Effects of Systemic Acidification of Mice with Sarcoma 180

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ABSTRACT

The effects of dietary-induced acidosis on the growth and rates of complete regression of Sarcoma 180 in mice have been studied. The experiments here reported have demonstrated that mineral acidification of laboratory food produces a late decrease in tumor growth and significantly increases the rates of complete tumor regression. Blood acid-base studies also demonstrate the effects of these diets in altering the acid-base balance, and seemingly, this is independent of starvation and/or ketosis. The relationships of such in vivo acid-base metabolic changes to the control of tumor metabolism are briefly discussed. A therapeutic potential for this preliminary approach is considered.

INTRODUCTION

The effects of starvation in retarding tumor growth are well known (4, 34). Similarly, the favorable influence of acidosis on tumor control and regression has long been recognized (1, 14, 26, 30, 41). Different acids added to diets have been shown to be effective in preventing liver cancer in rats (28), decreasing tumor growth in rabbits (41), and inhibiting the growth of solid tumors and leukemias in mice (1). Spontaneous regressions of the s.c. form of Sarcoma 180 transplanted to Swiss mice have been reported in up to 23%, varying with the different systems used by separate investigators (18, 40). Mice transplanted with this nonmetastasizing tumor presumably die of local necrosis and/or infection and secondary toxemia. Caloric restriction amounting to more than 33% of the food consumed by control groups has been shown to significantly retard the growth of this particular tumor (2), and this inhibition has been shown to be related to caloric restriction per se rather than to a deficiency of specific nutrients (3). A marked effect of lowering pH on the control of tumor glycolysis has been shown repeatedly in different neoplastic cells (10, 20, 35, 47). These facts have led many investigators to attempt to interfere with tumor growth through the use of specific inhibitors of tumor glycolysis (6, 21, 29). Unfortunately, in most of these studies the direct effects of environmental H+ concentration were not taken into account. The experiments reported here were planned and undertaken to delineate the interference of in vivo tumor glycolysis and tumor growth through a low systemic pH (32, 47), independent of starvation in the Sarcoma 180 tumor system.

MATERIALS AND METHODS

Three experiments were performed sequentially. Experiment 1 compared the rates of tumor growth and survival of normally fed mice with those of mice fed with quantitatively different acidified diets. Experiment 2 was conducted to determine the effects of different diets on blood acid-base status as well as the rates of growth of tumor-free mice subjected to different dietary measures. Experiment 3 was performed to confirm the findings of Experiment 1 and also to study the effects of acidification of both water and food on tumor growth.

Experimental Design

Experiment 1

Experiment 1 consisted of 3 different groups.

Group 1. The control group was composed of 20 mice. Nonacidified laboratory food and water were served ad libitum from Day 0.

Group 2. This group consisted of 40 mice implanted with tumor on Day 0 and subjected to 4 different periods of 2 N HCl food (Days 0 to 6, 7 to 9, 12 to 18 or 19, and 21 to 24 or 25). Between the "acidifying" periods the mice received nonacidified food (see Chart 1). Four of these mice were sampled on Day 5 to study their acid-base status.

Group 3. Forty mice received a 1 N HCl diet beginning the day of tumor transplant continuously throughout the duration of the experiment (72 days).

Concurrent with these studies, acid-base parameters were determined in 10 normal mice (Table 1, control group). Another 4 normally fed mice growing large tumors were studied to assess the effects of tumor growth on the acid-base balance (Table 1, 32 days after implantation).

Experiment 2

This experiment did not involve tumor transplantation and was conducted instead to determine the subacute (Day 5) and chronic (Days 14 and 32) effects of the 1 N HCl diet on acid-base balance, as well as the amount of food eaten by normally fed versus acid-fed mice. The rates of weight gain and growth of 1 N HCl-fed mice were compared to those of mice fed under the same conditions in Experiments 1 and 3 which carried a tumor transplant. This experiment involved 25 mice.

Experiment 3

This experiment was designed to confirm and extend Experiment 1. Four different groups with 20 mice in each group were transplanted with Sarcoma 180 on Day 0 and received the following diets.

Group 1. The control group received a normal diet plus tap water ad libitum.

Group 2. Group 2 received 1 N HCl food plus tap water ad libitum.

