Inhibitory Effects of D-Glucosamine on the Growth of Walker 256 Carcinosarcoma and on Protein, RNA, and DNA Synthesis

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SUMMARY

The effectiveness of D-glucosamine as an antitumor agent was tested on both rats and mice bearing transplantable tumors. Continuous tail-vein infusion of 0.42 g of glucosamine/kg/hr over a period of 36 to 36 hr resulted in a high rate of tumor regression in Charles River CD rats bearing i.m. Walker 256 carcinosarcoma (subline M-10). The number of tumor-free rats was dependent on the strain of rat used. Continuous s.c. infusion of glucosamine resulted in about 56% regression of s.c. Sarcoma 180 in mice.

Incorporation of lysine-14C, uridine-14C, and thymidine-14C into surviving tumor slices obtained from tumor-bearing rats treated in vivo with glucosamine showed 75 to 85% inhibition of RNA and DNA synthesis and slight inhibition of protein synthesis. The effect of glucosamine on the incorporation of the precursors was much less in normal tissues than in tumor tissue. The effects of glucosamine on acid-soluble nucleotides in neoplastic tissue were studied. It was found that the pool of UDP-N-acetylhexosamine is increased six-fold in Walker 256 carcinosarcoma in vivo but that the pools of other uridine nucleotides (e.g., UMP, UDP, and UTP) were significantly reduced.

The resistance of rats and mice successfully treated with glucosamine to a second syngeneic tumor graft suggests that glucosamine treatment may interfere with the tumor in such a way as to permit the host to respond successfully to the antigenic makeup of the tumor.

INTRODUCTION

The first observation that D-glucosamine has an antitumor effect was made by Quastel and Cantero (25). They found that daily injection of glucosamine into mice bearing Sarcoma 37 resulted in decreased cell mass and extensive hemorrhagic areas in the tumors. Although tumors did not undergo complete regression, the survival time of the treated animals was almost doubled. Tarnowski (30) reported that glucosamine slightly increased the survival time of mice bearing RC mammary tumors.

Fjelde et al. (9) observed that D-glucosamine inhibited the growth of human epidermoid carcinoma cells in tissue culture, with concentration of 1 mg/ml causing death of the cells within 24 hr. The i.p. administration of divided doses of D-glucosamine or 2-deoxy-D-glucose inhibited the growth of Walker 256 carcinosarcoma in rats without significant toxicity to the host (1). Tumor growth resumed when treatment was stopped and slowed when treatment was reinstituted.

Glucosamine in vivo exerted a cytotoxic effect on the Yoshida rat ascites sarcoma; in vitro, it inhibited anaerobic glycolysis of the tumor (16). Woodward et al. (32) have shown that D-glucosamine inhibits the growth of yeast cells. Ely et al. (8) reported that D-glucosamine and 2-deoxy-D-glucose inhibited the growth of embryonic chicken heart in tissue culture without altering cell viability.

Rubin et al. (26) showed that D-glucosamine, but not N-acetylg glucosamine, caused degeneration of Sarcoma 37 ascites tumor cells in tissue culture. While screening for chemical agents that would inhibit the growth of Leukemia L1210 in mice, Skipper and Thomson (29) also tested D-glucosamine. They found that single daily doses of 1500 mg/kg for 5 days caused no increase in the survival time of mice bearing Leukemia L1210.

Loss of viability and transplantability in several ascites tumor lines exposed to D-glucosamine in vitro has been demonstrated in our laboratory (4). The incorporation of labeled lysine, uridine, and thymidine into the fraction of tumor cells which is insoluble in TCA3 is significantly inhibited by D-glucosamine (2).

Large amounts of glucosamine have been administered i.v. to patients in glucosamine tolerance tests (10, 12, 15, 31). Much of the glucosamine appeared in the urine unchanged shortly after administration.

Even trace amounts of D-glucosamine14C are rapidly excreted in urine in healthy rats (13, 18) and in rats bearing experimental tumors (3). Such observations strongly suggest that effective blood and tissue levels are not maintained following single daily doses of glucosamine as used in most previous experiments (1, 7, 17, 18, 28, 30). In the present study, glucosamine was administered to tumor-bearing rats by continuous tail-vein or s.c. infusion in order to maintain high levels of glucosamine in the blood.

