Effects of Nandrolone Propionate on Experimental Tumor Growth and Cancer Cachexia

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We studied the tumor host response to excessive doses of an anabolic steroid (nandrolone propionate, 2.5 mg 20 g intraperitoneally every second day for 11 days) with respect to body composition and tumor cell kinetics in MCG 101 sarcoma–bearing mice (C57BL/6J) with progressive cachexia. Although survival and food intake were not affected, a significant weight gain was observed that was essentially attributed to water retention. Net protein content was increased only to a minor extent (15%), of which only the liver accounted for a significant part of the body compartments. Hepatic protein accumulation was obviously caused by decreased protein degradation, since hepatic RNA content was unchanged. After anabolic steroid administration, reduced histochemical staining of succinate dehydrogenase was observed in skeletal muscles rich in oxidative type 1 fibers, but it was not different from that of tumor-bearing control animals, which was also confirmed by measurements of citrate synthase and cytochrome *c* oxidase activities in skeletal muscle and liver tissue. The anabolic steroid had no significant effect on tumor growth in terms of weight progression, energy state, polyamine synthesis rate, cell division rate, and cell cycle cytocompartments. We conclude that anabolic steroid supplementation is not therapeutically beneficial in counteracting progressive weight loss in experimental cancer.

CANCER is mostly characterized by progressive host wasting, which is a result of a complex process involving anorexia and metabolic abnormalities in the host tissues. Circulating catabolic factors such as cachectin and interferon and tumor parasitization may well contribute to the negative energy balance and further impoverish the cancer-bearing host. The ultimate death is mostly a result of a metabolic derangement in which host tissues become short of energy for cellular survival. Clinically, the debility of the malnourished cancer patient is well recognized in terms of reduced muscle mass and fat tissue. Although exogenous supply of nutrition can rectify a negative balance,[1,2] it seems attractive to treat cancer patients with protein-synthesis promoters such as anabolic steroids primarily to restore muscle proteins, provided that tumor growth is not stimulated. In a previous study on metastatic cancer patients, beneficial effects on body weight were observed despite the fact that improved survival could not be achieved.[3] Other studies have more specifically demonstrated that anabolic steroids may induce an increase of muscular mass in nonmalignant conditions in humans.[4] The present study was performed to analyze the tumor host response to anabolic steroids with respect to body composition and tumor cell kinetics in tumor-bearing mice.

MATERIALS AND METHODS

Model

Three-month-old, weight-stable (20 to 25 g) female mice (C57BL/6J, Bomholtgård, Ry, Denmark) were used throughout. A poorly differentiated sarcoma (MCG 101) originally induced by methylcholanthrene was used as an isogenic tumor graft. Animals were anesthetized by intraperitoneal injection of pentobarbital 0.06 mg/g, and the tumor was then transplanted subcutaneously into the flanks under aseptic conditions. The tumor does not penetrate the abdominal wall or metastasize. Its influence on the host metabolism has been evaluated in a series of reports.[5,6] The tumor is palpable after 5 to 7 days and demonstrates an exponential growth rate until the tumor-bearing mice die with cachexia 15 to 17 days after tumor implantation.[7,8] Anorexia is demonstrable from days 9 to 10, and the cachectic reactions of the host to the tumor are considered similar to those observed in clinical cancer.[6,9]

Animals were kept in individual cages with coarse sawdust bedding and had free access to a Purina chow diet (ALAB, Stockholm, Sweden) and tap water unless otherwise stated. The ambient temperature was 25° to 26°C, and the mice were maintained on a 12-hour light/dark cycle.