Group 3. Group 3 received 1 N HCl food from Day 0. HCl (0.05 N) water was substituted for tap water beginning on Day 4 (25 ml of 1 N HCl to 500 ml of tap water).
Normal and acid diets were prepared by thoroughly grinding pelleted laboratory chow and mixing it with water or HCI solutions. Each 100 g of powdered food was evenly mixed with either 72 ml of tap water (control diet), 72 ml of 1 N hydrochloric acid (1 N HCI diet), or 72 ml of 2 N hydrochloric acid (2 N HCI diet). From the paste obtained, 4- to 4-cm pieces were separated and allowed to dehydrate at room temperature at least 6 days to recover initial dry weight. In Experiment 3, tap water was acidified with hydrochloric acid to a concentration of 0.05 N HCI while normal tap water was permitted in Experiments 1 and 2. Food and water were always allowed ad libitum. Mice started on their different diets from Day 0, i.e., immediately after tumor transplant and/or group separation.

Assessment of Acid-Base Balance

Venous blood (0.1 to 0.15 ml) from a superficial razor blade cut of the tail vein was collected in heparin-pretreated microcollection capillary tubes (Biochemical Instruments, Medfield, Mass.) tightly applied to the skin on the bleeding point and was immediately mixed using mixing fleas moved within the tube with a magnet. The tubes were closed with tube sealers and immediately stored in ice. Blood gas determinations: pH, PCO2, PO2, calculated bicarbonate, and base excess were performed within one-half hour of collection using a Corning Model 165 pH/Blood Gas Analyzer. Metabolic pH was calculated using a Blood Gas Calculator Type BGC (Radiometer, Copenhagen, Denmark). This pH value, corrected to a PCO2 of 40 mm Hg, represents the metabolic component of the pH, independent of respiration-induced changes. No significant changes in PO2 were observed in any of the treated groups.

Animal and Tumor Evaluation. Mice were periodically weighed, and all tumors were measured by calipers at the times indicated in Charts 1, 2, 4, 6, and 7. The product of the 2 largest perpendicular tumor diameters in sq mm measured by the same observer was used as an index of tumor mass (23).

RESULTS

Experiment 1—Effect of Acid Diets (1 and 2 N HCI) on Growth of Sarcoma 180 and on Growth and Survival of Sarcoma 180-bearing mice. Chart 1 shows the mean weights for all surviving mice in each of the different groups. Standard error of the mouse weights was less than 0.6 g in all experiments. The control group steadily gained weight from the beginning of the experiment. Animals in Group 2 lost an average of 7 g during the first 5 days on the double acidified (2 N HCI) diet. This diet proved intolerable after a few days, with mice of this group able to ingest only 15 to 20% of the amount of food consumed by controls. Mice under these conditions would rapidly become cachectic and die when their weight fell to 26 g, 26 to 28 g, and greater than 28 g. They were then reallocated into the different experimental groups to obtain as accurately as possible the same initial mean weights among the different groups of each experiment. Mice were lodged 5 to a cage. All the groups were maintained in the same room and subjected to the same environmental factors, e.g., temperature, light, and noise. An interval of 4 months elapsed between Experiments 1 and 2, and 2 months elapsed between Experiments 2 and 3. By the end of 72 days, all tumor-bearing animals had either died from tumor growth or had survived following complete regression of the transplanted tumor.

Diets

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Chart 1. Experiment 1. Means of mouse weight in the different groups. Group 1 represents the normally fed control group. Group 2 was subjected to 4 different periods on 2 N HCl diet. Time periods under arrows, acidification periods under 2 N HCl diet. Group 3 was maintained on 1 N HCl diet throughout the duration of the experiment. All tumor transplants were done on Day 0. (For further details, see text.)

Chart 2. Experiment 1. Mean tumor mass in the alive mice of the 3 groups of mice shown in Chart 1.

the control group, the mean tumor size of the living mice in the 2 N HCl Group 2 (111 sq mm) is clearly below (p < 0.05) the mean tumor size (298 sq mm) of the controls (Chart 2). Only 2 mice in the 2 N HCl acid group had died with measurable tumor growth by that time. Mice of Group 2 were fed with normal nonacidified food from Day 26, and the final rates of survival between Groups 1 and 2 is not different (Chart 3).

