Mechanisms of Cachexia Induced by T-Cell Leukemia in the Rat

S. Roe, A.L. Cooper, I.D. Morris, and N.J. Rothwell

Body wasting (cachexia) is a common feature of cancer and a major cause of morbidity and mortality. The mechanisms underlying cachexia are largely unknown, and studies in experimental animals have focused mainly on solid tumors. Therefore, the objective of the present study was to quantify and investigate cachexia in experimentally induced T-cell leukemia in the rat. Induction of leukemia by serial passage (injection of cervical lymph node suspension) resulted in a rapid increase in white blood cell (WBC) count, hypertrophy of the spleen (by day 11), and severe morbidity within 17 to 18 days. Body weight gain and food intake declined steadily in leukemic animals from day 12, although weight loss was significantly greater in pair-fed, nonleukemic animals. However, leukemic rats had a lower body fat content and higher water content than pair-fed animals on day 18, so the measurement of body weight significantly underestimated the severity of cachexia. Resting oxygen consumption ($\dot{V}O_2$), measured during the light phase, declined in pair-fed animals from day 13, but was elevated in leukemic rats on days 12 to 18 by 25% ($P < .05$, one-way ANOVA) compared with pair-fed rats and by 7% ($P < .05$, one-way ANOVA) relative to free-feeding controls. Hypermetabolism was associated with an increase in brown adipose tissue (BAT) activity (74% and 89%, respectively, $P < .05$, one-way ANOVA) in leukemic rats compared with control and pair-fed groups. Effects of leukemia on $\dot{V}O_2$ and BAT were prevented by administration of the adrenergic antagonist, propranolol. These results indicate that T-cell leukemia in the rat results in rapid and severe cachexia, which is largely due to marked hypophagia, but is also accompanied by inappropriately high rates of energy expenditure that are mediated by sympathetic activation of BAT thermogenesis.

CACHEXIA REPRESENTS a syndrome of homeostatic disturbances, of which the most prominent feature is weight loss. Unlike simple starvation, weight loss in cachexia results from complex interactions between the host and the tumor, causing early loss of both lean and fat tissue. This debilitating condition is a common feature of malignant disease and significantly increases both mortality and morbidity in cancer patients. The clinical features of cancer cachexia have been reviewed extensively, and include depletion of body fat and protein stores.[1-4]

Negative energy balance, which causes weight loss, results from a reduction in energy intake (decreased food consumption or impaired nutrient absorption) and/or an increase in energy expenditure. Clinical studies have yielded conflicting results on the mechanisms underlying cachexia, and have reported both reduced food intake and increased energy expenditure in cancer patients,[5-9] or in some cases, no changes in these parameters.[10,11] Measurements of energy balance in patients are extremely difficult, and results may be inaccurate, particularly when small differences in intake or expenditure over long periods contribute to the weight loss. Information on body composition usually relies on indirect measurements (eg, skinfold thickness), which may not be valid for patients with altered body composition, and continuous measurements of energy intake and expenditure are almost impossible to achieve in the clinical setting, particularly in children. These problems are further compounded by difficulties in interpretation of energy balance data from patients who are losing or have lost weight, in terms of obtaining comparable control data and correcting data for loss of lean body mass.

For these and ethical reasons, research on the mechanisms underlying cachexia has focused largely on experiments in animals with spontaneous or implanted solid tumors. Published results indicate that changes in energy balance are dependent on the type and severity of the tumor, but are often associated with both decreases in food intake and increases in energy expenditure[12-17]; the latter are dependent, at least in part, on sympathetic activation of thermogenesis in brown adipose tissue (BAT).[18]

Animal experiments on cachexia have relied almost exclusively on studies involving solid tumors, although leukemia is a common form of cancer (2.5% in males and 3.4% in females of all cases[19]), particularly in children, in whom cachexia is likely to have a more serious impact on subsequent growth and recovery. However, no data are available on its impact on energy metabolism either in patients or in experimental animals. Therefore, the objective of the present study was to quantify the time course and magnitude of cachexia in experimentally induced T-cell leukemia in the rat and to investigate the mechanisms underlying cachexia.