In all experiments, the anabolic steroid (Anadur, 1 mL contains nandrolone 3-(4-hexyloxypheny)-propionate 25 mg, preservative [phenylcarbinol] 50 mg, arachis oil to 1 mL, kindly supplied by Kabi Pharmacia Therapeutics, Helsingborg, Sweden) was administered in a dose of 2.5 mg/20 g intraperitoneally every other day to the study group. Treatment started with the first dose given on day 1 after tumor implantation and continued for 10 to 12 days. The dose was adjusted to the smallest amount producing a significant weight gain. Tumor-bearing control animals were sham-injected with arachis oil. In experiments to study the morphologic and mitochondrial enzyme histochemistry of skeletal muscle, non–tumor-bearing animals eating ad libitum were also used for comparative reasons. Animals were killed by cervical dislocation unless otherwise stated.

Experimental Protocol

The effects of nandrolone propionate on survival were studied. The time from tumor implantation to spontaneous death was registered for both study and control mice. Metabolic and tumor cell kinetic effects of nandrolone propionate were also examined. These experiments were performed 10 to 12 days after tumor implantation, when the tumor size was approximately 2 to 3 g.

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The Tumor-Bearing Host

Food intake and body composition. Individual food intake was measured continuously by daily weighing of food consumption. Body weight, tumor weight, and carcass weight (tumor-free body weight) were determined. Dry weight was determined after drying the body and the tumors separately in an oven at 80°C.[9] Lipids were extracted from the whole carcass with chloroform-methanol (1:1), ethanol-acetone (1:1), and then pure ether, which was allowed to evaporate.[9] The lipid-free dry weight represents whole-body protein content.[10]

In separate experiments, wet and dry weights were also measured for skeletal muscle, liver, spleen, and heart after removal of fat and connective tissue.

Nitrogen determination. Tissue nitrogen was analyzed with a modified micro-Kjeldahl technique using a Technicon Autoanalyzer (Stockholm, Sweden) after predigestion with sulfuric acid in a Tecator system (Höganäs, Sweden) with spectrophotometric determination as described by Tetlow and Wilson.[11]

Determination of hepatic RNA. RNA was determined according to the method reported by Munro and Fleck.[12] Liver tissue was immediately frozen in liquid nitrogen after excision. RNA was determined as absorbance at 260 nm. All samples were corrected for unspecific absorbance at 260 nm by protein determination. Calf thymus RNA was run as a standard, and the result was expressed as grams of RNA per kilogram wet tissue weight.

Morphologic and mitochondrial enzyme histochemical analysis. Skeletal muscles were morphologically investigated. The entire right hind leg was removed and frozen in a mixture of propane and propylene chilled by liquid nitrogen, with the foot perpendicular to the calf. Cross-sections of the muscle at the middle of the calf were cut in a cryostat, and enzyme histochemical staining of myofibrillar adenosine triphosphatase and succinate dehydrogenase[13] was performed to study the distribution of mitochondria in different muscle fiber types.

Cytochrome c oxidase activity. Activity in skeletal muscle and liver tissue was estimated polarographically as oxygen consumption, using a Clark oxygen electrode according to the method described by Tottmar et al.[14]

Citrate synthase activity. Activity in skeletal muscle and liver tissue was determined according to the method reported by Shepherd and Garland[15] in homogenates prepared in 10 vol Tris hydrochloride buffer (50 mmol/L), pH 8.2, containing EDTA (1 mmol/L) and $MgSO_4$ (5 mmol/L).

The Tumor

Ornithine decarboxylase. Ornithine decarboxylase ([ODC] a rate-limiting enzyme for polyamine synthesis) activity was determined according to the method reported by Edström et al,[16] which is a minor modification of the method described by Noguchi et al.[17] Scintillation counting was performed with an LKB Wallac 1215 Racbeta II liquid scintillation counter (Sweden), whereby the counts of the sample divided by the counts of the baseline sample gave the amount of $^{14}CO_2$ released per hour. Tissue activity of ODC could then be expressed as nanomoles CO_2 per hour per milligram protein.

