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Research Article

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Metabolic Effects of Cachectin/Tumor Necrosis Factor Are Modified by Site of Production

Cachectin/Tumor Necrosis Factor–secreting Tumor in Skeletal Muscle Induces Chronic Cachexia, While Implantation in Brain Induces Predominately Acute Anorexia

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Abstract

We have developed a murine model of wasting by injecting intracerebrally cells which continuously secrete h-cachectin/TNF (CHO-TNF) to: (*a*) determine the effects of cachectin/TNF produced continuously in the central nervous system (CNS), and (*b*) compare the metabolic effects of cachectin/TNF-secreting tumor in the brain to the cachexia caused by CHO-TNF tumor in peripheral tissue (IM). Intracerebral CHO-TNF tumors produced increased serum h-cachectin/TNF levels with lethal hypophagia and weight loss (mean survival time of 11 d); these changes were not observed in association with nonsecretory control brain tumors. The metabolic consequences of intracerebral cachectin/TNF production were indistinguishable from acute, lethal starvation: whole-body lipid content was decreased significantly but protein was conserved. Although intramuscular cachectin/TNF-secreting tumors caused similar increases of serum h-cachectin/TNF levels, profound anorexia did not develop; wasting developed after a longer period of tumor burden (50 d) with classical signs of cachexia (i.e., anemia and depletion of both protein and lipid). These studies provide a reproducible animal model of site-specific cytokine production and suggest that, regardless of serum levels, cachectin/TNF produced locally in brain influences both the rate of development of wasting and its net metabolic effects. (*J. Clin. Invest.* 1990. 86:2014–2024.) Key words: cancer • cytokines • tumor necrosis factor • body composition • AIDS • anorexia • brain tumor • angiogenesis

Introduction

Weight loss is frequently the most obvious manifestation of life-threatening illness. The syndrome of "cachexia" is characterized by weight loss, anorexia, anemia, and net losses of body protein and lipid; its prevalence exceeds 70% for certain types of cancer, and is even higher in patients suffering with AIDS (1–5). Cachectic patients may lose > 50% of their preillness body weight, and the onset of malnutrition and inanition accelerates their demise. Advances in nutritional therapy have done little to alter the poor prognosis of this wasting diathesis, and its pathogenesis remains incompletely understood (6, 7). It is known that normal regulation of food intake is severely deranged in cachexia; even when food is freely available the cachectic host does not consume enough to meet basal energy needs (1, 7, 8, 9).

One putative mediator of anorexia and increased catabolism is cachectin/tumor necrosis factor (TNF),[1] a pleiotropic cytokine that occupies a pivotal role as a mediator of infection and neoplastic disease (reviewed in references 10 and 11). Recent evidence suggests that cachectin/TNF is capable of mediating weight loss and altering normal metabolic priorities through its effects on both the central nervous system (CNS) and peripheral tissues. We and others have shown that repeated intraperitoneal administration of cachectin/TNF induces cachexia with a pattern of tissue wasting that includes whole-body protein depletion, unlike the protein-conserving pattern induced by simple caloric restriction (12, 13). Systemically administered h-cachectin/TNF also down-regulates skeletal muscle synthesis of actin and myosin (14), and alters gastrointestinal motility and absorptive function (15, 16). Oliff and co-workers (17) have induced cachexia in nude mice by intramuscular implants of a genetically engineered cell line that continuously secretes h-cachectin/TNF. Minute quantities of cachectin/TNF injected directly into the cerebral ventricles suppress food intake and alter the responsivity of specific hypothalamic neurons (18), and acute intravenous injection induces pyrexia via a hypothalamic mechanism (19). Finally, anticachectin/TNF antibodies attenuate anorexia and weight loss in animals chronically injected with h-cachectin/TNF (12) or given transplantable tumors (20). Although clinical studies have demonstrated elevated serum cachectin/TNF levels in samples from patients with diseases known to be associated with cachexia (e.g., cancer [21], AIDS [22], and parasitic infection [23]), no correlation of circulating cachectin/TNF levels to the severity of weight loss in cachectic patients has been found (24). Based on these and other data, we have suggested previously that the net metabolic effects of cachectin/TNF may depend in large part on the paracrine effects of cytokine tissue concentration achieved locally, and that organ-specific local action may outweigh the effects of circulating levels (10, 25).

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1. *Abbreviations used in this paper:* CHO, Chinese hamster ovary; PCA, parachloroamphetamine; TNF, tumor necrosis factor.

Until recently it was not possible to directly study the paracrine contribution of cachectin/TNF produced in specified tissue sites to the development of cachexia in a tumor-bearing animal model. The present study attempts to bridge this gap, and herein we describe the development of a reproducible model of continuous h-cachectin/TNF biosynthesis in brain by stereotactic injection of genetically engineered Chinese hamster ovary (CHO). Using this model, the metabolic effects of continuous "internal" exposure of the CNS to cachectin/TNF are characterized, and compared to those metabolic derangements induced by cachectin/TNF produced in peripherally implanted tumors. Evidence is provided that the predominate effect of continuous production of cachectin/TNF in the CNS is acute anorexia which causes an accelerated rate of wasting and death as compared to peripheral CHO-TNF tumor-bearing animals, even though serum levels of cachectin/TNF are similarly increased in both cases.

Methods

Reagents. Murine monoclonal anti-human-cachectin/TNF antibody (anti-h-cachectin/TNF mAb 3E3, kindly provided by M. Fournel, Miles Inc., Berkely, CA), was diluted in sterile saline for injection to a final concentration of 2 mg/ml. Murine IgG (Jackson Immunoresearch Laboratory, Bar Harbor, ME) was diluted in sterile saline to a final concentration of 2 mg/ml. Isophane insulin suspension USP 100 U/ml (Eli Lilly & Co., Indianapolis, IN) was diluted in sterile saline to a working concentration of 10 U/ml. Ibuprofen (Sigma Chemical Co., St. Louis, MO) and indomethacin (Sigma Chemical Co.) were diluted in ethanol:water (1:1) to final concentrations of 10 mg/ml. Parachloroamphetamine (PCA) (Sigma Chemical Co.) was prepared in a final concentration of 1 mg/ml with sterile saline.