MATERIALS AND METHODS

Animals. Rats were adult males weighing 320 to 380 g. Several strains were used including Charles River CD (of

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The abbreviation used is: TCA, trichloroacetic acid.
Sprague-Dawley descent). Sprague-Dawley, Wistar, and Schmidt (Sprague-Dawley). Mice were Swiss-Webster males weighing 35 to 40 g. The animals were maintained at 20° in a thermostatically controlled room with cycles of 12 hr each of light and darkness. They were fed with Purina chow and were allowed tap water ad libitum.

Isotopes. Lysine-14C with a specific activity of 250 mCi/m mole, uridine-14C with a specific activity of 50 mCi/m mole, and thymidine-14C with a specific activity of 30.1 mCi/m mole were obtained from the New England Nuclear Corp., Boston, Mass. For each experiment, the isotopic compounds were freshly dissolved at the appropriate concentration in Krebs-Ringer phosphate buffer.

Transplantation of Tumors. Charles River CD bearing the M-10 subline of the Walker 256 carcinosarcoma were originally obtained from Dr. Isidore Wodinsky of Arthur D. Little, Inc., Cambridge, Mass. Male rats bearing 6- to 7-day-old tumors served as donors; in some experiments, rats bearing the M-13 subline were also used. Donor animals were anesthetized with ether, and tumors were removed and transferred to a beaker served as donors; in some experiments, rats bearing the M-13 subline were also used. Donor animals were anesthetized with ether, and tumors were removed and transferred to a beaker containing Krebs-Ringer phosphate buffer. Each tumor was then dissected free of connective tissue, and pieces of tumor were placed in a hand-operated Potter glass homogenizer with a loosely fitting pestle. Ten parts (by volume) of sterile Krebs-Ringer buffer were added to 1 part (by weight) of tumor tissue, and the material was gently homogenized for 20 sec.

Microscopic examination of the homogenate, with trypan blue as a vital stain (4), demonstrated that 85 to 90% of the cells were intact and viable. An 0.15- to 0.2-ml sample of this cell suspension was implanted i.m. in the right thigh of each recipient rat.

Solid Sarcoma 180 tumors, originally obtained from Dr. E. Mihich at Roswell Park Memorial Institute, were maintained by weekly s.c. transplantation of 1- to 2-mm cubes of tissue from 8- to 10-day-old donor tumors into the right axillary region in recipient mice.

Infusion. Rats bearing 4- to 6-day-old i.m. Walker 256 carcinosarcoma were lightly anesthetized with ether, and the proximal part of the tail was shaved and painted with tincture of iodine. Then a 2-cm U-shaped incision was made, and the tail vein was exposed, ligated, and cannulated with PE-10 0.28 x 0.61 mm inner diameter and outer diameter) intramedic polyethylene tubing. A light 4.0-silk ligature was used to hold the cannula in the tail vein. The rat was lightly heparinized through the cannula with 0.5 mg of heparin, and the cannula was filled with dilute heparin solution (2 mg of heparin in 10 ml of sterile 0.9% NaCl solution) and clamped. The wound was closed with interrupted 3- silk sutures.

The rat was placed in a restrainer and allowed to recover. The cannula was then attached to a Harvard multispeed peristaltic pump or a Harvard multispeed syringe pump, and infusion was begun. During infusion, the animals had access to food and water ad libitum. At the end of infusion, the pump was stopped, the cannula was removed, the tail vein was ligated, and the wound was closed. Infusion rates greater than 1.5 ml hr resulted in destruction of the tail and gangrene, and rates greater than 2.5 ml/hr caused the death of the animals. Infusion rates of 0.6 to 1.2 ml/hr were used in the experiments to be described.

Several solvent systems were tested as vehicles for D-glucosamine HCl. These included distilled water, 0.9% NaCl solution, and Krebs-Ringer phosphate buffer. Best results were obtained when D-glucosamine HCl was dissolved in distilled water or 0.9% NaCl solution and the solution was adjusted to pH 4.0 to 4.5 with 1.0 N NaOH.