Bromodeoxyuridine administration and tumor tissue preparation for bivariate flow cytometric analysis. In vivo uptake of bromodeoxyuridine ([BrdU] no. B-5002, Sigma, St Louis, MO) into tumor DNA was accomplished by intraperitoneal injection of BrdU 0.1 mg/g body weight. The injection was given 3.5 hours before animals were killed. Solid pieces (~ 100 mg) of tumor tissue were fixed directly in 70% ethanol and stored at 4°C for at least 12 hours. Nuclei extraction, BrdU administration, and DNA staining were performed as described elsewhere.[18]

Specimens were analyzed on a FACScan flow cytometer (Becton Dickinson) connected to a Hewlett Packard 310 computer. The optic beam for simultaneous fluorescein and propidium iodide excitation was a 15-mW, 488-nm, air-cooled argon-ion laser. Wavelengths corresponding to the maximum emission were 525 nm (green fluorescence) and 610 nm (red fluorescence) for the respective fluorochromes.

Calculations of labeling index, DNA synthesis time (T_s), potential doubling time (T_{pot}), and DNA distribution were performed as described previously[18-20] using the Lysus II program, assuming a uniform distribution and a linear progression of cells throughout the S phase. At least 10,000 events were collected in list mode for these calculations (Fig 1). Briefly, T_s and T_{pot}, the cell division time not taking into account the cell loss, are derived from quantification of the relative movement (RM), which at time zero is assumed to be in mid–S phase and accordingly has a value of 0.5. RM will

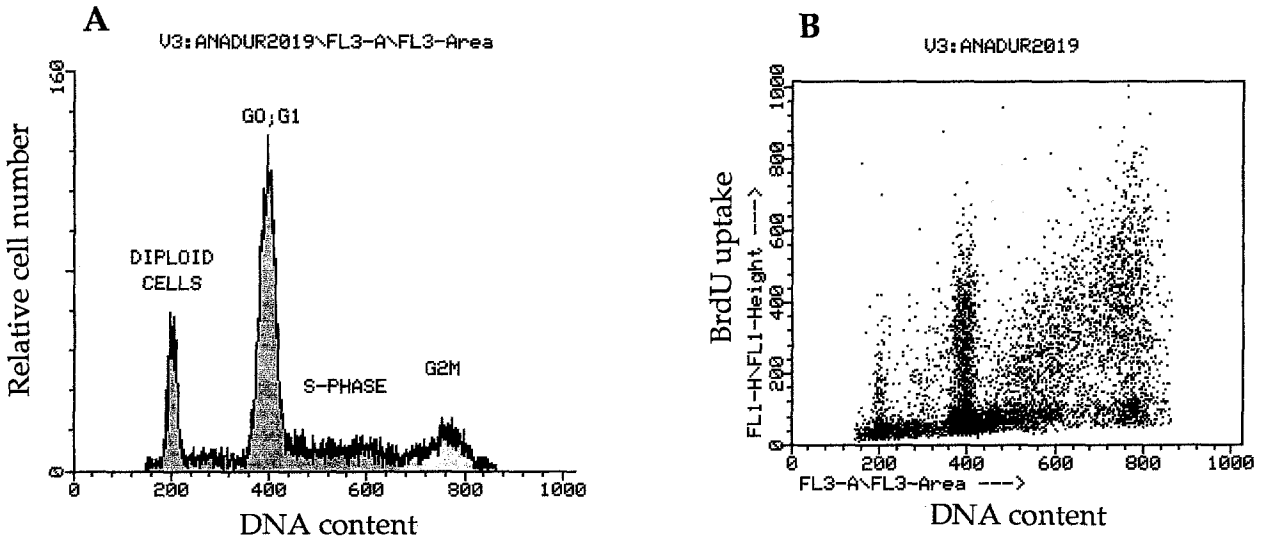

Fig 1. Representative flow cytometric distributions of (A) total DNA content and (B) corresponding BrdU fluorescence 3.5 hours after intraperitoneal BrdU injection. DNA content: 200 = diploid cells (supporting cells and inflammatory cells), 400 = G_{0-1}, and 800 = G_2M.