MATERIALS AND METHODS

Animals

All studies were performed on young adult (60 to 80 days old) male Piebald Variegated rats (supplied and bred by the Biological Services Unit, University of Manchester). The animals weighed 220 to 270 g and were fed a powdered diet (CRM Labsure, Kent, UK) and water. They were housed in pairs in wire-bottomed cages at 22°C with a 12-hour light/dark cycle (8 AM to 8 PM).

Induction of Leukemia

T-cell leukemia was induced as previously described,[20] and was maintained in Hooded Oxford rats by serial passage. One hundred microliters of a cell suspension (containing 1×10^5 cells) obtained

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from enlarged cervical lymph nodes of animals approaching the terminal phase of leukemia was injected intramuscularly into the hindlimb of recipient animals.[21] Control animals were injected with 100 μL 0.9% saline.

Body Weight, Food Intake, and Water Intake

Food and water intake were measured in pairs of animals (ie, per cage). Measurements of food and water intake and body weight were made daily between 7 and 8 AM. Food intake and any spillage were measured to the nearest 0.1 g; water intake and body weights were measured to the nearest gram. Pair-fed animals were given the same amount of food as eaten by leukemic animals on the previous day between 5 and 7 PM. Food was presented at this time to ensure that all animals ate during the dark cycle.

White Blood Cell Count and Spleen, Liver, and Cervical Lymph Node Weight

Blood was collected under halothane anesthesia into heparinized (10 U/mL) syringes by cardiac puncture, and the number of circulating white blood cells (WBCs) was measured by a Coulter Counter (Coulter Electronics, Bedford, UK). The animals were then killed by cervical dislocation, and the spleen, liver, and cervical lymph nodes were dissected out and weighed.

Oxygen Consumption

Resting oxygen consumption ($\dot{V}O_2$) was measured by indirect calorimetery[22] at 24°C for at least 2 hours (between 8 AM and 12 noon) or until constant steady-state values had been obtained. Rates of $\dot{V}O_2$ were corrected for standard temperature and pressure—dry and metabolic body size ($mL \cdot min^{-1} \cdot kg^{-0.75}$).

Colonic Temperature

Colonic temperature (Tc) was determined immediately after measurements of $\dot{V}O_2$ in conscious, hand-held, minimally stressed rats (between 10 AM and 12 noon) by means of a plastic-coated thermocouple, which was inserted into the rectum to a depth of 6 cm.

BAT Activity

Thermogenesis in the interscapular BAT depot was assessed from in vitro activity of the mitochondrial proton-conductance pathway by the binding of radiolabeled guanosine diphosphate ([GDP] 10 Ci/mmol; Amersham International, Buckinghamshire, UK) to isolated mitochondria. This protocol has been described in detail elsewhere.[23] In brief, isolated mitochondria were prepared by differential centrifugation in 0.2 mol/L sucrose and then incubated with radiolabeled GDP and either 2 or 200 μmol/L unlabeled GDP (the higher concentration determined nonspecific binding). Mitochondrial protein content was determined using a dye reagent method (Bio-Rad Laboratories, Watford, UK) with bovine serum albumin as the standard.

Propranolol

The effect of intraperitoneal injection of L-propranolol (10 mg/kg) on $\dot{V}O_2$ and BAT activity was assessed on days 13 to 16 after implantation in control, pair-fed, and leukemic animals.

Carcass Composition

Animals were killed by cervical dislocation, and the carcasses were stored at −20°C until analysis. The thawed carcasses were chopped into 5-cm^3 blocks, weighed, homogenized in a blender, and freeze-dried to constant weight (48 hours). Water content was determined from the difference in weight before and after drying. Body fat content was determined by automated Soxhlet extraction (petroleum ether extraction) on triplicate samples of freeze-dried homogenate.

Statistical Analysis

All values are expressed as the mean ± SEM. The data were analyzed using a paired or unpaired Student's *t* test for comparison of two groups, as appropriate, and a one-way ANOVA followed by Scheffé's post hoc test for three groups. Results from time-course experiments were analyzed using multiple ANOVA (MANOVA) to compare responses over time. Comparisons between groups at specific time points were analyzed using an unpaired Student's *t* test or one-way ANOVA followed by Scheffé's post hoc test for two and three groups, respectively. In all cases, two-tailed probabilities of less than 5% were considered statistically significant.