It was found that continuous restraint and infusion of rats for periods longer than 40 hr left the animals in poor condition and that such treatment for more than 48 hr often caused the death of the experimental animals.

Administration of D-glucosamine to mice by tail-vein infusion was unsuccessful because of the difficulty of cannulating the tail vein. Accordingly, continuous s.c. infusion was adopted for the experiments with mice. Mice bearing s.c. 4- to 8-day-old Sarcoma 180 were lightly anesthetized with ether, and an intramedic PE-10 cannula was inserted subdermally into the dorsal pelvic region. The mouse was placed in a restrainer, the cannula was attached to a Harvard multispeed syringe pump, and infusion was begun. An infusion rate of 0.085 to 0.140 ml/hr was used. Continuous infusion for longer than 18 hr caused the death of the mice. Some of the difficulty encountered with long infusion periods was due to immobilization, since mice that were immobilized but not infused also died in 24 to 30 hr.

Excretion of Hexosamine in Urine. Tumor-bearing rats, housed in metabolic cages, were infused with glucosamine (0.410 g/kg/hr for 32 hr). Urine samples collected at various times were centrifuged, and part of the clear supernatant was passed through a Dowex 50 H+ column to retain the hexosamine. The hexosamine was eluted with 1 N HCl and measured by the method of Good and Bessman (11).

Incorporation of Lysine-14C, Uridine-14C, and Thymidine-14C into the Acid-insoluble Fraction of Each of Various Rat Tissues after Glucosamine Infusion. The incorporation of L-lysine-14C, uridine-14C, and thymidine-14C into the insoluble fraction of each normal or neoplastic tissue was linear for at least 45 min. In the experiments to be reported, an incubation time of 30 min was adopted.

Tumor, liver, and kidney tissues from control rats and from rats treated with glucosamine were quickly removed and placed in beakers containing ice-cold Krebs-Ringer phosphate buffer. The tissue samples were then cut into thin slices, and 750 mg of wet tissue were incubated in 7.5 ml of pH 7.4 Krebs-Ringer phosphate buffer containing 2 mM glucose. After 2 min of equilibration at 37°, 0.25 μCi of lysine-14C, uridine-14C, or thymidine-14C was added, and incubation was performed in a Dubnoff incubator at 37° for 30 min. Reaction was stopped by adding equal volumes of 20% TCA to each flask. Acid-insoluble fractions were prepared for radioactivity study as described previously (2).

Isolation and Quantitative Measurement of UDP-N-Acetylhexosamine from Walker 256 Carcinosarcoma after Glucosamine Infusion. Six Charles River CD rats bearing 6-day-old Walker 256 carcinosarcoma were infused through the tail vein with 0.41 g of glucosamine/kg/hr for 32 hr. Thirty min before termination of glucosamine treatment, 20 μCi of uridine-2-14C in 0.5 ml of 0.9% NaCl solution were injected i.p. into each rat. At the end of perfusion, the animals were killed, and tumor tissue was rapidly removed and placed in a beaker...
Table 1

Effects of D-glucosamine on the growth of Walker 256 carcinosarcoma and on the survival of tumor-bearing adult Charles River CD rats

Treatment of experimental animals was begun 4 to 6 days after implantation of Walker 256 carcinosarcoma (sublines 10 and 13). Each compound was infused into the tail vein or through a cannula placed subdermally in the dorsal pelvic region. The rate of infusion of fluid was 0.8 to 1.1 ml/hr. At the end of the infusion, the cannula was removed, and the incision was closed. The animals were kept under observation for 8 to 10 weeks.