eventually reach a value of 1.0 when the only BrdU-labeled cells that have not yet divided now reside in G_2M. Thus, the increase in RM from 0.5 to 1.0 during the time from BrdU injection to tumor sampling represents T_s. T_{pot} can be calculated from the equation $T_{pot} = \lambda \times T_s$/labeling index, where λ is a correction factor for the age distribution of the tumor population and a value of 0.8 has been assumed.[18]

Energy status. Nonnecrotic parts of tumor tissue were rapidly frozen in liquid nitrogen and freeze-dried for 8 hours (LYOVAC GT 2; Leybold-Heraeus, Köln, Germany). Tumor tissue was then minced to a powder. Perchloric acid 0.285 mL (1.5 mmol/L) containing 1 mmol/L EDTA was added to 15 mg dry powder, and the extraction was performed by gentle agitation for 20 minutes on ice. The precipitate was separated by centrifugation and neutralized before injection onto the high-performance liquid chromatography column[21] (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was a prepacked reverse-phase C2/C18 Silica Column, Mino RPC S5/20 (5 μm, 4.6 × 200 mm). The elution medium mobile phase consisted of 0.1 mol/L ammonium dihydrogen phosphate buffer ($NH_4H_2PO_4$) with the pH adjusted to 6.0 with 3 mol/L ammonium hydroxide. The nucleotides, nucleosides and purine bases, adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate, inosine, and hypoxanthine were separated, and concentrations were determined as described in detail elsewhere.[22] The energy charge was calculated according to the equation reported by Atkinson[23]: energy charge = ([ATP] + 1/2[ADP])/([ATP] + [ADP] + [AMP]).

Statistics

Differences between two groups were tested with Student's *t* test. Results are presented as the mean ± SE; $P < .05$ is considered statistically significant.

RESULTS

Effects of Nandrolone Propionate on the Tumor-Bearing Host

The spontaneous survival time was not affected by the anabolic steroid. Thus, all animals died within 15 to 17 days after tumor inoculation. Food and water intake were not significantly changed in these animals during the study period as compared with sham-injected tumor-bearing animals.

Body weight increased gradually from day 3 to day 10, at which time the difference was 2 g (Fig 2) in comparison to sham-injected animals. This difference was primarily attributed to water retention (85%), but also to protein accumulation (15%; Fig 3). Lipids were not changed. Relative protein accumulation was found in liver tissue in terms of dry weight and nitrogen content (Fig 4A and B), whereas RNA content in liver tissue (Fig 4C) and hepatic mitochondrial enzyme activity, determined as activities of citrate synthetase and cytochrome *c* oxidase, were not changed (Fig 5). None of the corresponding enzyme activities were changed in either skeletal muscle, heart muscle (not shown), or spleen (not shown) as compared with those in sham-injected tumor-bearing animals.

The enzyme histochemical staining of succinate dehydrogenase showed patches of reduced enzyme activity in many type 1 muscle fibers in the deep portion of the lateral head of the gastrocnemius muscle of most tumor-bearing mice as

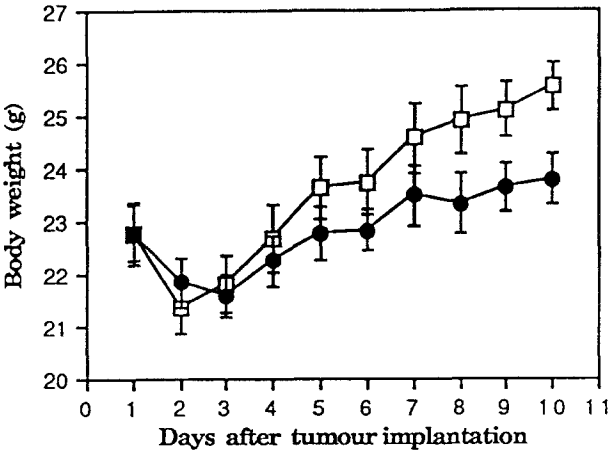

Fig 2. Development of body weight after tumor implantation in (□) mice treated with injections of nandrolone propionate and (●) sham-injected controls. n = 10 animals in each group (mean ± SEM).

compared with non–tumor-bearing animals (Fig 6). The same change was seen in the soleus muscle of occasional tumor-bearing animals. This change was generally restricted to these muscles, which are rich in oxidative type 1 fibers. It was not observed in the muscles of normal mice. Tumor-bearing animals that had received the anabolic steroid demonstrated the same type of change. There was also an apparently reduced cross-sectional area of muscle fibers in tumor-bearing mice (Fig 6), although morphometric analysis was not performed.