Analytical methods. A sandwich-type ELISA that has been previously described by our laboratory (26) was employed to detect human cachectin/TNF. Cachectin/TNF bioactivity was determined by modification of the standard L929-cell cytotoxicity assay (27). Serum glucose and triglyceride concentrations were determined using standard automated methodology (Hitachi 717, Boehringer Mannheim Inc., Houston, TX). Serum thyroxine was measured by radioimmunoassay using antibody-coated tubes provided as a kit (ICN Biochemical, Horsham, PA). All samples were assayed in duplicate or triplicate.

Cell culture. The cachectin/TNF-secreting cell line was developed and kindly provided by Dr. A. Oliff (Merk Sharp & Dohme Research Laboratories, West Point, PA). A mammalian expression vector containing the human cachectin/TNF gene was transfected into Chinese hamster ovary (CHO) cells and a cell line isolated that constitutively secreted cachectin/TNF (CHO-TNF) (17). As a control, a CHO cell line was isolated that had been transfected with the same expression vector but devoid of the cachectin/TNF insert (17). Both cell lines were maintained in DMEM supplemented with 10% FCS at 37°C in a humidified atmosphere of 5% CO_2/95% air. The CHO-TNF cells constitutively secreted cachectin/TNF as measured both by ELISA and L929-cell cytotoxic activity. In our hands, the findings of the original description of these cells was confirmed: the CHO-TNF cell line produced significant quantities of cachectin/TNF (1.5–3.0 μg cachectin/TNF per 10^6 cells per 24 h), and the CHO control line did not.

Brain and hindlimb tumor cell implantation. The right anterior brain region was selected for tumor implantation because this is a neurologically "silent" area; tumor growth there in rodents has not been found to produce acute signs of neurological impairment (28). On the day of brain tumor implantation, cells were trypsinized from the culture flasks and resuspended in fresh medium (final concentration of 40–60 × 10^6 cells/ml). Intracerebral (IC) injection of cells was performed using a Hamilton syringe and a 23-gauge Luer-type beveled needle that was fitted with a plastic sheath designed for an injection depth 3.5 mm from the skin surface. Mice were anesthetized with methoxyflurane and the head securely fixed in a holder. The midline was marked with a felt-tip pen, and the injection site marked at a point 1 mm posterior to the outer canthus of the right eye and 3 mm lateral from the midline. During injection the needle was advanced in a plane perpendicular to the skull up to the hub of the needle guard (3.5 mm deep to the skin). This approach delivered cells into the desired anatomic region in a reproducible manner as confirmed by brain histology (below). Mice injected by this method tolerated injection volumes of 15–30 μl, and intracerebral injection of cells was readily performed with negligible morbidity and mortality related to the procedure. Intramuscular (im) hindlimb injections were routinely performed in awake, restrained mice using a Hamilton syringe fitted with a 23-gauge needle; cells were resuspended to a final concentration of 200–250 cells/ml. The leg was manually immobilized and the needle tip inserted directly into the thickest part of the gastrocnemius-soleus muscle group. Control animals received injection of medium alone for the individual experiments as indicated.

Animal study protocols. Male nude (nu/nu) mice (n = 178) weighing 19–22 g were purchased from a commercial supplier (Harlan Sprague Dawley, Inc., Indianapolis, IN) and housed four, five, or six animals per cage in a temperature controlled room (25°C) on a 12-h light/dark cycle. All experimental protocols were approved by the Rockefeller University Animal Care and Use Committee. Animals were provided water and a standard pelleted diet (Purina Lab Chow) *ad libitum* for 7–14 d before use in any experiment. Studies were performed using groups of animals numbering four to six as indicated for the individual experiment. Body weights were recorded daily at 9 AM and food intake estimated by weighing the residual food for individual cages. Brain tumor–bearing animals were studied for a 10-d period; intramuscular tumor–bearing animals were studied for periods of either 10 or 50 d. Monitoring of brain tumor–bearing animals revealed no neurological signs of paralysis, ataxia, or seizure. Results from a representative survival analysis of animals injected intracerebrally or intramuscularly are shown in Table I. These data suggested that mortality was related to the number of CHO-TNF cells injected intracerebrally, and since animals with 2 × 10^6 succumbed very rapidly, the lesser dose of 1 × 10^6 cells was used in all subsequent studies. (Injection of fewer cells did not reliably induce tumor growth.) To generate appropriate control groups for the hypophagic CHO-TNF brain tumor group, pair-fed control animals were allowed daily access

Table I. Survival after Sham or CHO Cell Injection in Nude Mice

	n	Cells injected	Mean survival time
			d
Sham-NI	12	0	60.0*
IC-CHO	12	1 × 10^6	14.0*
IC-TNF	16	1 × 10^6	11.1±0.4
IC-TNF	15	2 × 10^6	8.1±0.5
Sham-PF	10	0	10.8±0.7
IM-TNF	11	1 × 10^7	60.0*

* Animals were sacrificed at the time indicated as outlined in Methods. Mean±SEM.

Sham-NI, controls injected intracerebrally with carrier alone; IC-CHO, injected intracerebrally with CHO cells that did not secrete cachectin/TNF; IC-TNF, injected intracerebrally with CHO-TNF cells; Sham-PF, injected intracerebrally with carrier medium and subsequently pair-fed to the quantity of food consumed by the IC-TNF (1 × 10^6 cells) animals; IM-TNF, injected intramuscularly with CHO-TNF cells.

to the quantity of food consumed by the matched CHO-TNF brain tumor group on the previous day.

Upon completion of the observation period, animals were anesthetized with methoxyflurane and blood obtained by cardiac puncture. In the course of the experiments, animals noted to be moribund (unreactive to environmental stimulae or having agonal respirations) were sacrificed by carbon dioxide narcosis. Necropsies were performed and tissues fixed in a 10% buffered formaldehyde solution, routinely processed, embedded in paraffin, sectioned at 6 μm, stained with hematoxylin and eosin, and examined by light microscopy.