RESULTS

Experiment 1: Time Course of Changes in Disease Status in Leukemic Rats

Rats (initial weight, 246 ± 4 g; n = 34) were divided into two weight-matched groups and injected with either 0.9% saline (control) or a suspension of leukemic cells. Animals were killed on days 11, 14 (n = 6), and 16 (n = 5), and the number of circulating WBCs and spleen weight were determined. In a separate experiment, control and leukemic animals (n = 6 per group) were killed on day 15; the liver and cervical lymph nodes were removed and immediately weighed.

Separate studies have shown that all animals become severely moribund 18 to 19 days after implantation, but for the present study, they were killed before this time. Circulating WBC and spleen weights were similar in control animals on days 11, 14, and 16 (Fig 1). However, leukemic animals displayed marked increases in circulating WBC count and spleen weight, which were significantly greater than those of control animals at all time points ($P < .001$, unpaired Student's *t* test). Liver and cervical lymph node weights were also significantly increased in leukemic animals compared with controls (1.4- and 3.7-fold, respectively, $P < .01$, unpaired Student's *t* test).

Experiment 2: Comparison of Body Weight and Food Intake in Leukemic, Pair-Fed, and Control Rats

In a preliminary study, body weight and food intake were compared in leukemic and control animals. Leukemic animals displayed a marked loss in body weight and food intake on days 14 to 17 after implantation as compared with controls (day 17, 11% and 81%, respectively; data not shown). To establish the contribution of food intake to body weight loss in leukemic rats, a group of pair-fed nonleukemic animals were compared with free-feeding control and leukemic rats. Thirty-two rats with an initial weight of 224 ± 5 g were divided into three weight-matched groups: freely fed control (n = 10), pair-fed nonleukemic control (n = 12), and leukemic (n = 10) rats. Pair-feeding was achieved using data from previous studies to predict the intake of leukemic rats on each day. This amount of food was presented to the pair-fed animals, but was adjusted on the following day to correct for any deviations from the

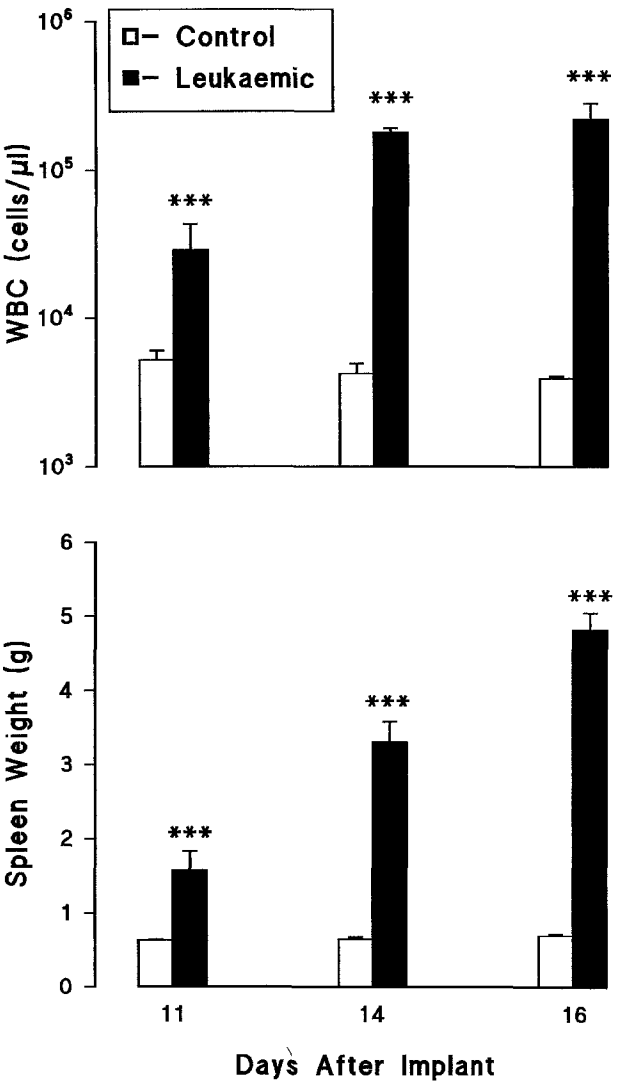

Fig 1. WBC count and spleen weight of control and leukemic rats on days 11, 14, and 16 after implantation. Values are the mean ± SEM (n = 5 to 6). ***$P < .001$ *v* control, unpaired Student's *t*-test.