Effects of the Anabolic Steroid on the Tumor

Effects of the anabolic steroid on the tumor in terms of weight, ODC activity, cell kinetic measurements of BrdU uptake, and energy status are shown in Table 1. Thus, in animals treated with the anabolic steroid, tumor weight, ODC activity, cell division rate, and energy status were not significantly changed.

DISCUSSION

This study describes the effects of nandrolone propionate administration on survival and on metabolic tumor host

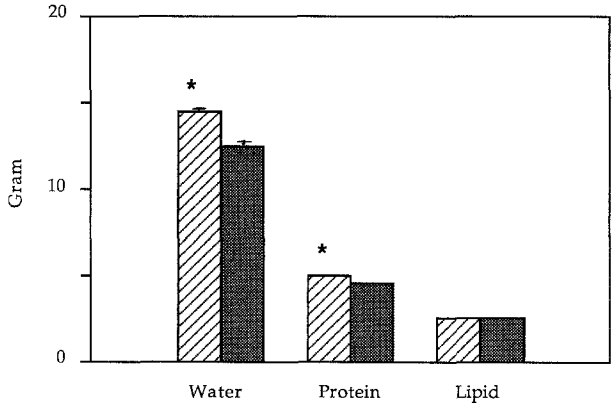

Fig 3. Body composition in freely fed (▨) tumor-bearing mice treated with nandrolone propionate and (■) tumor-bearing controls. n = 10 animals in each group (mean ± SEM). *$P < .05$.