Group 3 (1 N HCl) behaved in a different manner. Mice in this group initially lost an average of 4 g in weight (Chart 1, day 4), but by Day 35, with no change in diet, the mean weight of the surviving mice had become identical to that of controls. Mice of Group 3 showed tumor growth rates only slightly less than that of controls during the time of maximal growth of Sarcoma 180 (Chart 2, Days 5 to 12), but thereafter the rate of tumor growth was considerably slower. In the control group, one tumor failed to grow and another 3 spontaneously regressed (15%), while all the tumors in Group 3 took and grew. However, the proportion of mice exhibiting complete regression of tumors was considerably higher in the 1 N HCl-fed group than in controls. Finally, 50% of the transplanted mice in Group 3 survived without any evidence of presence of tumor by the end of the experimental period. Tumors of mice in this 1 N HCl-fed group also began to kill their hosts at a later date than in the controls (Chart 3). Life tables using a Breslow test showed a significant difference in survival between the groups (p < 0.001). The final survival rate between the controls (4 of 20) and the 1 N acid group (20 of 40) show a statistically significant difference at the p < 0.01 level using a χ² test.

Experiment 2-Effect of Acid Diet (1 N HCl) on Growth of Normal Swiss Mice. Chart 4 shows the growth of 2 groups of Swiss mice, with almost identical initial mean weights, which were not subjected to tumor transplants. One was normally fed, and the other one was fed with 1 N HCl food from Day 0. Chart 5 shows the amount of food eaten by the mice and the days in the normally fed and 1 N HCl-fed mice without transplanted tumors of Experiment 2. There was a progressive,
Effects of Systemic Acidification with Sarcoma 180

Chart 3. Experiment 1. Life tables and final survival of mice in the control, 2, and 1 N HCl groups shown in Charts 1 and 2.

Chart 5. Experiment 2. Food consumption per mouse and day of the same 2 groups of mice shown in Chart 4 on control and 1 N HCl food. This chart also shows the linear adjustment of mice to the acidified dietary conditions.

Chart 6. Experiment 3. Gravimetric measurements of groups of mice on control and 1 N HCl food, showing no demonstrable differences between the 2 differently fed groups.

linear adjustment to consumption of single-acidified food from Days 0 until 6. Food consumption after Day 5 showed no demonstrable differences between the 2 differently fed groups. Chart 5 also shows an occasional but very significant variation in daily food consumption affecting different cages at different days, a feature which is as yet unexplained.

Experiment 3-Effect of Acid Diets (1 and 2 N HCl) and Acidified Water (0.05 N HCl) on Growth of Sarcoma 180 and on Growth and Survival of Sarcoma 180-bearing Swiss Mice. This experiment was conducted to confirm the results obtained in Experiment 1, while attempting to still further increase the rates of "spontaneous" tumor regression through the addition of acidified water at different times during the experimental period. Once more, the final survival and complete regression of tumors in the group receiving 1 N HCl acidified food plus normal water reached 50%. Despite the fact that 5 of 20 mice (25%) in the control group enjoyed complete spontaneous regressions of tumors (maximal size reached by these 4 tumors, 215 to 260 sq mm) and despite the smaller number of mice in the 1 N HCl group of this experiment, life table analyses of survival were again significantly in favor of the acid diet animals (p < 0.05 using the Breslow Test) (Chart 8). The addition of HCl acid to drinking water to 0.05 normal concen-
tration beginning Day 4 or 14 did not further increase the rate of tumor regression. Acidification of water from Day 4 proved to be intolerable for the mice; this group behaved similarly to the 2 N HCl diet group of Experiment 1. Chart 6 also shows that the addition of acidified water on Day 4 increased the weight loss observed during the first 12 days of the experiment. Similarly, the addition of 0.05 HCl water on Day 14 (Group 4) decreased the subsequent growth rate of that cohort; but, in contrast to the Day 4 acidification group, this group of mice survived the late acidification of water.

The final survival of Groups 2 and 4 in Experiment 3 (1 N HCl food and 1 N HCl food plus 0.05 HCl water after Day 14) was very similar (Chart 8), and the later addition of acidified water did not improve the final results. This suggests that the relative acidification which induces tumor regression must be present during the period of maximal tumor growth between Days 5 and 12. The growth patterns of the tumors in the control groups of Experiments 1 and 3 are slightly different. Observed initial tumor growth for all the groups in Experiment 3 appeared to be faster than in Experiment 1 (Charts 2 and 7), perhaps the result of the larger mass inoculated in Experiment 3. Whatever the cause, this initial growth rate seemed to make no difference in the outcome of similarly treated groups. An unusual course was observed in 3 mice of Group 4. Two tumors initially grew to a considerable extent (160 and 168 sq mm), slowly regressed to 30 and 80 sq mm, respectively, and then regrew, killing their hosts as they reached 506 and 442 sq mm in size. A third tumor grew to 30 sq mm during the first initial days and then "spontaneously" regressed completely by Day 14. However, it too recurred killing its host when it reached 420 sq mm.