Body composition analysis. Carcass water, lipid, and protein were determined by established methods (12, 20). Briefly stated, after necropsy the headless, tumor-free carcasses were eviscerated, the heart included with the carcass, and the wet carcass weight recorded. Carcasses were frozen in dry ice, ground to a fine powder in an blender, and dried to a constant mass at 80°C (3–4 d). Lipid was extracted from the dried specimens by sequential exhaustive extraction using chloroform:methanol (1:1), ethanol:acetone (1:1), and diethyl ether. After taring, an aliquot of the lipid-free extract was analyzed for total nitrogen by Dumas methodology using an automated elemental analyzer (model 240, Perkin-Elmer Corp., Norwalk, CT). A factor of 6.25 was used to convert total carcass nitrogen to total carcass protein (12).

Statistical methods. Data are expressed as mean±SEM. Differences in body composition and serum chemistries were analyzed for statistical significance by analysis of variance (ANOVA).

Results

Intracerebral injection of cachectin/TNF-secreting cells. To develop a model whereby the effects of cachectin/TNF produced continuously in the brain might be reproducibly studied, we first examined the feasibility of inducing the growth of CHO tumors in nude mouse cerebrum. Animals received injections of 1×10^6 CHO-TNF or control CHO cells into the neurologically silent right anterior hemisphere, and 10 d later were euthanized for necropsy. Histological examination showed the development of intracerebral CHO tumors in all animals injected with either the CHO-TNF or CHO control cell line (Fig.

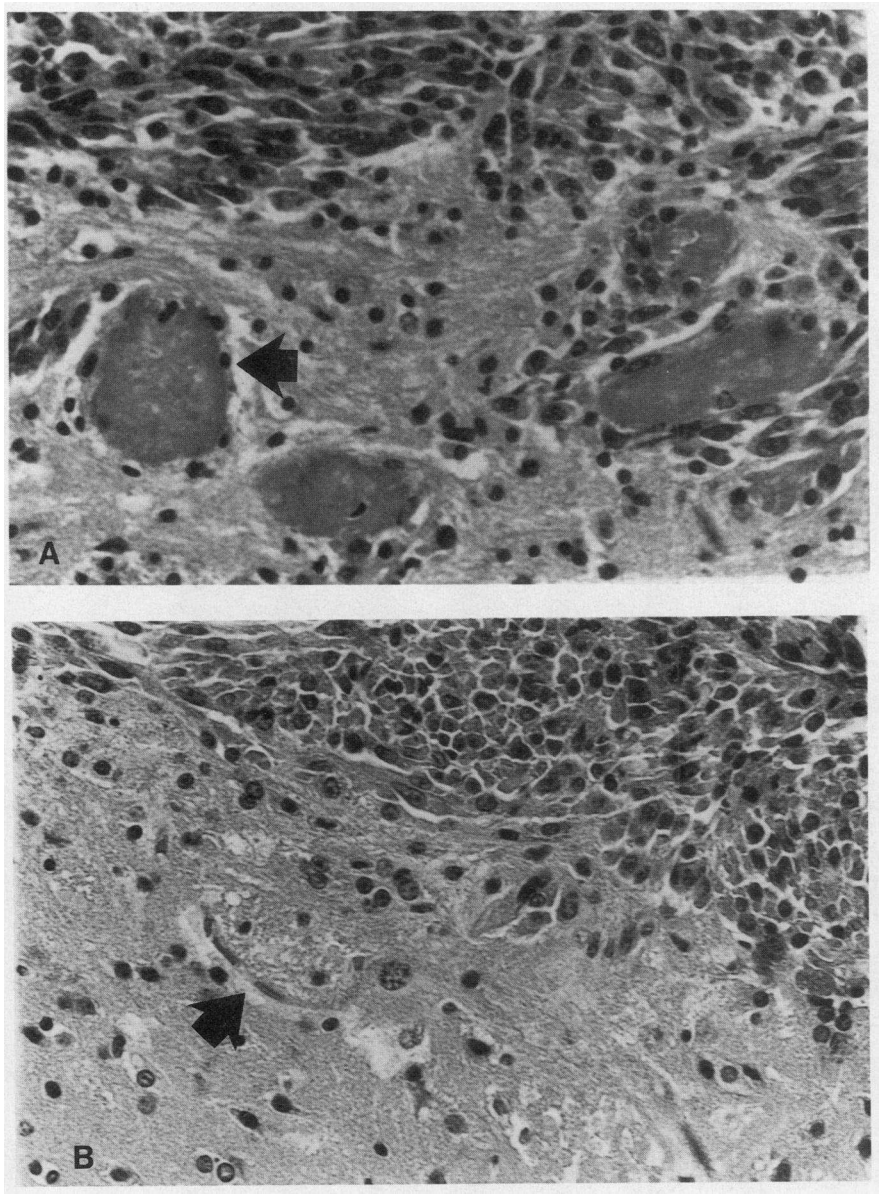

Figure 1. (*A*) Representative photomicrograph of the cachectin/TNF-secreting tumor (CHO-TNF) in brain. There is increased cellularity in the brain adjacent to the tumor, and numerous dilated thrombosed blood vessels are shown (*arrow*). Hematoxylin and eosin stain, original magnification 160×. (*B*) Photomicrograph of the control (nonsecreting) CHO tumor in brain. Note the virtual absence of a parenchymal response as compared to *A* and the normal parenchymal vasculature (longitudinally-sectioned capillary, *arrow*). Hematoxylin and eosin stain, original magnification 160×.

1). Both cell lines produced viable intraparenchymal tumors of similar size (~ 3 × 1 × 1 mm) extending from the pial surface into the deep gray matter of the right anterior hemisphere with occasional tumor deposits noted in the leptomeninges distant from the injection site.

Significant histological differences were associated with cachectin/TNF-secreting tumors as compared to nonsecreting controls. Whereas control CHO brain tumors led to minimal to undetectable degrees of gliosis and neovascularity (Fig. 1), the parenchymal response to CHO-TNF tumor growth was notable for peritumoral gliosis, dilated blood vessels with plump endothelial cells, and focal thrombosis in nearby microvasculature. Neovascularity was significantly increased in the peritumoral regions as compared to the nonsecretory controls. Focal margination of inflammatory cells was present within vascular lumena in the vicinity of the CHO-TNF tumor. Neither distant metastases nor significant intracerebral tumor necrosis were observed in brain-injected animals with tumors from either cell line (CHO or CHO-TNF).

