Effect of Urease Injections on Ehrlich Ascites Tumor Growth in Mice (36097)

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Mice injected with crystalline jack bean urease have shown altered incorporation of ^{3}H-thymidine into DNA of the ileum, colon, and spleen and in the total DNA content of spleen and colon (39). Injection of this enzyme causes hydrolysis of endogenous urea with the liberation of ammonia. Ammonia (*i.e.*, sum of NH_3 and NH_4^+) is toxic to living organisms and impairs cellular metabolism (22, 29, 30). Urea hydrolysis *in vitro* resulting in the formation of 125–175 μg of NH_3–N/ml of incubation medium altered ^{3}H-thymidine incorporation into DNA of rat thymus slices but not in Ehrlich ascites tumor (EAT) cells (39). NH_4Cl, in concentrations of 25 m*M*, decreased RNA, DNA, and protein synthesis, glucose metabolism, and O_2 uptake by EAT cells *in vitro* (40). Presented herein are data on growth and development of EAT after intraperitoneal (ip) or subcutaneous (sc) inoculation of the tumor cells in mice given injections of crystalline jack bean urease.

Methods. Male Swiss Webster mice[1] were the experimental animals in all experiments. They were caged in groups of 4 or 5 in air-conditioned quarters and, unless specified otherwise, were provided with commercial food pellets and tap water *ad libitum*. The method for extraction and recrystallization of jack bean urease [urea amidohydrolase (EC 3.5.1.5), 1 unit = quantity of enzyme activity that liberates 1 mg of NH_3–N from a urea phosphate buffer in 5 min at 20° at pH 7.0] has been described (32). The enzyme was dissolved in sterile 0.9% NaCl and standardized for desired activity immediately before use. Control animals always received comparable volumes of physiological saline at the time of urease injections. All volumes injected were 0.2 ml or less. Cultures of EAT cells were maintained by sterile transfer to normal mice of ascitic fluid obtained on days 8 to 10 after tumor-bearing mice had been inoculated with EAT. Experimental groups were injected sc on the back of the neck region or ip with fresh ascitic fluid obtained from tumor-bearing mice. Cell counts were made using a hemacytometer. Animals were killed by guillotine and dissected immediately. Organs and solid tumors were blotted on paper toweling and weighed. Body weights were determined daily on individual animals and feed intakes were determined with animals fed ground laboratory pellets from containers designed to minimize feed wastage. Ammonia was determined on protein-free extracts of blood and ascitic fluid prepared immediately after the samples were collected into ice cold test tubes (3). Serial samples of blood from living animals were drawn from the tail vein. Colonic temperatures were obtained by a probe attached to a telethermometer[2].

The influence of urease injections upon EAT cell growth was studied in two sets of experiments. In the first of two experiments with mice given EAT cells sc, each of 24 mice were given 12×10^6 cells in ascitic fluid obtained on day 8 after inoculation. Twelve were injected ip with 0.4 units of urease 12 and 24 hr thereafter. When the above animals and their saline-injected counterparts were killed, 7 normal mice of the same age and weight were also killed for comparative data. A second experiment was conducted with 24 animals given, ip, undiluted ascitic fluid which had been obtained on day 9 after inoculation and contained 6×10^6 cells/mouse. One half of this group were given 0.1 unit of urease ip at 12 hr before, and 12 and

[1] Blue Spruce Farms, Altamont, NY.

[2] YS 1 probe attached to telethermometer (Yellow Springs Instrument Co., Inc.).

TABLE I. Body Weights and Fresh Tumor and Organ Weights in Normal Mice and Mice Inoculated sc with Ehrlich Ascites Tumor Cells and Injected ip with Saline or 0.4 units of Urease.[a]

	Normal mice (7)	Tumor-bearing mice	
		Saline (12)	Urease (11)
Body wt (g)			
Initial		21.2 ± 0.3	21.5 ± 0.3
Day 7		28.8 ± 0.5[b]	24.6 ± 0.7[c]
14		32.4 ± 0.7[b]	28.5 ± 0.9[c]
21	33.7 ± 0.5	34.6 ± 0.8[b]	30.6 ± 0.9[c]
Tumor wt (mg)		1022 ±295[b]	410 ± 88[c]
Liver			
mg	1455 ± 43[b]	1929 ± 72[c]	1529 ± 60[d]
mg/g of body wt	43.1 ± 1.0[b]	55.7 ± 1.1[c]	50.2 ± 1.2[d]
Spleen			
mg	90 ± 3.2[b]	157 ± 18.5[c]	111 ± 9.1[d]
mg/g of body wt	2.7 ± 0.1[b]	4.5 ± 0.4[c]	3.6 ± 0.2[d]
Brain			
mg	465 ± 7.4[b]	371 ± 34[c]	368 ± 22.7[c]
mg/g of body wt	13.8 ± 0.3[b]	10.7 ± 1.0[c]	12.3 ± 1.0[b,c]
Kidney			
mg	539 ± 12.4[b]	531 ± 19.7[b]	470 ± 24.4[c]
mg/g of body wt	16.0 ± 0.3[b]	15.4 ± 0.5[b]	15.1 ± 0.6[b]