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of rats</th>
<th>Route</th>
<th>Rate (g/kg/hr)</th>
<th>Duration (hr)</th>
<th>Total dose (g/kg)</th>
<th>No. of rats surviving tumor-free for at least 8 weeks</th>
<th>Mean survival time of rats dying with tumors (days ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>62</td>
<td>Tail vein</td>
<td>0.9</td>
<td>32</td>
<td>28.8</td>
<td>5</td>
<td>29.5 ± 5.8</td>
</tr>
<tr>
<td>Distilled water, pH 4 to 4.5</td>
<td>5</td>
<td>Tail vein</td>
<td>0.278</td>
<td>32</td>
<td>9.2</td>
<td>0</td>
<td>36.8 ± 7.0</td>
</tr>
<tr>
<td>D-Glucosamine dissolved in distilled water, pH 4 to 4.5</td>
<td>6</td>
<td>Tail vein</td>
<td>0.362</td>
<td>32</td>
<td>11.6</td>
<td>2</td>
<td>38.9 ± 6.2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Tail vein</td>
<td>0.416</td>
<td>18</td>
<td>7.5</td>
<td>0</td>
<td>31.2 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Tail vein</td>
<td>0.416</td>
<td>32–36</td>
<td>13.3–15.6</td>
<td>73</td>
<td>64.5 ± 8.2</td>
</tr>
<tr>
<td></td>
<td>81</td>
<td>Tail vein</td>
<td>0.416</td>
<td>32–36</td>
<td>13.3–15.6</td>
<td>3</td>
<td>61.5 ± 11.9</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>Subdermal</td>
<td>0.416</td>
<td>32–36</td>
<td>13.3–15.6</td>
<td>0</td>
<td>27.1 ± 1.8</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>6</td>
<td>Tail vein</td>
<td>0.416</td>
<td>32</td>
<td>13.3</td>
<td>0</td>
<td>30.8 ± 6.2</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>6</td>
<td>Tail vein</td>
<td>0.416</td>
<td>32</td>
<td>13.3</td>
<td>0</td>
<td>30.8 ± 6.2</td>
</tr>
</tbody>
</table>

a Rate in ml/hr.
b Dose in ml.

containing ice-cold 10% TCA. After homogenization, centrifugation, and neutralization, the acid-soluble fraction was directly applied to a Dowex 1-formate (200 to 400 mesh) resin column. The column was washed with distilled water, and then a 4-step gradient elution was performed with 4.0 N formic acid, 0.2 M ammonium formate in 4.0 N formic acid (Tube 110 on), 0.4 M ammonium formate in 4.0 N formic acid (Tube 210 on), and 1.0 M ammonium formate in 4.0 N formic acid (Tube 285 on). Purification, identification, and quantitative measurement of acid-soluble nucleotides were performed as described previously (5). The control animals were infused with 0.9% NaCl solution for 32 hr, but everything else involving them was the same as for the experimental animals.

Other Analysis. Glucose was determined by the glucose oxidase method of Saifer and Gerstenfeld (27), and clinical chemistry was performed according to standard methods as reported previously by Mihich et al. (22–24).

RESULTS

Table 1 shows the effects of infusing glucosamine into Charles River CD rats bearing 4 to 6-day-old Walker 256 carcinosarcoma. Two groups of controls were used; they were untreated tumor-bearing rats and tumor-bearing rats infused with distilled water. Infusion of tumor-bearing rats with 0.278 to 0.362 g of glucosamine/kg/hr for 32 hr brought about a consistent reduction in tumor size for the first 4 to 7 days after glucosamine treatment. After this, however, there followed a rapid growth of the tumor, with only a moderate increase in survival time. When the tumor-bearing Charles River rats were subjected to tail-vein infusion with 0.416 g of glucosamine/kg/hr for 32 to 36 hr, a large proportion of the tumors regressed, and a pronounced increase in survival time of the animals that developed tumors was observed. Infusion s.c. of glucosamine at the same concentration in a marked inhibition of tumor growth, but complete regression occurred in few animals. N-Acetylglucosamine or D-glucose administered by tail-vein infusion had no effect on the growth of Walker 256 carcinosarcoma in Charles River rats.

