted controls preferentially deplete lipid stores to meet daily energy requirements (Table II). During cachectin-induced anorexia, however, endogenous protein stores are depleted in a manner quantitatively similar to that noted in disease-related cachexia (26). While previous investigators have reported that cachectin does not directly induce protein breakdown in isolated skeletal muscle in vitro (27), the present data suggest that net whole-body protein erosion is enhanced by the systemic administration of cachectin.

The present data do not clarify to what degree cachectin-induced cachexia is mediated via enhanced whole-body energy expenditure, centrally mediated anorexia, or altered peripheral metabolism. Cachectin is known to induce the release of IL-1 from endothelial cells and macrophages (28, 29), and IL-1 may cause anorexia (30). IL-1 is known to induce catabolic hormone release (ACTH and corticosterone), and these factors in turn might contribute to accelerated catabolism (31, 32). In addition, cachectin may also induce increased levels of glucagon, cortisol, and the catecholamines (17). While these changes in hormonal status may be indirectly stimulated by cachectin, direct cellular injury to gastrointestinal epithelial and endothelial cells has been observed after injection of a single sublethal dose of cachectin (16, 33). Further investigation is therefore needed to delineate the multiple contributory mechanisms of cachectin to cachexia.

Tolerance developed to the anorexigenic effects of recombinant human cachectin during repeated intraperitoneal injections in the rat, and this may explain why previous investigators have failed to observe sustained cachexia during
cachectin injection protocols (34, 35). The biochemical mechanisms involved in this tachyphylaxis remain unclear. Tolerance to other hormones (e.g., insulin, IL-1) has been attributed to altered intracellular second messenger responses, downregulation of plasma membrane receptors, and the appearance of circulating inhibitors, antibodies, or specific carrier proteins. Mice injected with endotoxin-activated conditioned medium from murine macrophages also exhibit tolerance to the anorexigenic effect when given at low doses, but not at higher doses (12). Oliff et al. (21) recently observed severe cachexia in nude mice bearing tumors secreting recombinant human cachectin/TNF, but not in controls bearing non–cachectin-secreting tumors. They did not observe tachyphylaxis in a carefully done study, and postulated that the continual presence of cachectin in the circulation may be necessary to induce persistent cachexia. Further studies of the kinetics of cachectin appearance and clearance during cachexia are needed to better understand the role of this factor in both experimental models and disease states.

Macrophage products have been implicated as mediators of the anemia associated with malignancy or chronic inflammation, but the etiology of decreased RBC mass is not fully understood (36, 37). Cachectin/TNF inhibits hematopoietic precursor cell proliferation, and directly inhibits the expression of erythroid burst-forming units in vitro (38–40). Cachectin/TNF is also capable, however, of stimulating the production of granulocyte/monocyte colony-stimulating factor, and can directly induce the differentiation of hematopoietic stem cells (41–43). Thus, while cachectin might influence hematopoiesis through both stimulatory and inhibitory activities, the present study demonstrates that the net effect of chronic cachectin exposure in vivo is hypoproducive anemia. Anemia has also been observed after TNF infusions in cancer-bearing man (44), suggesting the importance of this response in human disease.

Leukocytosis is a hallmark of chronic inflammation and in part represents the mobilization of leukocytes necessary to promote tissue-specific host defense mechanisms and inflammation. A large body of evidence supports previous suggestions that cachectin/TNF is a central mediator of inflammation: in addition to being a potent pyrogen, cachectin is capable of inducing PGE-2 and collagenase release, activating leukocytes, stimulating expression of activation antigens on endothelium, and enhancing leukocyte chemokinetics (reviewed in references 45, 46). The elevated numbers of circulating leukocytes after chronic cachectin exposure in the present study likely reflect chronic systemic inflammation and toxicity. It seems probable that other humoral products of the inflammatory response participate as mediators of the wasting cachectic state. However, the multiple systemic derangements of homeostasis induced by cachectin are striking, and suggest a means of therapeutic intervention by antagonism of this proximal mediator.