Acid-Base Metabolic Data. Acid-base data obtained at Day 0 for 10 normal Swiss mice in Experiment 1 are summarized in Table 1, control groups. Venous samples for determination of acid-base status were also obtained from 4 different mice of Group 2 (2 N HCl) on Day 5. The results obtained after 5 days of semistarvation on 2 N HCl diet are summarized in Table 1, 2 N HCl group. The chronic effects of growing tumors on the acid-base balance of the mice were assessed in 4 mice with large tumors (Table 1, 32 days after implantation). These larger neoplasms induced a degree of chronic metabolic acidosis for which the mice attempted to compensate through hyperventilation. A similar pattern was seen in the most acidotic mouse of Group 2.
on 1 N HCl diet (Table 2, control group). Despite the relatively small number of animals sampled, the difference between the means of metabolic pH of normally fed mice with active tumor growth (pH 7.17) and controls (pH 7.325) is marked enough to be statistically significant (p < 0.05).

Acid-base determinations performed during Experiment 2 (Table 2, control group) demonstrate a degree of acidosis appearing within 5 days of 1 n HCl acid feeding. Table 2, control group also shows that these differences between normally fed and acid-fed mice are maintained after 14 days. However, at the end of 32 days (Table 2, group controls at Day 32 and 32 days on 1 n HCl acidified food) these differences have largely disappeared. At that time, both groups also show a higher mean pH, possibly related to aging (see Ref. 16; Table 2). It is also important to note that after the initial diet-induced weight loss had been restored (see Chart 4, Day 14), an acidosis of a metabolic origin still persisted (Table 2, control group at Day 0 and 1 HCl group at Days 5 and 14 of acidified food), indicating a continued effect of these diets on acid-base homeostasis independent of starvation. In this regard, none of the mice fed with 1 n HCl diets exhibited acetonuria at any time using a sensitive colorimetric method (Acetest; Ames Co., Elkart, Ind.), while this procedure could occasionally detect a tract of acetonuria in mice of the control groups.

**DISCUSSION**

The importance of a persistently elevated pH to cancer growth and progression has been known for many years (26, 27, 30, 50). The relationship of these clinical observations to the characteristics of cancer cells has recently been stressed (16). Further evidence indicates that severe metabolic acidosis could very well have been responsible for some of the spontaneous regressions seen in this or other types of animal tumors. However, it is also likely that many animals die secondary to lactic acidemia before tumor regression can take place.

Severe chronic lactic acidosis is rarely if ever compatible with life in clinical situations. However, it has been suggested that the cancer patient is always a patient with "potential" lactic acidosis (45). This metabolic derangement seems to arise more frequently in laboratory animals transplanted with a variety of tumors, in which high levels of lactic acid have been reported (5, 43). The data in Table 1, 32 days after implantation shows a significantly lower pH in mice carrying large Sarcoma 180 tumors as compared to normal controls (metabolic pH 7.17 versus 7.325). Although direct lactic acid measurements were not performed, it is very likely that the chronic acidemia was mediated by this organic acid which was in turn induced by the transplanted tumor itself. A pathological accumulation of lactic acid could very well have been responsible at least in part for some of the spontaneous regressions seen in this or other types of animal tumors. However, it is also likely that many animals die secondary to lactic acidemia before tumor regression can take place.

Starvation is known to be a significant inhibitor of tumor growth (4, 36). Despite the difficulties in separating the effects of starvation ketosis from those of a directly induced mineral metabolic acidosis in these animal experiments, we must stress the complete lack of ketonuria observed in the successfully treated 1 n HCl acidotic groups, while an occasional trace of acetone was detected in the normally fed mice. This finding agrees with other publications which have shown that the induction of mineral acidosis, apart from decreasing the formation of organic acids such as lactic and pyruvic acids, can also suppress the production of ketone bodies through a direct antiketogenic effect (13, 24). Starvation-induced ketosis was

<table>
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<th>Table 2</th>
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<td>1 n HCl group, Day 14 of acidified food</td>
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<td>Control group at Day 32</td>
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<td>1 n HCl group, Day 32 of acidified food</td>
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*Means and ranges of the acid-base balance of transplanted mice fed with normal and 1 n HCl food after 5 and 14 days. The acidifying properties of 1 n HCl diet become evident after the original weights have been regained by Day 10 (Charts 4 and 5). A respiratory compensation through hyperventilation (low PCO2) occurred in 3 of 5 mice after 5 days on 1 n HCl diet.