[a] Each animal was injected sc with 12×10^6 EAT cells and given 0.4 unit of urease ip at 12 and 24 hr thereafter. Number of mice in parentheses.

[b,c,d] Differences in data with different superscripts are statistically significant ($p < .05$).

24 hr after EAT cell injection.

Subsequently 18 mice were given ip 0.2 ml of undiluted ascitic fluid obtained on day 8 after EAT inoculation. One half of this group received 0.2 units of urease/mouse 12 hr before and at 12, 24, and 36 hr after inoculation. Nine additional animals given only saline were included for comparison.

Body weight, food intake, and colon temperatures were studied in mice carrying the ascitic form of the tumor. Two injections of urease (0.2 unit/12 hr interval) were given ip to animals on day 6 after tumor inoculation. Colonic temperatures were recorded at the time of the first urease injection and at 3, 13, 15, 24, 32, and 48 hr thereafter. Body weights were recorded at 0, 24, and 48 hr. Food was available from 6 to 12 and 33 to 48 hr after the first urease injection. Colon temperatures were also determined on other tumor-bearing and normal mice.

Blood serum and ascitic fluid ammonia, hematocrit, and organ weights were determined on mice carrying the ascitic form of the tumor and injected with 0.2 unit of urease/mouse on days 6 and 7 after tumor inoculation. Body weights were followed daily for 5 days after the second urease injection and the animals were killed on day 12 after tumor inoculation. Packed tumor cell volume was determined on ascitic fluid centrifuged at 2000*g*. There were 5 animals/treatment group. Groups of 3 mice treated identically were also injected ip with ^{3}H-thymidine[3] on day 6 after EAT cell inoculation. Ascitic fluid (0.2 ml/mouse/day) was drawn daily for cellular DNA-bound ^{3}H-thymidine radioactivity (38). DNA was determined by the diphenylamine method (2).

Four mice inoculated ip with EAT cells were injected ip with 0.5 ml of 0.5 *M* NaCl or the same volume of 0.5 *M* NH_4Cl solution

[3] Specific activity, 6.0 Ci/mmole; purchased from Schwarz Bioresearch, Inc., Orangeburg, NY.

on day 9 after receiving EAT ip. ^{3}H-Thymidine[3] was given ip in 0.1 ml of water 1.5 hr afterwards. Samples of 0.2 ml of ascites fluid were aspirated from all mice at 2, 8, 24, and 48 hr following injection of ^{3}H-thymidine and cell smears were prepared. Fixation, staining, and autoradiography were then performed according to Zajicek *et al.* (37). One thousand cells were counted per slide to determine percentage of labeled cells.

Results. The average solid EAT tumor weight measured 3 weeks following sc inoculation of ascites in mice given 0.4 units of urease twice was 40% of that for their saline-treated controls (Table I). Wet weight of spleen and liver per gram of body weight were significantly increased in tumor-bearing mice compared to normal mice, and urease treatment caused this increase to be significantly reduced ($p<.05$). The presence of the tumor caused a 22% reduction in brain weight ($p<.05$), which was reduced only 11% in mice given urease. The change in weights of liver, spleen, and brain per unit of body weight in urease-injected mice were 56, 52, and 50% lower than in saline-injected tumor-bearing animals, respectively, compared with normal mice. These differences are comparable to those observed in tumor weight. The average gains in body weight during the 3 week experimental period were 13.4 and 9.1 for saline- and urease-injected tumor-bearing animals, respectively. In a second experiment carried out in identical manner when the dosage was reduced to 0.2 unit of urease/injection, tumor weight was reduced 24%, which was not significant. Other changes following this dosage of urease were not significant.