Previously we have shown that cachectin is capable of inducing acute hypodynamic shock and severe tissue injury in normal animals (16, 17). The pattern of tissue inflammation after cardiovascular collapse and shock is different, however, from the present observations after chronic administration of cachectin/TNF. After acute cachectin-induced shock, critical organ injury is very similar to acute lethal endotoxemic lesions and primarily involves the kidneys, lungs, adrenals,
and gastrointestinal tract (16, 17). After chronic injections of cachectin, however, the kidneys and lungs were relatively spared, and significant abnormalities in the liver, heart, spleen, and omental adipose were observed. Periportal inflammation and increased liver weights were observed in cachectin-treated animals. This latter observation is characteristic of other cachexia syndromes, and in the present study is likely due to several factors. Extramedullary hematopoiesis typically occurs in rats during anemia, and could contribute to increased total organ weight. Extensive inflammation with accumulation of inflammatory cells and associated edema also contribute to increased organ weight. Since cachectin/TNF also stimulates increased total liver protein synthesis and is capable of directly stimulating increased acute phase protein biosynthesis (30, 47, 48), the increased liver weights in the present study are consistent with observations in other inflammatory states where hepatic parenchymal protein accrual likely represents an adaptive acute phase response.

Cachectin-induced cardiac lesions have not been observed previously, but other evidence implicates cachectin as a mediator of cardiac dysfunction. Cardiac output declines sharply after the infusion of cachectin in dogs, and the falling cardiac output is not restored by either intravenous fluid administration or significant elevations of circulating catecholamines (17). Furthermore, cardiac function is significantly improved during primate bacteremia by passive immunization with anticachectin mAbs (19). Specific alterations of skeletal muscle resting membrane function and energy metabolism have also been observed in response to cachectin infusion (10, 11). The pathogenesis of the peculiar cachectin-induced subendothelial inflammation is unclear.

Cachectin-induced depletion of the marginal zone of the splenic lymphoid follicles has also not been previously observed. In normal rats, the marginal zone consists largely of B cells that express a variety of activation antigens. The specific depletion of activated lymphocytes (Fig. 4) may represent mobilization of these activated immunoresponsive cells as a part of cachectin-induced inflammatory responses. Another interesting feature of splenic histology was the apparent absence of blood forming units in the spleens of the cachectin-treated anemic rats. The number of erythroid forming units in both hepatic and splenic tissue is characteristically increased during anemia in rodents. Whereas some compensatory extramedullary hematopoiesis occurs in the liver after cachectin-induced anemia, the lack of a parallel splenic response may contribute to the development of anemia.

Blood monocytes obtained from cancer patients show enhanced cachectin responses (49), and several laboratories have reported increased serum cachectin/TNF levels in association with a variety of diseases including malaria and Leishmaniasis (50), meningococcal septicemia (51), acute renal allograft rejection (52), prolonged critical illness and sepsis (53); and in volunteer subjects given small doses of intravenous endotoxin (54). Serum cachectin levels during some human cachectic states may well be below the limits of detection for currently available assays, and further study is needed to clarify the role of endogenous cachectin production in the development of cachexia in man.

The identification of a host factor that is sufficient to induce cachexia, anemia, leukocytosis, and inflammation in a variety of tissues advances our understanding
of the systemic responses to invasion and injury. Cachectin-mediated anorexia and alterations of cellular metabolism may contribute to many of the nutritional features observed in cancer cachexia, trauma, and inflammation. Since protection against cachectin-induced cachexia is conferred by the administration of anticaechectin antibodies in this study, cachectin antagonists may be of use during the nutritional therapy of some cachexia syndromes.

Summary

Cachexia is a potentially lethal syndrome of unknown etiology characterized by anorexia, weight loss, and protein wasting that frequently complicates the treatment of chronic inflammation and cancer. Cachectin/TNF was isolated during the search for a humoral mediator of cachexia and found to stimulate the breakdown of energy stores from adipocytes and myocytes in vitro, but the chronic effects of the monokine in vivo are not known. Sublethal doses of recombinant human cachectin administered twice daily for 7–10 d caused cachexia in rats, as evidenced by reduced food intake, weight loss, and depletion of whole-body lipid and protein stores. Significant anemia is also observed and found to be the result of decreased red blood cell mass, not expanded plasma volume. Leukocytosis and histopathological evidence of tissue injury and inflammation are observed in several organs, including omentum, liver, spleen, and heart. These data suggest that the exposure of the normal host to cachectin is capable of inducing a pathophysiological syndrome of cachexia, anemia, and inflammation similar to that observed during inflammatory states or malignancy.

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