intake of leukemic rats. Body weight was measured from days 5 to 17, and food and water intake from days 7 to 17.

Food intake of pair-fed animals matched that of leukemic animals throughout the experiment, apart from a transient increase on day 11 in leukemic rats ($P < .05$, one-way ANOVA; Fig 2). Food intake of pair-fed and leukemic animals was significantly less than that of free-feeding control animals from days 13 to 17 ($P < .05$, one-way ANOVA, and $P < .001$, MANOVA).

The body weight of control animals fed ad libitum increased throughout the experiment (body weight gain on day 17, 42 ± 3 g; Fig 2). Leukemic and pair-fed animals gained weight at a rate similar to that of controls over days 5 to 10. Thereafter (days 11 to 15) the body weight of leukemic animals stabilized, but pair-fed animals lost weight from day 13 despite a food intake similar to that of leukemic animals. The body weight change of pair-fed animals was significantly greater than that of control animals over days 13 to 17 ($P < .05$, one-way ANOVA). By day 17, the body weight of leukemic animals had declined and was significantly less than that of control animals ($P < 0.05$, one-way ANOVA). However, pair-fed animals lost significantly more weight than leukemic rats (days 13 to 17, $P < .05$, one-way ANOVA). Thus, by day 17, leukemic animals had gained 18 ± 6 g since day 0 (implantation), whereas pair-fed animals had lost 9 ± 4 g over this time.

The water intake of all animals was similar until day 10 (data not shown). On days 11 and 12, water intake of leukemic animals increased transiently (19% to 23%) above that of pair-fed and control animals. Thereafter, water intake of leukemic and pair-fed animals was similar but significantly less than (58%) that of control animals.

Experiment 3: Effect of Leukemia on $\dot{V}O_2$, Tc, and Carcass Composition

$\dot{V}O_2$ and Tc for individual rats were recorded on days 8 to 18 following implantation in freely fed control, leukemic, and pair-fed nonleukemic control animals (n = 6 per group;