Since glucosamine may provoke hyperglycemia (6, 20, 21), the levels of blood glucose and free glucosamine in the blood of rats during treatment were measured. Chart 1 shows that infusion of glucosamine at a rate of 0.41 g/kg/hr induced a significant increase in blood glucose levels during the infusion period. Blood glucose levels returned to normal, however, by 24 hr after cessation of treatment. Free glucosamine could not be detected in the circulating blood in animals before treatment, but showed a continuous increase during infusion (Chart 1). No free glucosamine could be detected in the circulating blood 24 hr after termination of treatment. Chart 2 shows the cumulative urinary excretion of glucosamine in a typical infusion experiment and indicates that 70% of the administered glucosamine is excreted in the urine within 3 days after...
the end of the infusion period. Chart 2 also indicates that there was no upset in acid-base balance as reflected by the pH of urine during or after the infusion. The infusion of glucosamine caused a transient decrease in serum bicarbonate, but no change in blood pH and no significant change in plasma electrolytes; there were transient increases in blood urea nitrogen and serum glutamic oxalacetate transaminase. It would thus appear that the effects of glucosamine on tumors were not the result of damage to the host as measured by these parameters.

A group of 22 rats successfully treated with glucosamine and a control group of rats of the same age were implanted with Walker 256 carcinosarcoma (subline M-10). In this experiment all of the control rats died with tumor. Although the presence of the tumor was initially apparent in each reimplanted animal, only 4 of the reimplanted rats died of tumors, and then only after a prolonged time. The other reimplanted rats rejected the tumor within 6 to 14 days after implantation.

Data presented in Table 2 demonstrate that the strain of rats has a significant influence on the response of Walker 256 tumors to D-glucosamine. The highest rate of tumor regression and the greatest increase in survival time following treatment was noted in Charles River CD and Schmidt rats after glucosamine treatment.

**Effects of D-Glucosamine on the Survival of Swiss-Webster Mice Bearing s.c. Sarcoma 180.** The effects of D-glucosamine on tumor growth and host survival were also studied in mice bearing Sarcoma 180. Administration of the compound by tail-vein infusion was not feasible in mice because of the difficulty of cannulating the tail vein. Accordingly, continuous s.c. infusion was done. The effects of s.c. infused D-glucosamine in mice bearing Sarcoma 180, as measured by tumor regression and survival time, are presented in Table 3. Infusion of the tumor-bearing mice with glucosamine at a rate of 0.64 g/kg/hr for 12 hr on Days 4 to 7 and 10 to 14 following tumor implantation resulted in tumor regression in 56% of the mice, with a significant increase in survival time. Single infusion at a rate of 0.64 or 0.83 g/kg/hr for 12 hr on any one of Days 4 to 7 was ineffective.

A group of mice successfully treated with glucosamine was rechallenged with new Sarcoma 180 implants. Of the 24 mice given reimplantations, 15 rejected the tumor. The remaining 8 died with tumor without significant increase in survival time, while all of the control group of mice given transplants of Sarcoma 180 died with tumor.

**Cytological Changes Caused by D-Glucosamine in Normal and Neoplastic Tissues in Vivo.** The effects of glucosamine on the morphology of normal and neoplastic tissues were studied in both mice and rats bearing tumors. For cytological examination, rats bearing Walker 256 carcinosarcoma were treated with 0.42 g of glucosamine/kg/hr for 18 to 32 hr. Mice bearing solid Sarcoma 180 were treated with 0.64 g/kg/hr for 12 hr. At the end of treatment, a number of animals were anesthetized, and various organs were subjected to gross examination. In every case, extensive hemorrhagic areas were observed in the encapsulated Walker 256 tumor, along with softening at the periphery of the tumor. Hemorrhage was less severe in the intradermal Sarcoma 180 tumors.