**Means and ranges for the different acid-base values of mice of Charts 4 and 5 at Day 32. The acidifying effects of 1 n HCl diet seem to have decreased significantly, but some differences still persist.**
S. Harguindeguy et al.

Chart 8. Experiment 3. Life tables and final survival of mice in the control and treated groups also shown in Charts 6 and 7.

not demonstrable by Acetest during the first 5 days of the experiments. A complete adjustment to the acidified dietary conditions occurred between Days 4 and 5 (Chart 5), and after this time, we would not detect any differences in eating patterns or food consumption among the normally fed and the 1 N HCI-fed animals. It is an unlikely possibility that the results at the end of 72 days were affected by the initial loss of 4 g body weight in the 1 N HCI group during the first 5-day period of dietary adjustment or by depletion of some specific nutrient during this time (3). While a caloric restriction of 33% has been reported to be necessary to retard the growth of Sarcoma 180 (2), this degree of caloric deficit could have occurred only during Days 1 and 2 of these experiments (Chart 5). Also, the initial observable growth of tumors from Days 5 to 10 is practically of the same magnitude in the controls and the 1 N HCI-fed groups of Experiment 1 (Chart 2).

An apparent loss of effect in the acidifying properties of the 1 N HCI diet in the middle stages of the experiment (Table 2, controls at Day 32 and 32 days on 1 N HCI acidified diet) can explain the fact that no more than 50% of the animals showed complete regression of tumor growth. More severe degrees of acidosis were unsuccessfully attempted through the addition of more acid to the food (Chart 1, 2 N HCI diet) or by acidification of drinking water in addition to the acid diet shortly after tumor inoculation (Chart 6, Group 3). Both measures exceeded the capacity of mice to adjust to such acidifying changes (Charts 3 and 8). The addition of acidified water after Day 14 (Chart 6, Group 4) did not further increase the final rates of tumor regression (Chart 8, Group 4). It is possible that different methods of inducing more severe acidosis, which do not create starvation conditions, will be able to further increase rates of complete regression of this or other transplanted animal tumors.

Another interesting feature was observed by comparing the slopes of mice weight in the control groups of Experiments 1 and 2 and the rates of weight "regain" after Day 5 of the 1 N HCI-fed Groups 3 and 2 of Experiments 1 and 2, respectively (Charts 1 and 4). After the first 5 days, at a time when this tumor is already visible and palpable s.c., the Sarcoma 180 transplant produces a rapid negative effect on the normal growth and weight gain of mice, a situation simulating the "anorexia-cachexia" syndrome seen in humans with advancing cancer.

These acid-base considerations strongly suggest that a persistent low pH could prove to be synergistic with chemotherapeutic drugs such as 5-fluorouracil, prednisolone, methotrexate, 6-mercaptopurine, cyclophosphamide, and actinomycin, all of which have been shown to be inhibitory of tumor glycolysis or to affect glycolysis and/or respiration (22, 33, 46, 48). Conversely, the stimulatory effects of insulin (17, 49), glucose (42), and high pH (35) on malignant cell growth, glycolysis, and DNA synthesis could be utilized alone or in combination to stimulate cancer cell activity for controlled periods of time to find a higher specificity for anti-DNA chemotherapeutic agents.

Immunological factors could have also affected the results of these studies. The relationships of H+ environmental changes to cell-mediated immune defense cannot be integrated at the present time because of lack of evidence. A few publications, however, have demonstrated the importance of H+ environment on the glycolysis and function of mature granulocytes and macrophages in inflammation (19, 37) and also in malignant disease (9, 19, 26, 37). Furthermore, a low and falling pH seems to create appropriate environmental conditions for macrophage function in the inflammatory process (25).

In conclusion, total body acidification seems to be a promising avenue in antagonizing tumor growth in animals and its effects seem to be independent of starvation-ketosis. Acidosis per se is not likely to favor solid tumor growth, and it has the potential to delay it at least in some cases. Conversely, environmental field alkalosis per se seems likely to promote malignant growth (16). These concepts are applicable without regard to location or histological type of solid tumor and as such should be considered in any attempt to control or prevent neoplasia.

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