To confirm that h-cachectin/TNF was being produced by the genetically engineered cells in vivo, blood was obtained by cardiac puncture 10 d after intracerebral injection of CHO-TNF cells and the serum prepared. ELISA performed on these sera revealed increased levels of circulating h-cachectin/TNF (0.66±0.22 ng/ml). The circulating form of cachectin/TNF was bioactive as confirmed by serum assay using the standard L929-cell cytotoxicity bioassay (data not shown). As anticipated, no cachectin/TNF was detected by ELISA or by L929 bioassay performed on specimens obtained from animals bearing the control CHO cell brain tumor.

Separate groups of animals were injected intramuscularly with 1×10^7 CHO-TNF cells, and over the subsequent 4–8 wk, large hindlimb tumors (2.3±0.5 g) developed. Serum assayed for human-cachectin/TNF by ELISA had elevated levels 10 d (0.44±0.17 ng/ml) and 50 d (0.48±0.13 ng/ml) after intramuscular injection; these levels do not differ significantly from the IC-CHO-TNF tumor-bearing animals. Complete necropsy performed in six animals bearing intramuscular CHO-TNF tumors for 50 d revealed evidence of pulmonary metastases in two, and pulmonary, hepatic, and renal metastases in one. Histological evidence of central necrosis was noted in the large primary tumors, and viable tumor cells were present at the tumor margins. Brain histology was normal, and there was no evidence of gliosis, inflammation, neovascularity, or metastasis.

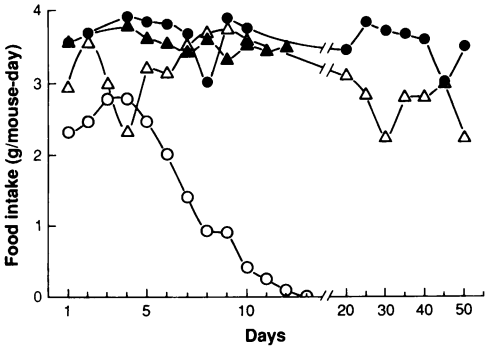

Figure 2. Food consumption. On day zero, all mice were injected with carrier or CHO cell suspension according to the following: *Solid circles* (n = 12) received carrier medium alone injected into brain; *open circles* (n = 17) received 1×10^6 CHO-TNF cells in brain; *solid triangles* (n = 15) received 1×10^6 CHO cells in brain; *open triangles* (n = 11) received 1×10^7 CHO-TNF cells injected into hindlimb skeletal muscle.

Induction of hypophagia and weight loss by cachectin/TNF-secreting brain tumor. We and others have previously shown that cachectin/TNF acts peripherally to increase cellular catabolism in adipocytes (29, 30) and myocytes (31), and induces anorexia and pituitary hormone release by a direct effect on the CNS (18, 32). Having established that CHO-TNF tumors located in either brain or skeletal muscle induced similar increases in the circulating levels of h-cachectin/TNF (above), we wondered whether the production of relatively large amounts of cachectin/TNF in brain would trigger a markedly different metabolic response as compared to its effects when produced in peripheral muscle tissue. In agreement with the observations of Oliff and colleagues (17), hypophagia and weight loss were not observed in control animals injected intramuscularly with the nonsecretory control CHO cells; they continued to grow and gain weight normally after tumor implantation (Table II). Mice injected intramuscularly with cachectin/TNF-secreting cells, however, failed to gain weight normally and after 4–6 wk lost body weight. Mean daily food intake decreased slightly as compared to carrier-injected controls within the first 10 d after injection, then normalized for several weeks, and finally decreased to a rate ~ 25% less than carrier-injected controls (Fig. 2). Thus, these observations confirmed that continuous production of cachectin/TNF in

Table II. Changes in Body Weight of Nude Mice after Injection with Either CHO or CHO-TNF Cells, or Carrier Medium Alone

	Day 2	Day 4	Day 7	Day 10	Day 20	Day 30	Day 40	Day 50
				Body wt (% of preinjection baseline weight)				
IC-TNF (n = 22)	97±1	95±4	85±3	72±2	—	—	—	—
Sham-PF (n = 11)	94±3	94±6	88±7	76±6	—	—	—	—
IC-CHO (n = 24)	103±4	105±5	103±5	103±5	—	—	—	—
IM-TNF (n = 11)	101±1	104±2	104±4	100±3	99±2	93±3	94±5	89±3
IM-CHO (n = 6)	103±4	111±4	115±5	113±4	128±5	135±4	139±3	141±5

IC-TNF, injected intracerebrally with 1×10^6 CHO-TNF cells; Sham-PF, injected intracerebrally with carrier medium alone and subsequently pair-fed to the quantity of food previously consumed by the IC-TNF group; IC-CHO, injected intracerebrally with 1×10^6 nonsecreting CHO cells; IM-TNF and IM-CHO refers to animals injected intramuscularly with 1×10^7 CHO-TNF or CHO cells, respectively.

hindlimb muscle prevents normal weight gain and eventually induces wasting. This pattern was then compared to the effects induced by cachectin/TNF secreted in CNS tissue.

By contrast, anorexia and weight loss developed within 7 d when the cachectin/TNF-secreting cell line was implanted in the brain: food intake fell to zero g/mouse-day (Fig. 2) and total body mass declined 28% from preinjection weight (Table II). Anorexia and weight loss in these brain tumor–bearing mice were not due to the nonspecific effects of CNS tumor growth, because they were not observed during the growth of similar sized CHO tumors that did not secrete cachectin/TNF (Fig. 2; Table II), or after injection with other cell lines including murine and human glial cells (data not shown). These CHO-TNF brain tumor–bearing animals had a wasted, poorly groomed appearance, and gross evidence of weight loss (Fig. 3). With progressive depletion of subcutaneous fat stores the interscapular region became depressed and the individual ribs could be visibly discerned. Quite unexpectedly, they also developed increased body hair in the regions overlying their abdomens, shoulders, and flanks. In most cases hair growth was first visible on the abdomen within 7 d, and persisted throughout the experimental period. Hair growth did not occur in control mice implanted with CHO cell tumors that were devoid of the h-cachectin/TNF gene, or in pair-fed controls, suggesting that hypertrichosis was a specific response to the production of cachectin/TNF in brain. The appearance of body hair was not directly attributable to increased serum cachectin/TNF levels, because animals with intramuscular CHO-TNF tumors also had increased serum cachectin/TNF levels but did not show signs of new hair growth.