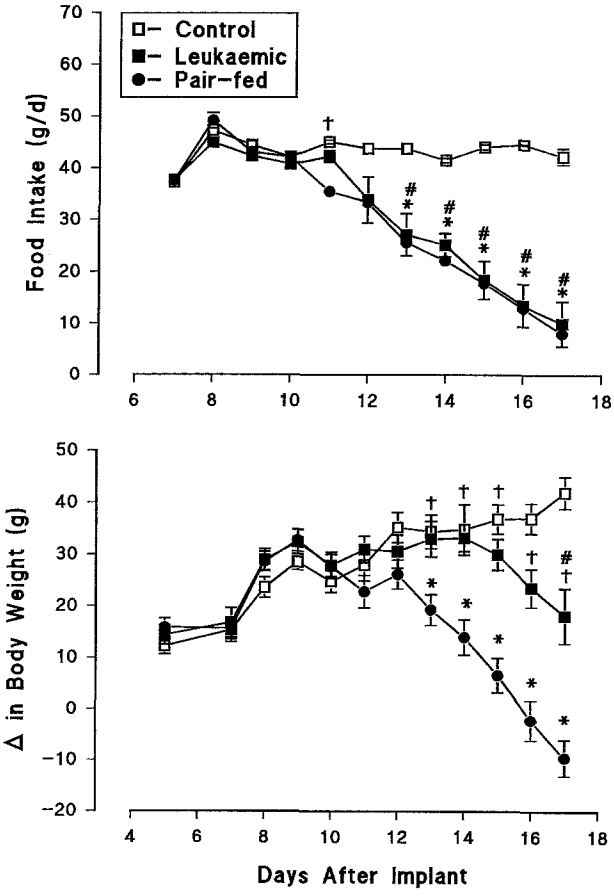

Fig 2. Food intake and body weight change of control, pair-fed, and leukemic rats. Values are the mean ± SEM (n = 5 to 6 per pair of animals for food intake and n = 10 to 12 per animal for body weight). *$P < .05$, pair-fed *v* control; #$P < .05$, leukemic *v* control; †$P < .05$, leukemic *v* pair-fed (one-way ANOVA and Scheffé's post hoc test). Responses of the 3 groups were significantly different over the experiment for both parameters ($P < .001$, MANOVA).

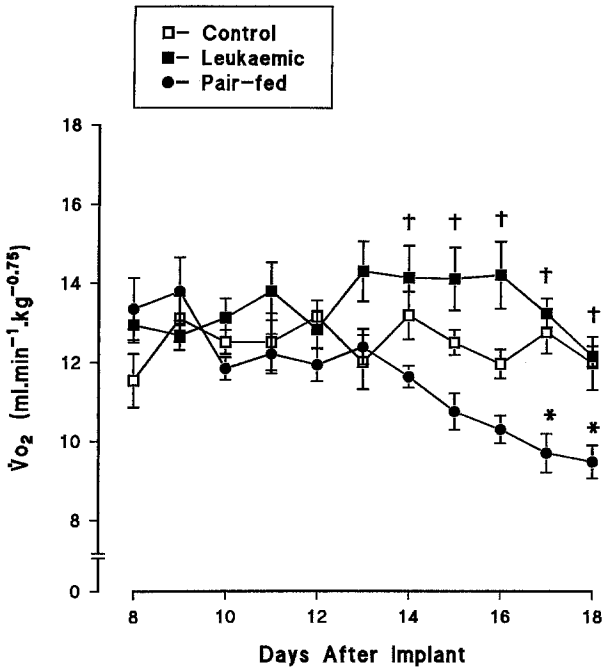

Fig 3. Resting $\dot{V}O_2$ in control, leukemic, and pair-fed rats. Values are the mean ± SEM (n = 6). *P < .05, pair-fed *v* control; †P < .05, leukemic *v* pair-fed (one-way ANOVA and Scheffé's post hoc test). Responses of the 3 groups were significantly different over the experiment (P < .01, MANOVA) for $\dot{V}O_2$.

initial body weight, 269 ± 3 g). On day 18, the animals were killed and carcasses were stored for analysis.

Resting $\dot{V}O_2$, corrected for body size, was similar in all groups over days 8 to 12 (Fig 3), and in control animals it was stable for the remainder of the experiment. On day 14, $\dot{V}O_2$ of control animals was 7% lower than that of leukemic rats but 11% greater than values for pair-fed animals. Thus, leukemic animals exhibited an 18% increase in $\dot{V}O_2$ compared with the pair-fed group, and this difference was maintained over days 15 and 16 (P < .05, one-way ANOVA). Thereafter, $\dot{V}O_2$ declined in the leukemic group to values similar to those of control animals. However, leukemic animals had a significantly increased $\dot{V}O_2$ compared with pair-fed animals on days 17 to 18 (P < .05, one-way ANOVA), and this was also significantly reduced in pair-fed animals compared with controls (P < .05, one-way ANOVA). Over the duration of the experiment, the pattern of $\dot{V}O_2$ of the three groups was significantly different (P < .01, MANOVA). Tc of control animals remained constant over days 8 to 18; Tc of leukemic and pair-fed animals was similar to control values until day 12. Thereafter, on days 12 to 15, Tc of leukemic rats increased, while that of pair-fed animals gradually declined, as compared with controls. Thus, Tc of leukemic animals was significantly increased by 0.7°C as compared with Tc of pair-fed animals on day 15 (P < .05, one-way ANOVA). On days 17 to 18, Tc of leukemic animals declined to values comparable to those of pair-fed animals (day 18: control 36.6 ± 0.4°C, pair-fed 34.9 ± 0.1°C, and leukemic 35.1 ± 0.7°C; data not shown).