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**Table 2**

<table>
<thead>
<tr>
<th>Rats</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>Infusion</th>
<th>No. of rats surviving tumor-free for at least 8 weeks</th>
<th>Mean survival time of rats dying with tumors (days ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charles River CD</td>
<td>Control</td>
<td>62</td>
<td></td>
<td>5</td>
<td>29.5 ± 5.8</td>
</tr>
<tr>
<td>D-Glucosamine</td>
<td>81</td>
<td></td>
<td>0.416</td>
<td>32–36</td>
<td>64.5 ± 8.2</td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td>Control</td>
<td>14</td>
<td></td>
<td>4</td>
<td>32.2 ± 5.9</td>
</tr>
<tr>
<td>D-Glucosamine</td>
<td>26</td>
<td></td>
<td>0.394</td>
<td>36</td>
<td>53.4 ± 7.6</td>
</tr>
<tr>
<td>Wistar</td>
<td>Control</td>
<td>14</td>
<td></td>
<td>0</td>
<td>27.3 ± 7.0</td>
</tr>
<tr>
<td>D-Glucosamine</td>
<td>17</td>
<td></td>
<td>0.391</td>
<td>36</td>
<td>30.9 ± 8.7</td>
</tr>
<tr>
<td>Schmidt Sprague-Dawley</td>
<td>Control</td>
<td>16</td>
<td></td>
<td>1</td>
<td>29.3 ± 7.9</td>
</tr>
<tr>
<td>D-Glucosamine</td>
<td>18</td>
<td></td>
<td>0.402</td>
<td>36</td>
<td>31.7 ± 7.3</td>
</tr>
<tr>
<td>Holtzman Sprague-Dawley</td>
<td>Control</td>
<td>15</td>
<td></td>
<td>0</td>
<td>31.9 ± 6.2</td>
</tr>
<tr>
<td>D-Glucosamine</td>
<td>16</td>
<td></td>
<td>0.394</td>
<td>37</td>
<td>45.4 ± 8.1</td>
</tr>
</tbody>
</table>

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Chart 2. Cumulative excretion of hexosamine in urine (●) and pH of urine (▲) of 6 rats bearing 4-day-old Walker 256 carcinosarcoma and infused with D-glucosamine (0.42 g/kg/hr for 32 hr). During and after infusion, the rats were housed in separate metabolic cages. Bars, ± S. D.
Table 3
Effects of s.c. glucosamine infusion of the growth of s.c. implanted Sarcoma 180 in adult Swiss Webster mice

Treatment of experimental animals was begun 4 or 5 days after tumor implantation. Each compound was infused through a cannula placed s.c. in the left dorsal region. The rate of infusion of fluid was 0.124 ml/hr, and infusion was continued for 12 hr on each day of treatment. At the end of the infusion, the cannula was removed. The animals were kept under observation for 8 to 10 weeks.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Day of treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Infusion</th>
<th></th>
<th>No. of mice surviving tumor-free for at least 8 weeks</th>
<th>Mean survival time of mice dying with tumors (Days ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48</td>
<td></td>
<td></td>
<td>4</td>
<td>31.9 ± 4.8</td>
</tr>
<tr>
<td>0.9% NaCl solution</td>
<td>4–7 and 10–13</td>
<td>0.124&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.9%</td>
<td>0</td>
<td>23.1 ± 2.7</td>
</tr>
<tr>
<td>Glucosamine, dissolved in</td>
<td>4–7 and 10–14</td>
<td>0.41</td>
<td>4.92</td>
<td>2</td>
<td>40.9 ± 3.9</td>
</tr>
<tr>
<td>distilled water and adjusted to</td>
<td>5</td>
<td>0.64</td>
<td>7.78</td>
<td>1</td>
<td>37.0 ± 3.2</td>
</tr>
<tr>
<td>pH 4 to 4.5</td>
<td>5</td>
<td>0.64</td>
<td>7.78</td>
<td>49</td>
<td>49.3 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.83</td>
<td>9.96</td>
<td>2</td>
<td>16.1 ± 9.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> The tumor was implanted on Day 0.<br>
<sup>b</sup> Rate in ml/hr.

Table 4
Incorporation of lysine-<sup>14</sup>C, uridine-<sup>14</sup>C, and thymidine-<sup>14</sup>C into the acid-insoluble fraction of liver, kidney, and tumor tissues in rats bearing Walker 256 carcinosarcoma, before and after treatment with glucosamine

Of 15 Charles River rats bearing 6-day-old Walker 256 carcinosarcoma perfused through the tail vein with 0.42 g of glucosamine/kg/hr for 32 hr, 5 were killed on Day 0 (immediately after the end of perfusion), 5 were killed on Day 1 (24 hr later), and 5 were killed on Day 6. 5 rats bearing untreated 7-day-old Walker 256 carcinoma served as controls. Slices of liver, kidney, or tumor tissue were incubated with labeled lysine, uridine, or thymidine, and incorporation of the labeled compounds into the TCA-insoluble fraction of each tissue was determined as described in the text.