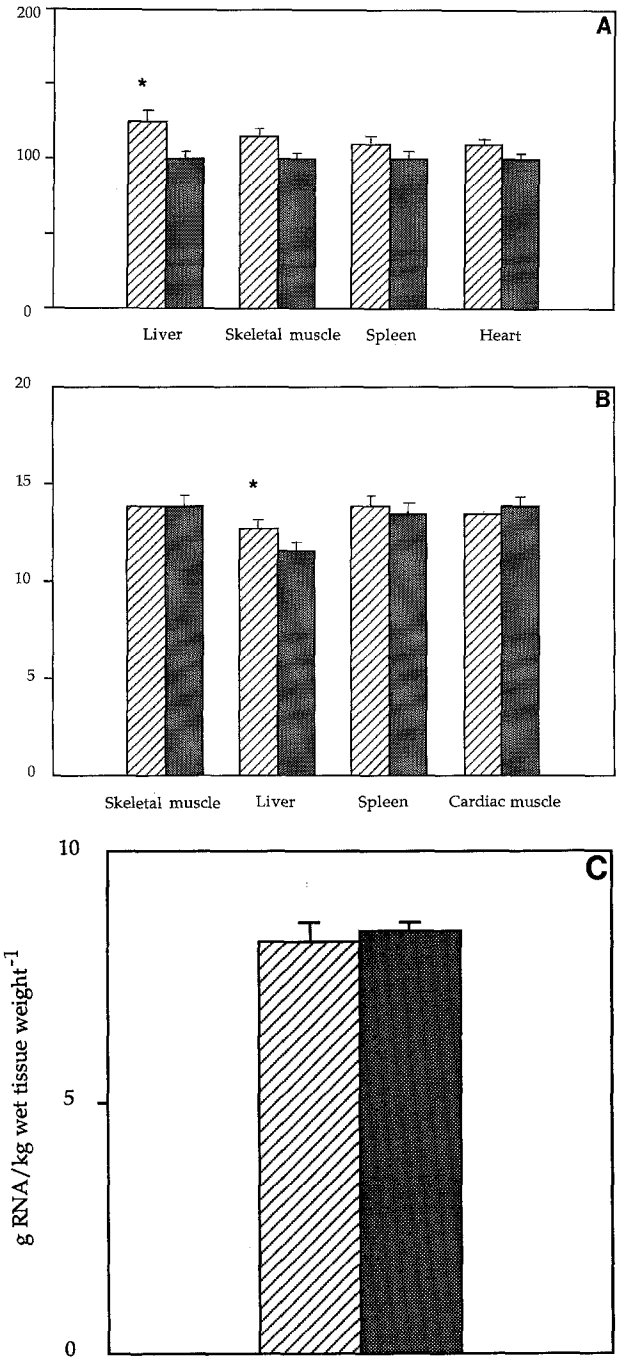

Fig 4. (A) Relative dry weight (g) of visceral organs and skeletal muscle from (▨) tumor-bearing mice treated with nandrolone propionate. (■) Tumor-bearing controls = 100%. n = 10 animals in each group. (B) Nitrogen content (g/100 g tissue) in visceral organs and skeletal muscle. n = 10 animals in each group. (C) RNA content (g/kg wet tissue weight) in liver tissue from nandrolone propionate–treated mice (n = 8) and sham-injected tumor-bearing controls (n = 6). Mean ± SEM.

reactions of weight-stable mice with a methylcholanthrene-induced sarcoma. Nandrolone propionate is an anabolic steroid with a relatively long duration of action[24] primarily consisting of androgenic and nitrogen-retentive properties. The anabolic steroid dose administered every second day in this study was adjusted to the smallest amount generating a significant increase of tumor host weight during a study period of 10 to 12 days after tumor inoculation. This observation time was chosen because loss of carcass weight after that is well recognized and the tumor burden will subsequently reduce the motile activity of the host.[25,26] The cumulative amount of nandrolone propionate administered was equivalent to that taken in uncontrolled use by body builders.[27]

A significant gain of total body weight was observed without an effect on dietary intake, survival, or tumor net weight. The weight gain was essentially attributed to water retention of the host tissues, and protein mass accounted for only 15%. A significant increase of protein mass was observed in liver tissue. We have previously reported that even in untreated tumor-bearing animals there is a significant enlargement of liver tissue,[6,26] which probably constitutes an immunologic response to this immunogenic MCG 101 sarcoma. The additive increase of hepatic protein and nitrogen content in anabolic steroid–treated animals was obviously due to a decreased degradation rate of structural proteins, since hepatic RNA content was not changed in these animals as compared with tumor-bearing controls. It was obviously not a consequence of a hepatotoxic effect,[28] as shown by the unchanged activities of hepatic mitochondrial enzymes.

The enzyme histochemical studies demonstrated a pronounced focal loss of succinate dehydrogenase activity in type 1 muscle fibers and generally smaller muscle fiber diameter in gastrocnemius muscle from anabolic steroid–treated and control tumor-bearing animals as compared with healthy non–tumor-bearing animals (Fig 6). Although there was a trend toward increased dry weights of skeletal muscle and heart tissue, as well as of the spleen, for mice treated with the anabolic steroid, compared with control