We performed additional experiments to determine whether intracerebral injection of anti-h-cachectin/TNF monoclonal antibody would alter the course of CHO-TNF brain tumor-induced hypophagia, weight loss, or hair growth. As is shown in Table III, intracerebral injection of monoclonal anti-h-cachectin/TNF antibody (40 μg IC on alternate days), but not pooled murine IgG in control animals, conferred a slight attenuating effect on the development of hypophagia in CHO-TNF brain tumor-bearing animals on days 7–12 after tumor implantation. Administration of anti-cachectin/TNF antibody did not abrogate the development of weight loss (Table III) or hair growth as compared to nontreated or IgG-injected controls.

Because many of the biological effects of cachectin/TNF in host tissues are attributable to secondary endogenous mediators (10), we performed the following experiments to assess the potential contribution of other factors to the hypophagia induced by intracerebral production of cachectin/TNF. One possibility was that cachectin/TNF mediated hypophagia by inducing anterior hemisphere serotonin release. This was examined by pretreating animals with parachloroamphetamine (PCA) using an established protocol that ablates serotoninergic

Figure 3. Wasting and increased body hair (hypertrichosis) induced by cachectin/TNF-secreting tumor in brain. 10 d before this picture both animals were injected with CHO-TNF cells: the animal on the right received 1×10^6 cells into the brain; the animal on the left received 1×10^7 cells into hind limb skeletal muscle. Although serum TNF levels were similar in the two animals, note the presence of hair in the CHO-TNF brain tumor-bearing animal. Non–tumor-bearing pair-fed controls also develop a wasted appearance similar to the IC-CHO-TNF animals, but do not develop increased hair growth (not shown).

Table III. Food Intake in Intracerebral Tumor-bearing Animals and Controls Given Anti-Cachectin/TNF mAb, Parachloroamphetamine, Insulin, Indocin, or Ibuprofen

	n	Day 1–2	3–4	5–6	7–8	9–10	11–12	Wt (% of preinjection)
		Food consumed* (*g/mouse*)						
Sham-Nl	12	7.2	8.1	7.6	6.7	7.7	7.2	103
IC-TNF	17	4.8	5.6	4.5	2.3	1.3	0.65	68
IC-IgG	5	5.3	6.7	3.7	1.6	0.5	0.5	73
IC-mAb	11	5.1	6.9	4.8	4.0	3.9	4.2	65
Sham-PCA	6	8.0	6.9	7.7	7.7	7.4	7.1	102
TNF-PCA	6	3.2	3.1	3.3	2.6	3.4	Dead	57
Indocin	6	3.0	0.95	Dead	—	—	—	95
Ibuprofen	6	1.6	1.1	Dead	—	—	—	85
NPH	11	4.5	6.0	5.1	3.7	3.2	4.8	85

* Food consumption data per 48 h is given for animals grouped 5–6 per cage as indicated in Methods. Sham-nl, normal controls injected intracerebrally with medium alone; IC-TNF, mice with CHO-TNF brain tumor; IC-IgG, normal murine IgG intracerebrally administered on alternate days to IC-CHO-TNF mice; IC-mAb, anti-cachectin/TNF mAb intracerebrally administered on alternate days to IC-CHO-TNF mice; Sham-PCA and TNF-PCA, PCA (10 mg/kg i.p.) injected daily for 2 d; 5 d later, intracerebral injection with carrier medium or CHO-TNF cells performed; Indocin, Ibuprofen, IC-CHO-TNF animals treated daily with either agent administered i.p. as described in text; NPH, IC-CHO-TNF animals treated twice daily with NPH-insulin as described in text.

neuronal function (33). As is shown in Table III, controls given PCA had a rate of daily food consumption that was similar to normal controls (not receiving PCA). However, PCA pretreatment did not protect against the development of hypophagia when animals were subsequently injected with a CHO-TNF brain tumor, suggesting that serotonin was not a predominate secondary mediator in this model. Eicosanoids have also been implicated as mediators of cachectin/TNF toxicity (34) and IL-1–induced hypophagia (35), and inhibitors of cyclooxygenase (indocin and ibuprofen) have been used to inhibit these effects. In the present studies, however, when used in doses that were protective against IL-1–induced anorexia (indocin 3 mg/kg twice daily; ibuprofen 20 mg/kg twice daily) (35), these agents did not attenuate the hypophagia or weight loss induced by CHO-TNF brain tumor (Table III). For unknown reasons, mortality was increased in CHO-TNF brain tumor–bearing animals receiving these agents (Table I).

Because these studies did not provide evidence for a predominate role of serontonin or eicosanoids in the development of intracerebral cachectin/TNF-induced hypophagia, we next considered whether cachectin/TNF rendered the CNS incapable of responding to hyperphagic mediators. Insulin is one such hyperphagia-inducing factor that stimulates the lateral hypothalamic feeding center (36) and ameliorates anorexia in rats given intraperitoneal cachectin/TNF (13). To assess the effects of insulin in the present model, NPH-insulin was administered (20 U/100 g body weight, subcutaneously, twice daily [13]) to CHO-TNF brain tumor–bearing animals and food intake measured. As is shown in Table III, insulin administration attenuated the development of hypophagia, led to a modest increase in daily food consumption, and reduced the magnitude of weight loss; survival and hair growth were unchanged as compared to controls. Serum glucose levels measured 10 d after CHO-TNF cell injection were decreased in the insulin-treated animals (43±4 mg/dl) as compared with animals not treated with insulin (54±14 mg/dl), but this difference was not statistically significant. Thus, these experiments indicated that intracerebrally-produced cachectin/TNF did not prevent the CNS from responding to the hyperphagic effects mediated by insulin.