<table>
<thead>
<tr>
<th>Labeled compound</th>
<th>Tissue</th>
<th>Incorporation of compound (dpm/10 mg of protein ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver Day 0 Day 1 Day 6</td>
</tr>
<tr>
<td>Lysine-&lt;sup&gt;14&lt;/sup&gt;C</td>
<td>Liver 364 ± 23 436 ± 33 259 ± 14 229 ± 21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney 389 ± 39 365 ± 20 420 ± 66 398 ± 81</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor 6150 ± 52 4998 ± 139 5161 ± 163 3218 ± 241</td>
<td></td>
</tr>
<tr>
<td>Uridine-&lt;sup&gt;14&lt;/sup&gt;C</td>
<td>Liver 145 ± 11 138 ± 22 147 ± 15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney 163 ± 18 145 ± 27 153 ± 21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor 1230 ± 86 178 ± 17 184 ± 31</td>
<td></td>
</tr>
<tr>
<td>Thymidine-&lt;sup&gt;14&lt;/sup&gt;C</td>
<td>Liver 112 ± 6 52 ± 9 63 ± 13 58 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney 128 ± 16 41 ± 7 54 ± 12 75 ± 18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor 4247 ± 69 1196 ± 150 992 ± 107</td>
<td></td>
</tr>
</tbody>
</table>

apparent when infusion was performed for a shorter period of time.

The tumor-bearing mice infused with glucosamine were studied similarly. Massive hemorrhagic areas were apparent in the Sarcoma 180 tumors after treatment. Microscopic examination of the Walker 256 carcinosarcoma and Sarcoma 180 tumors obtained at the end of glucosamine treatment showed marked shrinkage of the nuclei, retraction of the cytoplasm, and advanced eosinophilia. A few days after treatment, extensive necrosis of tumor tissues was apparent. This was followed, in most cases, by autolysis.

Simultaneous gross examination of major organs, such as the liver, spleen, lungs, heart, kidney, and large and small intestines, revealed no signs of toxic effects of glucosamine treatment in the tumor-bearing rats and mice.

Effects of Glucosamine on the Biosynthesis of Protein, RNA, and DNA in Normal and Neoplastic Tissues in Vivo.

The experiments just described show that exogenous glucosamine inhibits the growth of experimental tumors in rats and mice. Previous experiments have demonstrated that transplantability, viability, and biosynthesis of protein, RNA, and DNA in Sarcoma 180 ascites cells are inhibited in vitro by glucosamine at concentrations above 20 mM (2, 4).

The effects of therapeutic levels of D-glucosamine on the subsequent in vitro biosynthesis of protein, RNA, and DNA in normal and neoplastic tissues obtained from tumor-bearing rats were studied. In these experiments, 20 rats bearing i.m. Walker 256 carcinosarcoma were infused with glucosamine (0.42 g/kg/hr for 32 hr). After the end of treatment, 5 rats were killed immediately (Day 0), 5 were killed on Day 1, and
5 were killed on Day 6. A group of 5 rats bearing Walker 256 carcinosarcoma served as controls.

Slices of liver, kidney, and tumor were incubated in the presence of labeled precursors in the absence of glucosamine, as already described ("Materials and Methods"). A suggestion that glucosamine has a selective effect on nucleic acid synthesis in vivo was apparent in this study. Data presented in Table 4 show that RNA and DNA synthesis by the Walker tumor was markedly reduced at the end of glucosamine infusion but that the incorporation of lysine-14C into tumor tissue was somewhat reduced only after several days. Although glucosamine treatment in vivo had little effect on protein and RNA synthesis in liver or kidney tissue, incorporation of thymidine-14C into the acid-insoluble fractions of liver and kidney tissues was inhibited by glucosamine treatment in vivo.

Chart 3 shows the elution pattern of the acid-soluble nucleotides obtained from Walker 256 carcinosarcoma from a control animal and from an animal treated with glucosamine for 32 hr at a rate of 0.42 g/kg/hr. The most striking difference exhibited by the treated Walker 256 tumor as compared with the control is the tremendous increase in the peak representing UDP-N-acetylhexosamine.