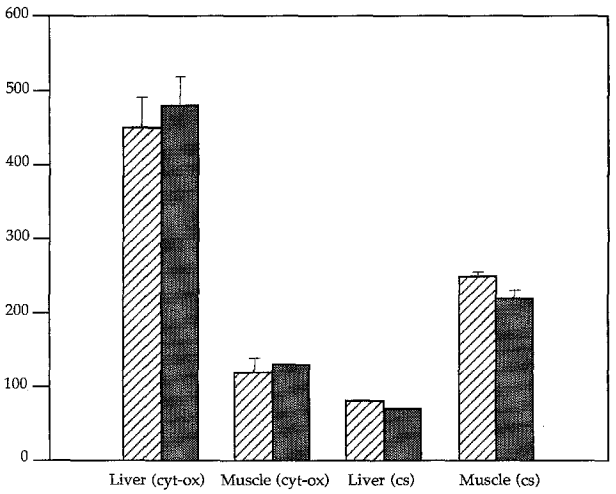

Fig 5. Citrate synthase ([CS] mmol 5,5 dithiobis-(2-nitrobensoic acid) (DNTB) · min^{-1} · kg $protein^{-1}$) and cytochrome *c* oxidase ([cyt-ox] mmol 02 · min^{-1} · kg $protein^{-1}$) activities in liver and skeletal muscle tissue from (▨) nandrolone-treated tumor-bearing mice and (■) tumor-bearing controls. n = 10 animals in each group (mean ± SEM).

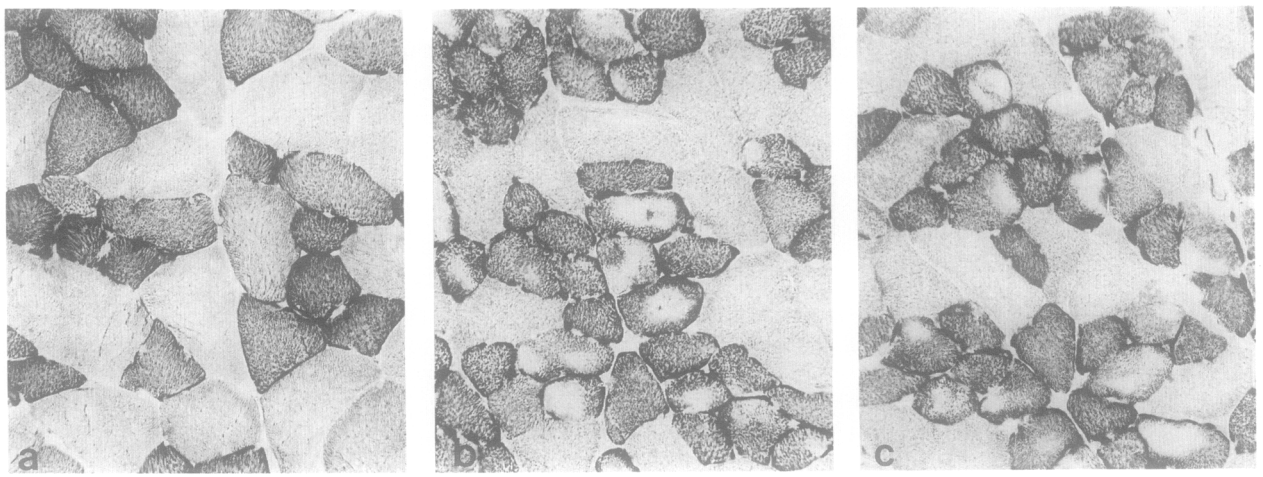

Fig 6. Cross-sections of deep portion of lateral head of gastrocnemius muscle after enzyme histochemical staining of the mitochondrial enzyme succinate dehydrogenase. (a) Normal muscle showing small, dark, type 1 muscle fibers with high enzyme activity and large, pale, type 2 muscle fibers with low enzyme activity. (b) Muscle of tumor-bearing mouse showing focal loss of enzyme activity in type 1 muscle fibers and generally smaller muscle fiber diameter as compared with the normal muscle in (a). (c) Muscle of tumor-bearing mouse treated with anabolic steroids showing the same type of changes as in the untreated mouse in (b).