Body composition analysis. The effects of semistarvation on body protein and lipid content have been repeatedly described (12, 37, 38), and based on these and other studies it has been suggested that starvation-elicited responses differ from the metabolic changes of cachexia. During unstressed starvation, lipid is utilized preferentially to protein, which tends to be conserved, but in cachexia there is net catabolism of both lipid and protein. In the present studies, body composition of pair fed controls (Table IV) agrees with the well-known consequences of partial starvation: near-lethal starvation induced a depletion of lipid but no significant change in whole-body protein content. The compositional changes in the anorectic, weight-losing CHO-TNF brain tumor animals revealed a similar pattern of lipid mobilization and protein retention (Fig. 4). As expected, control CHO brain tumor–bearing animals (which gained weight) did not have significant losses of body lipid or protein. In addition, we compared the body composition of animals with more chronic weight loss induced by intramuscular cachectin/TNF-secreting tumors to the composition of animals with brain tumor. Although the two groups of animals had similar levels of circulating cachectin/TNF, and similar degrees of body weight loss (27% after correction for tumor weight), the peripheral tumor-bearing animals had chronic losses of both whole-body lipid and protein (Table IV). Thus, although the CHO-TNF leg tumor–bearing animals did not develop profound hypophagia (Fig. 2), and did not rapidly succumb to the effects of starvation (Table I), they did develop chronic losses of whole-body protein and lipid (Fig. 4) which are characteristic of cachexia. These responses differed markedly from the acute starvationlike changes induced by brain-

Table IV. Body Composition

	Start wt	Final wt	Nontumor carcass	Water	Protein	Lipid	Tumor wt
	g	*g*	*g*	%	*g*	*g*	*g*
Sham-Nl (n = 12)	27.4±0.6	29.2±0.8	17.2±0.5	63.8±0.6	3.3±0.1	2.2±0.1	NA
IC-CHO (n = 11)	25.2±0.7	25.0±0.5	16.6±0.9	65.0±0.1	3.1±0.1	2.0±0.2	NA
IC-TNF (n = 10)	25.6±0.9	20.1±0.5*	12.5±0.5*	67.8±0.7*	2.9±0.1	0.6±0.1*	NA
Sham-PF (n = 10)	25.5±0.7	20.3±0.6*	12.9±0.3*	68.7±1.2*	2.9±0.1	0.5±0.2*	NA
IM-TNF (n = 11)	27.2±0.5	23.2±0.8*	10.6±0.7*	70.6±0.4*	2.1±0.1*	0.5±0.1*	2.3±0.5

* $P < 0.05$ vs. sham-nl. Sham-Nl, injected intracerebrally with carrier medium alone; IC-TNF or IC-CHO, injected intracerebrally with 1×10^6 CHO-TNF or CHO cells, respectively; Sham-PF, injected intracerebrally with carrier medium alone and subsequently pair-fed to the quantity of food peviously consumed by the IC-TNF group; IM-TNF, injected intramuscularly with 1×10^7 CHO-TNF cells, and sacrificed for carcass analysis after ~ 50 d (when nontumor body weight losses approximated the losses achieved in the CHO-TNF brain tumor-bearing group).

secreted cachectin/TNF, and suggested that the tissue site of cachectin/TNF production influenced both the rate of development of wasting, and the type of tissue depleted during tumor-associated wasting.

Blood and serum analysis. Investigation of differences between centrally and peripherally produced cachectin/TNF included analysis of hematocrit, glucose, triglyceride, and thyroxine which are summarized in Table V. In agreement with previous reports describing the development of anemia in animals with peripheral CHO-TNF tumors (39) or after chronic, intermittent h-cachectin/TNF injections (12, 40), animals with intramuscular hind limb CHO-TNF tumor developed a progressive decline in hematocrit. Hematocrit did not decline, however, in either pair-fed controls or CHO-TNF brain tumor–bearing animals. Serum glucose and triglyceride levels were decreased in pair-fed controls and all tumor-bearing animals with wasting as compared to controls. Thyroxine levels were decreased significantly in all animals that had weight loss (IC-TNF, Sham pair-fed, IM-TNF), and in agreement with the previously described effects of cachectin/TNF on the thyroid axis (41, 42), the lowest levels were observed in animals with the most severe weight loss in association with elevated cachectin/TNF levels (IC-TNF, IM-TNF).

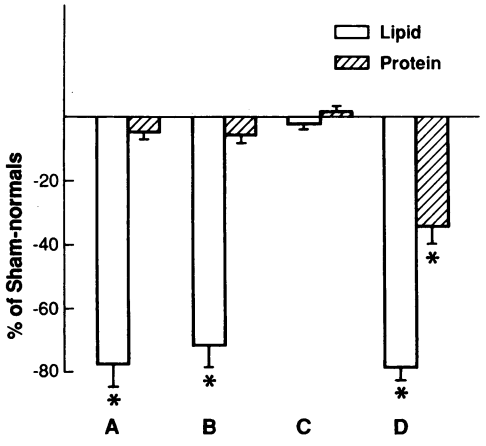

Figure 4. Body composition of experimental animals expressed as ratio to normal controls. To correct for slight differences in starting weights, lipid and protein content was computed as a percentage of starting body weight and plotted as the ratio to normal (see Table IV). (*A*) Sham-injected pair-fed controls, 10 d (n = 10); (*B*) CHO-TNF brain tumor-bearing animals, 10 d (n = 10); (*C*) CHO brain tumor-bearing animals, 10 d (n = 11); (*D*) CHO-TNF leg tumor-bearing animals with comparable wasting to the IC-TNF animals, 50 d (n = 11). *$P < 0.05$ vs. sham-injected controls.

Discussion

Blood levels of h-cachectin/TNF were similarly elevated by tumor growth in either brain or skeletal muscle, but both the rate of tissue losses and the net metabolic effects differed markedly. When cachectin/TNF was produced in the CNS it induced acute, profound anorexia and a metabolic state that, by analysis of survival, body composition, organ histology, and serum chemistries, resembled the response to acute, lethal protein-calorie starvation. By contrast, when cachectin/TNF was produced in peripheral tissues it triggered the gradual development of cachexia with anemia and losses of protein and lipid tissue. These data give evidence that the site of tissue

Table V. Packed Cell Volume, and Serum Glucose, Triglyceride, and Thyroxine Levels

	Hematocrit	Glucose	Triglyceride	Thyroxine
	%	*mg/dl*	*mg/dl*	*nmol/liter*
Sham-Nl	44.7±1.4 (11)	156.1±16.5 (11)	106.0±10.4 (10)	37.8±2.1 (16)
IC-CHO	46.5±2.1 (14)	116.0±11.8 (15)	103.7±11.9 (9)	24.5±1.9* (13)
IC-TNF	47.2±1.8 (8)	54.1±13.6* (7)	79.0±7.6 (7)	5.95±1.5* (7)
Sham PF	45.3±1.1 (9)	49.9±8.7* (9)	66.2±9.2* (9)	22.4±5.4* (6)
IM-TNF (10 d)	39.2±1.0* (5)	61.0±5.6* (5)	47.4±3.4* (5)	22.7±5.2* (5)
IM-TNF (50 d)	26.7±4.5* (8)	92.0±17.1* (7)	76.3±10.4 (7)	11.7±4.1* (7)

* $P < 0.05$ vs. sham-nl controls.
Abbreviations as described in Tables III and IV.
Numbers in parentheses, mice per group.

cachectin/TNF production influences the net metabolic effects of tumor-associated wasting.