Quantitative determination of the uridine nucleotides separated from the pooled peaks of Chart 3 by paper chromatography is shown in Table 5. Data obtained from this experiment reveal an increase of more than 6-fold in the UDP-N-acetylhexosamine pool in glucosamine-treated Walker 256 carcinosarcoma. This increase was accompanied by a slight decrease in the pools of UMP and by somewhat larger decrease in the pools of UDP and especially of UTP. Accumulation of UDP-N-acetylglucosamine also occurs in other experimental tumors exposed to glucosamine in vitro (5, 14).

**DISCUSSION**

After the original observations by Quastel and Cantero (25) that exogenous glucosamine inhibits the growth of transplanted tumors in mice, many attempts were made at testing D-glucosamine as a possible antitumor agent against various experimental tumors. Results were generally inconclusive (1, 7, 17, 18, 19, 28, 30). The apparent lack of inhibitory effects now appears to be due to the rapid excretion of glucosamine and the resulting failure to maintain sufficiently high glucosamine levels in the blood for long enough times when the agent is administered periodically. In the present study, glucosamine was administered by continuous tail-vein or s.c. infusion in order to achieve continuing exposure to high concentrations of glucosamine.

The rate of administration of glucosamine is obviously critical. Continuous tail-vein infusion of 0.42 g/kg/hr for 32 to 36 hr resulted in a high rate of tumor regression in Charles River CD rats bearing Walker 256 carcinosarcoma. If only somewhat less was administered (0.28 to 0.36 g/kg/hr for 32 hr), tumor growth was inhibited for only 4 to 7 days before...
beginning again. The period of administration is also critical, since administration of 0.42 g/kg/hr for only 18 hr had a limited effect. Finally, as Table 2 shows, the results vary with the strain of rat used.

Sarcoma 180 implanted s.c. in mice also responds to treatment with glucosamine. Here, because of the difficulties encountered in tail-vein infusion, continuous s.c. infusion of glucosamine was adopted. Best inhibition of tumor growth was achieved by multiple infusion of 0.64 g/kg/hr for 12 hr with infusions on Days 4 to 7 and 10 to 14. Under these conditions, about 56% of the treated tumor-bearing mice showed regression of their tumors.

The resistance of rats and mice successfully treated with glucosamine to a second implantation of the tumors suggests that glucosamine may have an injurious action on the tumor such that the host can respond successfully if the antigenic makeup of the tumor is sufficiently different from that of the host. Strain differences in response to glucosamine could reflect differences in antigenic makeup or in capacity to respond to antigenic challenge. Glucosamine inhibited the growth of subline M-10 of Walker 256 carcinosarcoma in Charles River CD rats at a blood glucosamine level that apparently did not seriously affect the well-being of the host animal, except for producing transient hyperglycemia and increases in blood urea nitrogen and serum glutamic oxalacetate transaminase.

Glucosamine may have a selective effect on nucleic acid synthesis in vivo, since it was observed at the end of glucosamine treatment that the synthesis of RNA and DNA, but not of protein, in the tumor tissue was significantly inhibited. Glucosamine treatment had no significant effect on protein and RNA synthesis in liver and kidney tissue. The incorporation of thymidine-14C into liver and kidney DNA, however, was inhibited somewhat by glucosamine treatment in vivo. These findings are in accord with observations made in vitro, where it was observed that D-glucosamine had a much smaller inhibitory effect on the incorporation of the labeled precursors into normal tissues than on that into neoplastic tissues (2).

N-Acetylglucosamine did not inhibit tumor growth in rats. The most reasonable explanation of the difference between the effects of the two compounds is a difference in the permeability of neoplastic cells to glucosamine and N-acetylglucosamine. Indeed, such evidence has been obtained in vitro with Sarcoma 180 ascites tumor cells (2).

REFERENCES

Inhibitory Effects of d-Glucosamine on the Growth of Walker 256 Carcinosarcoma and on Protein, RNA, and DNA Synthesis

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