tumor-bearing animals, statistical significance was not reached. This is in accordance with previous studies on catabolic patients[29,30] receiving anabolic steroids and tumor-bearing animals receiving growth hormone.[31] Although anabolic steroids can improve nitrogen balance during the first few days after minor operations in well-nourished patients, this effect seems to be transitory.[32] It is therefore likely that muscular effects can only be demonstrated if much greater doses are administered,[28,33] as in this study, and if the exposed individuals perform active muscular training concomitant with a significantly increased protein intake.[34] In this context, previous studies have demonstrated that exogenous insulin supplementation to tumor-bearing mice in vivo did not affect muscle RNA or protein content, although the sensitivity and responsiveness to insulin in vitro was retained.[35] On the other hand, insulin has been reported to reverse tumor-induced biochemical abnormalities and weight loss in tumor-bearing animals.[36] Additional compositional analysis demonstrated a preservation of host nitrogen.[37] However, in these studies it cannot be excluded that the improved food intake induced by insulin was the primary explanation for the absence of malnutritional findings. Whatever the mechanism of action of insulin, it does not seem to be a limited capacity for protein synthesis, since nutritional supplementation can reverse a negative energy balance in experimental cancer,[2,38] as well as in cancer patients.[1]

Table 1. Tumor Response to Nandrolone Propionate Treatment

	Nandrolone Propionate (n = 10)	Controls (n = 10)	*P*
Wet weight (g)	2.26 ± 0.28	2.40 ± 0.28	NS
Dry weight (g)	0.38 ± 0.06	0.40 ± 0.05	NS
Cell kinetics			
ODC activity (nmol/CO_2/h/mg protein)	1.8 ± 0.7	1.6 ± 0.8	
NS			
Labeling index	23.8 ± 2.2	24.5 ± 2.8	NS
T_s (h)	10.0 ± 1.0	9.2 ± 0.5	NS
T_{pot} (h)	35.0 ± 2.8	33.7 ± 4.4	NS
Cell cycle cytocompartments			
$G_{0\text{-}1}$ (%)	52.7 ± 1.9	55.7 ± 1.2	NS
S (%)	33.4 ± 1.7	30.8 ± 1.6	NS
G_2M (%)	15.2 ± 1.0	14.9 ± 0.6	NS
Energy state			
ATP	4.58 ± 0.65	4.58 ± 0.49	NS
ADP	3.93 ± 0.36	3.40 ± 0.26	NS
AMP	2.67 ± 0.19	2.18 ± 0.10	NS
IMP	2.25 ± 0.20	1.96 ± 0.13	NS
Hypoxanthine	1.66 ± 0.22	1.33 ± 0.09	NS
Inosine	3.98 ± 0.40	3.48 ± 0.26	NS
Energy charge	0.59	0.62	

Abbreviation: IMP, inosine monophosphate.

Tumor net weight was not affected by nandrolone treatment. Although insulin can stimulate tumor growth in vitro,[39] the effects of nandrolone did not differ in this respect from previous reports about the in vivo effects of other anabolic hormones such as insulin,[35-38,40] growth hormone,[31] and hydrocortisone[41] on tumor growth. However, tumor net weight is the result of the size and cell-division rate of the growth fraction, as well as the fractional cell death. This means that significant changes in these parameters might be observed despite the fact that tumor net weight is unchanged. In the present study, T_{pot} and the size of the growth fraction (labeling index of BrdU uptake) were 35 hours and 24%, respectively. The percentage of S-phase cytocompartment was somewhat higher than that of BrdU-labeled cells, which was probably due to the fact that not all cells in the S-phase fraction were actively synthesizing DNA. Since the tumor net weight and size and cell-division rate of the growth fraction thus were not changed, it could be assumed that the fractional cell death was not changed either. Previously,[20] we calculated the fractional cell death to be approximately 34%. This means that the tumor doubling time could be estimated to be 53 hours in the present study, which is consistent with previous

estimations based on continuous weighing of our experimental tumor.[42]

The observation of an unchanged ODC activity and energy charge in the tumor further supports the view of an unaffected tumor growth and substrate availability for the tumor.[20,43-45]

In conclusion, the most important observation in this study is that the anabolic steroid produced water retention in the tumor-bearing animal without providing the host with meaningful nitrogen storage. For this reason, we do not consider anabolic steroids therapeutically beneficial for counteracting the progressive weight loss in experimental tumors, and probably not in clinical cancer.

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