On day 18, leukemic and pair-fed rats weighed less than controls (Table 1; P < .01 and P < .001, respectively, one-way ANOVA). Although the total body weight of the pair-fed group was not significantly different from that of leukemic rats, the reduction in body weight from day 0 was significantly greater than that of leukemic animals (P < .001, one-way ANOVA). Leukemic and pair-fed animals both had a lower carcass fat content than control animals (P < .01, one-way ANOVA), but body fat content as a percentage of body weight was significantly reduced only in leukemic animals (P < .01, one-way ANOVA). The absolute mass of body water (in grams) of control animals was significantly greater than that of leukemic or pair-fed animals (P < .01, and P < .001, respectively, one-way ANOVA). However, the percentage water content was significantly elevated only in leukemic rats (P < .001, one-way ANOVA, *v* control and pair-fed groups).

Experiment 4: Effect of Propranolol on $\dot{V}O_2$ and BAT Activity

Resting $\dot{V}O_2$ values were measured over days 13 to 16 after implantation in control, leukemic, and pair-fed animals (n = 8). At these times, $\dot{V}O_2$ in leukemic animals was significantly increased compared with values obtained in pair-fed animals. After resting measurements of $\dot{V}O_2$ had been obtained (~2 to 3 hours), the animals were injected with L-propranolol and then returned to the calorimeter for another 2-hour period.

In separate experiments, animals (n = 4 to 7 per group) were killed on day 15 for assessment of BAT activity, 14 hours after injection of vehicle or propranolol. Animals were killed by stunning and cervical dislocation, and the interscapsular BAT depot was dissected and stored at −70°C until analysis.

The effect of propranolol on $\dot{V}O_2$ in control, leukemic, and pair-fed animals is shown in Fig 4. Resting $\dot{V}O_2$ was elevated in leukemic rats by 7% compared with control

Table 1. Body Weight and Fat and Water Content in Control, Leukemic, and Pair-Fed Animals on Day of Death (day 18)

	Final Body Weight (g)	Change in Body Weight (g)	Body Fat Content		Body Water Content	
			g	%	g	%
Control	306 ± 5	34 ± 3	57.9 ± 3.9	18.9 ± 1.2	200.6 ± 3.0	65.7 ± 0.4
Pair-fed	251 ± 3†	−20 ± 3*	34.3 ± 2.1*	13.6 ± 0.8	166.4 ± 1.7*	66.2 ± 0.4
Leukemic	269 ± 9‡	5 ± 5§‖	28.1 ± 6.6‡	10.1 ± 2‡	189.9 ± 5.1‡	70.6 ± 0.6§‖

NOTE. Body fat and water content are expressed as total content (g) and as % of body weight. Values are the mean ± SEM (n = 6).

*P < .01, †P < .001: pair-fed *v* control.

‡P < .01, §P < .001: leukemic *v* control.

‖P < .001, leukemic *v* pair-fed.