It is interesting that serum concentrations of cachectin/TNF reached similar levels within 10 d in animals with either brain or leg tumors, even though 10 times more cells were injected intramuscularly. Because some tumor necrosis was observed in animals with peripheral but not CNS tumors, this likely reduced the number of functional secretory cells in the peripheral tumor-bearing groups. The blood brain barrier was disrupted in the presence of CHO-TNF brain tumor, as evidenced by the proliferation of abnormal peritumoral capillaries, and the extravasation of ^{125}I-albumin (data not shown, Tracey and Cerami) which likely contributed to the release of cachectin/TNF into the peripheral circulation from the CNS.

Effects of continuously produced cachectin/TNF in the CNS. We and others have shown that systemically administered r-h-cachectin/TNF induces weight loss, anemia, and anorexia (12, 13, 43, 44). Plata-Salaman and co-investigators (18) reported that minute doses administered intracerebroventricularly-induced hypophagia and suppressed the activity of glucose-sensitive neurones in the lateral hypothalamic area. More recently, Bodnar and others (45) observed hypophagia and a rapid egress of intracerebro-ventricularly administered cachectin/TNF from the CNS to the circulation. However, chronic intermittent injections of cachectin/TNF can lead to the development of tolerance or "tachyphylaxis" to its anorectic effects (12, 44, 46), and may not reproduce the biology of cytokines produced locally and continuously in tissues. The results of the present studies indicate that the continuous "overproduction" of cachectin/TNF in brain induces a profound suppression of food intake. This hypophagia can not be ascribed to a nonspecific effect of brain tumor because it did not occur in association with similar sized tumors that lacked the inserted gene for h-cachectin/TNF, and was attenuated by intracerebral injection of a neutralizing monoclonal h-cachectin/TNF antibody.

Because cachectin/TNF-induced hypophagia was also attenuated by insulin, the mediation of anorexia did not occur via irreversible suppression of CNS responsiveness to exogenous hyperphagic stimulae. While these studies did not provide direct evidence of a predominate role for either cyclo-oxygenase products or serotonin as secondary mediators of anorexia, other mediators may have participated in the development of anorexia. One candidate factor is IL-1, which is induced by cachectin/TNF (47), sensitizes the host to cachectin/TNF (48), and mediates anorexia (35, 49). Because indocin and ibuprofen abrogate the development of IL-1-induced hypophagia (35) but did not alter the development of anorexia in animals with brain-produced cachectin/TNF, it is likely that different mechanisms are operative in the present model, or as suggested by Plata-Salaman (18), intracerebral cachectin/TNF is directly anorexigenic. Additional studies are needed to localize the site(s) of action of cachectin/TNF in brain, and to identify the specific mechanism of action in the mediation of anorexia.

That inanition contributes significantly to the death of animals exposed to brain-produced cachectin/TNF is suggested by the observation that pair-fed control animals, whose food intake was limited to that quantity consumed previously by the CHO-TNF brain tumor-bearing animals, succumbed at similar times (Table I). However, CHO-TNF brain tumor-bearing animals treated with either anti-TNF mAb or insulin had improved food consumption but no increased longevity. Thus, although the precise cause of death is unclear, these data suggest that other factors contribute to the lethality of intracerebrally-produced TNF.

In addition to inducing starvation, centrally-produced cachectin/TNF stimulated hair growth in nude mice (Fig. 3). The hairless defect in nu/nu mice is the result of abnormal keratinization of the hair shaft; although the hair follicles appear normal, the shafts are defective and break off at the skin (50). To our knowledge, the only previous references to this singular observation in nude mice have described increased body hair induced by oral cyclosporine A (50, 51). Hair growth in the present model cannot be attributed to the direct effects of increased serum h-cachectin/TNF levels, because these were also increased in mice with peripherally-located tumors that never developed increased hair growth. While we cannot explain the mechanism of this hair growth, it is interesting to note that acquired increases of body hair have been observed in patients with anorexia nervosa (52), or tumors originating from a variety of tissues (53). The latter syndrome, termed hypertrichosis lanuginosa, has been observed in association with weight loss, anorexia, and pituitary dysfunction, and although an unidentified humoral mediator has been implicated, its etiology is unknown (54). The data described herein suggest that local production of h-cachectin/TNF in brain may have triggered the systemic release of another factor that induced hair growth. In agreement with previous studies indicating that the blood-brain barrier is relatively impermeable to systemically administered cachectin/TNF (55), brain histology from cachectin/TNF leg tumor-bearing animals (with high serum h-cachectin/TNF levels) did not show evidence of cachectin/TNF-mediated inflammation. These data therefore support the conclusion that brain tissue levels in animals exposed to peripherally-produced cachectin/TNF were too low to mediate either profound anorexia or hair growth.

The present studies also provided a model for direct analysis of the local inflammatory effects of cachectin/TNF in brain. The histology of the parenchyma adjacent to intracerebral CHO-TNF tumors showing neovascularity, margination of inflammatory cells, and focal thrombosis (Fig. 1) agrees with the known inflammatory effects of locally produced cachectin/TNF. These properties include the potential for inducing endothelial leukocyte adherence molecule (ELAMs), increased capillary permeability to albumin, increased procoagulant activity, margination of leukocytes, and stimulation of angiogenesis (reviewed in references 10 and 11). In addition to its direct inflammatory effects, cachectin/TNF triggers the biosynthesis of other cytokines (e.g., IL-1, IL-6, PDGF) that mediate inflammation, and although the contribution of these secondary mediators to the systemic effects observed is unknown, this will be the subject of future investigations. Overproduction of cachectin/TNF in brain has been implicated in the pathogenesis of meningitis (56–58) and cerebral malaria (59); and anorexia invariably accompanies these infections. It is hoped that future studies of the role of cachectin/TNF-mediated brain inflammation in other intracerebral diseases (e.g., AIDS) may provide insight into the pathogenesis of associated hypophagia and weight loss.