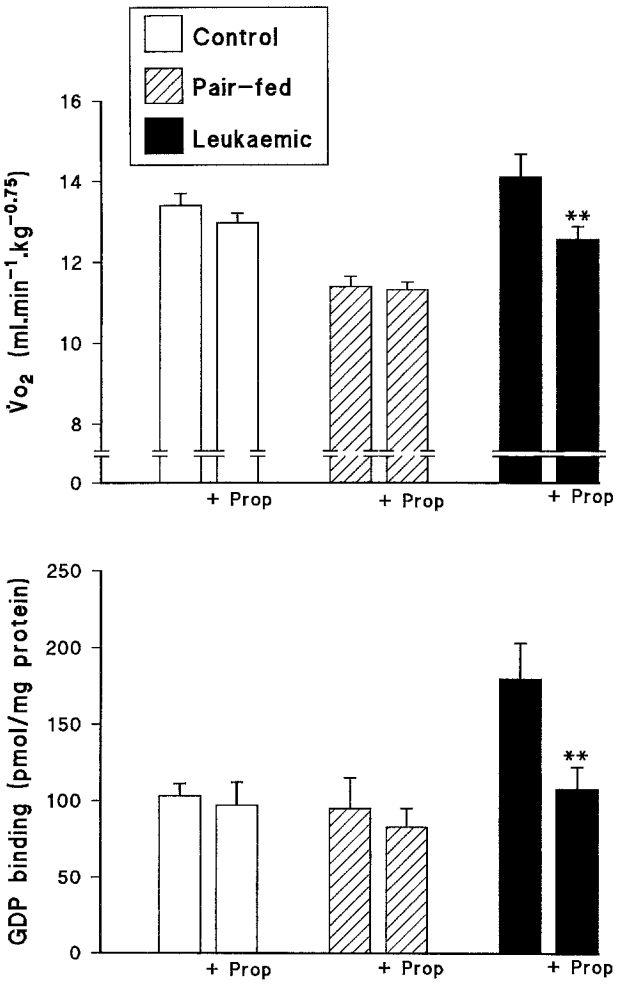

Fig 4. Effect of L-propranolol (10 mg/kg intraperitoneally) on resting $\dot{V}o_2$ and BAT activity determined by GDP binding in control, leukemic, and pair-fed rats. Values are the mean ± SEM (n = 4 to 7). **$P < .01$, paired and unpaired Student's t test.

animals and 24% compared with the pair-fed group ($P < .01$, one-way ANOVA). Propranolol had no effect on $\dot{V}o_2$ of either control or pair-fed animals, but significantly reduced (11% ± 2%) $\dot{V}o_2$ in leukemic animals ($P < .01$, paired Student's t test).

The thermogenic activity of BAT (assessed in vitro by GDP binding) was similar for control and pair-fed animals irrespective of whether they were treated with propranolol (Fig 4). Leukemic animals injected with vehicle exhibited increased BAT activity compared with nontreated control or pair-fed groups (74% and 89%, respectively, $P < .05$, one-way ANOVA). Propranolol treatment significantly reduced BAT activity in leukemic animals by 40% ($P < .01$, unpaired Student's t test).

DISCUSSION

The T-cell leukemia used in this study was identified after injection of ^{185}W tungsten trioxide into rats,[24,25] and has been subsequently characterized.[20] We have shown that implantation of these cells into rats results in rapid disease progression, as indicated by significant increases in the circulating WBC count and liver and lymphoid organ weights. The rat T-cell leukemia shows features common to human leukemias, particularly acute lymphoblastic leukemia, in which high blast cell counts and enlargement of these organs have been reported.[20,26,27] Although few data exist for these patients, cachexia has been observed in various forms of human leukemia.[28] All animals appeared healthy until day 16, after which they demonstrated lethargy and poor coat appearance. Experiments were usually terminated on day 17 to 18, when rats appeared moribund and wasted. The magnitude and time course of increases in WBC count were consistent both within and between separate experiments (data not shown).

The data shown in Fig 2 indicate that the pair-feeding regimen was successful, since food intakes were closely matched in pair-fed and leukemic animals throughout the course of the experiment. Up to and including day 12, the body weight change was also similar in these two groups, but thereafter declined more rapidly in pair-fed than in leukemic rats. This somewhat surprising result indicates that feeding efficiency was increased and energy expenditure was reduced in leukemic animals compared with pair-fed controls. This is in contrast to studies on animals with solid tumors,[29,30] which often report reduced energetic efficiency. However, this assumption is contradicted by measurements of oxygen consumption (see below) and carcass composition. Although pair-fed rats lost more weight than leukemic animals, their body fat content was slightly (but not significantly) higher. Thus, measurement of body weight or weight change in leukemic animals did not accurately reflect changes in body energy content. Leukemic rats showed higher body water content than pair-fed animals (23 g excess), which masked the loss of body weight. Measurements of body composition did take account of increased leukemic cell mass of the lymphoid organs, particularly the spleen (days 14 to 17, 10-fold; Fig 1), which may have contributed to the increase in body weight. Thus, measurements of body weight significantly underestimated loss of body energy stores in T-cell leukemia in the rat, and the onset of cachexia may have occurred earlier than would be assumed from measurements of body weight.