Whole-body metabolic response to intramuscular or intracerebral production of cachectin/TNF. Attempts to delineate the role of cachectin/TNF in human cachexia have been hampered by an inability to correlate circulating cachectin/TNF

levels with the manifestations of weight loss or cachexia (Manogue, K., unpublished observations, and 24). These observations previously led us to suggest that the net metabolic effects of cachectin/TNF might not be predominately dependent upon its circulating effects, but rather upon its paracrine action in specific tissues (10, 25). The present study now provides data confirming that although serum levels may be increased significantly by tumor growth in either brain or peripheral tissue, the net effects of CNS-derived cachectin/TNF significantly accelerates the rate of development of wasting, and leads to death before the development of cachexia.

The metabolism of starvation is characterized by mobilization of whole-body lipid and relative sparing of protein; it has been teleologically reasoned that this adaptive response allows the hungry animal to remain mobile in search of food. The metabolic responses to brain-derived cachectin/TNF were strikingly similar to those observed in starved, pair-fed controls. By contrast, animals with cachectin/TNF-secreting leg tumors that developed similar losses of body weight over a longer period of time (50 d) did not develop absolute hypophagia, but did become cachectic with net depletions of both lipid and protein tissues. We believe that the CHO-TNF brain tumor–bearing animals succumbed before significant protein losses and cachexia developed, and that the presence of metastases in 50% of the leg tumor–bearing animals may have contributed to the development of cachexia. It is interesting to note that although serum cachectin/TNF levels were significantly increased, protein losses did not develop in animals within the first 10 d after implantation of cachectin/TNF-secreting tumors in either brain (Fig. 4) or leg (not shown). This differs somewhat from previous studies showing increased whole-body protein losses induced by exposure to cachectin/TNF within 7–10 d (12) that occurred in association with coordinate decreases in muscle mRNA levels for myofibrillar proteins (14). The nature of the mediator(s) of whole-body protein losses induced in these latter animals is unknown, but previous studies suggest that it is not attributable to a direct effect of cachectin/TNF because incubation of skeletal muscle preparations with h-cachectin/TNF did not induce proteolysis (60). Flores and co-workers (61) observed acutely increased rates of muscle proteolysis within hours after infusion of cachectin/TNF. The present data suggest that cachectin/TNF-induced protein losses were not mediated by thyroxine, but the role of other catabolic glucose counter-regulatory hormones (e.g., cortisol, catecholamines) is unknown. Considering these and other data (12–14, 62, 63) indicating that systemic cachectin/TNF induces acute mobilization of both lipid and protein, it is likely that the pattern of combined lipid and protein loss in animals bearing peripheral cachectin/TNF-secreting tumors represents cytokine-induced mobilization of energy stores, and that other factors predominate in animals with intracerebrally-produced cachectin/TNF.

As occurs in cachexia, animals chronically exposed to cachectin/TNF by intraperitoneal injection or intramuscular cachectin/TNF-secreting tumor developed a hypoproliferative anemia associated with an increased rate of red blood cell degradation (12, 39, 40). The present model of peripheral cachectin/TNF production is consistent with these previous studies, and shows a decline of hematocrit within 10 d. The development of anemia within 1 wk of cachectin/TNF exposure is the result of decreased red blood cell mass, is not attributable to an expansion of the plasma volume, and is associated with increased red cell breakdown and decreased synthesis (12, 39, 40). For unknown reasons, anemia did not develop in animals with CHO-TNF brain tumors which also had elevated levels of circulating cachectin/TNF, but these data again suggest that the site of tissue production influences the systemic responses to this cytokine. It is conceivable that starvation induced by centrally produced cachectin/TNF also induces other factors that modulate the rates of red blood cell turnover, but additional studies are needed to define these hematologic responses in this model.

Role of cachectin/TNF production in diseased CNS. The role of brain-derived cachectin/TNF in human anorexia is unknown. Results from recent clinical studies indicate that cachectin/TNF is produced intracerebrally during meningitis in humans: elevated levels are readily detectable in the cerebrospinal fluid and serum, and these correlate to the severity of the infection (56–58), but whether the accompanying anorexia is directly attributable to intracerebral cachectin/TNF is unknown. Although peripheral blood leukocytes obtained from patients with malignancies produce increased amounts of cachectin/TNF (64), other studies of serum from patients with cancer-associated cachexia have failed to detect consistently elevated serum levels (24). The present model perhaps offers some insight into this discrepancy between circulating levels and the development of wasting, and suggests that the site of production of cachectin/TNF, and not the circulating level, ultimately determines the net metabolic result. Moreover, the paracrine effects of this cytokine are capable of dominating the host response, and of mediating either host starvation or cachexia. It will be interesting to apply this information towards the pathogenesis of other illnesses such as AIDS or anorexia nervosa, where the role of intracerebrally-produced factors that mediate anorexia and weight loss have been proposed but not identified. For instance, infection caused by the human immunodeficiency virus is characterized by extensive CNS disease (65), and recently cachectin/TNF has been detected in sera from these patients (22). Because brain astrocytes can be induced to secrete cachectin/TNF by neurotropic viruses (66), and HIV-infected cells also secrete cachectin/TNF (67), the present data suggest that local production of this cytokine in brain might contribute to the development of anorexia and wasting that characterizes infection with HIV.

In summary, the studies described here provide a reproducible, useful experimental animal model whereby the systemic effects of cachectin/TNF produced in the CNS or in peripheral tissues can be evaluated. Intracerebrally-produced cachectin/TNF induces hypophagia and lethal starvation, whereas peripheral tissue production induces cachexia. Thus, the net effects of cachectin/TNF may be profoundly effected by its concentration in vital tissues (e.g., CNS), regardless of the prevailing blood concentration. It is hoped that this experimental approach will advance our understanding of the pathogenesis of cachexia, and foster the development of new therapeutic approaches for the management of patients with disease-induced starvation and wasting.

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