Body composition data indicate that fat-free dry mass (calculated by the difference between body weight and measured fat and water content) was comparable in pair-fed and leukemic animals (leukemic, 54 ± 5 g; pair-fed, 50 ± 3 g), which suggests that body protein was spared largely at the cost of fat. However, gastrocnemius muscle mass was reduced in leukemic rats compared with control rats (24%, $P < .001$, unpaired Student's t test), indicating redistribution of protein. For example, loss of skeletal muscle protein may have been accompanied by increased hepatic and spleen mass, due to acute-phase protein synthesis and WBC mass.

Total daily energy expenditure was not determined in the present study, but resting oxygen consumption, measured for periods of several hours during the daytime, declined steadily in pair-fed rats during the period when food intake was restricted (day 13 onward; Fig 3). This finding is

consistent with the known reduction in energy expenditure and increased efficiency of energy utilization during food restriction, due in part to inhibition of diet-induced thermogenesis.[31] In direct contrast, resting oxygen consumption increased in leukemic rats from day 12 to values slightly greater than in controls but significantly higher than in pair-fed animals. For example, on days 15 and 16, $\dot{V}O_2$ corrected for body size was elevated in leukemic rats by 13% to 19% versus controls and by 31% to 38% versus pair-fed rats. It is highly unlikely that these differences were due to changes in physical activity, since all animals were quiet or sleeping in the calorimeters and leukemic animals appeared less active than other groups at all times. Data for oxygen consumption were expressed per unit metabolic body size, but absolute rates of oxygen consumption or $\dot{V}O_2$ were also increased to a similar extent in leukemic versus pair-fed rats (data not shown).

In the absence of continuous, 24-hour measurements of $\dot{V}O_2$, it is not possible to extrapolate results for oxygen consumption to total energy expenditure, and it is possible (but unlikely) that measurements of nighttime $\dot{V}O_2$ may have revealed a different pattern. Body temperature was also significantly increased in leukemic rats on day 15, but declined on the last 2 days when they appeared unwell. Although the elevation in body temperature could have resulted from an infection or leukemic fever, it may be dependent on $\dot{V}O_2$, since the increase in temperature coincided with the period of increased $\dot{V}O_2$. Any increase in metabolic rate not accompanied by increased heat loss will result in an increased temperature and may have occurred because of an inappropriate change in heat loss. A similar observation has also been made in moribund mice with malaria.[32]

The hypermetabolism apparent in leukemic animals was also accompanied by a marked increase in the thermogenic activity of BAT, the principal site of nonshivering thermogenesis in small rodents.[33] Elevated metabolic rates during disease are frequently associated with increased BAT activity,[18] at least in experimental animals, and this has also been observed in cachectic tumor-bearing rats.[29] BAT activity of children with solid tumors is also significantly increased,[34] indicating that BAT thermogenesis may contribute to the altered energy balance observed in humans and experimental animals with malignant disease.

The hypermetabolism and increased BAT activity observed in leukemic animals were both inhibited by administration of the adrenoceptor antagonist, propranolol. Sympathetic activation of BAT thermogenesis has also been implicated in the responses to pyrogens[18] and in the hypermetabolism apparent in patients with solid tumors.[35]

Overall, the results of this study indicate that T-cell leukemia in the rat provides a useful experimental model for the study of leukemia. A disadvantage, in common with the majority of animal tumors, is the rapid progression of disease as compared with malignancy in humans. Reduced food intake appears to be the primary cause of weight loss, but energy expenditure is also inappropriately high in leukemic animals and appears to be mediated, at least in part, by sympathetic activation of BAT thermogenesis. These data also raise several important points about interpretation of studies on cachectic animals that may be relevant to clinical research. First, measurements of body weight alone can be misleading: because of underlying changes in body composition, the extent of cachexia may be severely underestimated. Second, it is often inappropriate to compare body weight, food intake, and metabolic rate of cachectic animals or patients versus freely feeding controls—comparison with a pair-fed group may be more appropriate. Finally, even when food intake is markedly reduced, this may not be the only mechanism leading to weight loss.

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