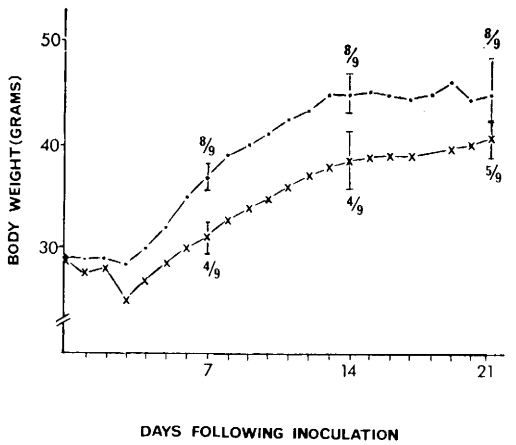

FIG. 1. Body weights of mice and the fraction showing the ascitic form of the tumor: Urease (0.2 unit/injection) was injected at 12 hr before and at 12, 24, and 36 hr after tumor cell inoculation. Ratios were determined by inspection and verified by necrospsy on Day 21; saline (.—); urease (X—).

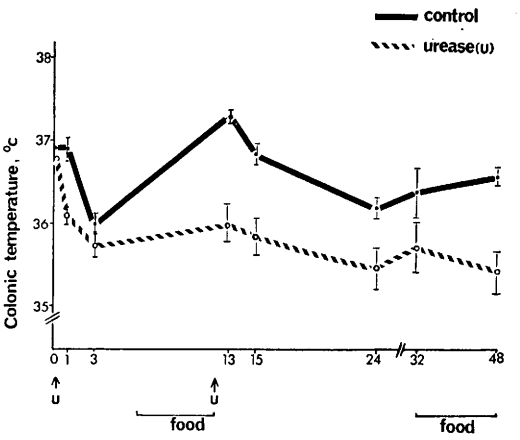

FIG. 2. Colonic temperatures of mice injected twice with 0.2 unit of urease (U) ip on day 6 after EAT cells were injected ip: Food was available at the times indicated. The number of animals in the saline-injected group at 0, 24, and 48 hr were 8, 8, and 5. Corresponding numbers for the urease-treated group were 8, 6, and 5.

Three animals of a group given 0.2 unit of urease at 12 hr before and 12, 24, and 36 hr after ip inoculation of EAT cells died within 48 hr after the last urease injection. Eight of 9 mice in the saline-treated group showed ascitic tumors at 21 days; whereas 5 of 9 showed ascitic tumors in the urease-treated group. Figure 1 shows that urease-treated animals weighed less throughout the 3 week observation period. Experiments in which 0.1 unit of urease/injection was given ip showed no effect on ascitic tumor development.

Mice bearing ascitic tumors for 8 days and injected twice with 0.2 unit of urease at a 12 hr interval showed a significant reduction in colonic temperature, the difference being a maximum of 1.58° at 13 hr (Fig. 2). The average initial body weight (g) of the control

TABLE II. Serum and Ascitic Fluid Ammonia, Hematocrits, and Organ Weights in Mice with Ascitic Form of EAT After Saline or Urease Injections.

Parameter[a]	Treatment	
	Saline	Urease
NH_3–N (μg/ml)		
serum	2.2 ± 0.9	1.7 ± 0.6
ascitic fluid	3.3 ± 1.0	6.8 ± 1.2[b]
Cell/ascitic fluid ratio	0.34 ± 0.1	0.53 ± 0.1
Hematocrit	46.2 ± 1.3	45.0 ± 2.0
Wet wt (mg)		
Spleen	84 ± 12	84 ± 9
Liver	1722 ± 110	1642 ± 100
Kidney	314 ± 16	362 ± 40
Body wt (g)		
Day 6	36.9 ± 0.7	38.2 ± 1.5
7	40.7 ± 0.9	36.9 ± 2.8
8	42.2 ± 1.0	36.7 ± 2.1[b]
9	44.0 ± 1.0	38.4 ± 3.3[b]
10	45.5 ± 1.2	40.0 ± 3.6
11	46.5 ± 1.3	42.2 ± 3.7
12	47.2 ± 1.5	42.2 ± 4.0

[a] Values determined on day 12 after EAT cell inoculation ip. Urease (0.2 unit/injection) given on days 6 and 7 after tumor inoculation. Mean ± SE of 5 animals/treatment.

[b] Difference between treatments is statistically significant ($p < .05$).

group was 39.8 ± 1 compared to 39.9 ± 1.1 in the urease-treated animals. At 48 hr after the first urease injection, the controls averaged 37.8 ± 0.9 g in weight and the urease group 33.3 ± 0.9 g ($p<.05$). Total feed consumed in g for the combined periods of measurement averaged 3.9 per control animal compared to 1.3 for the urease-treated group. Wet spleen weight averaged 106 ± 13 and 87 ± 6 mg for the controls and urease-injected animals, respectively. Average dry spleen weights differed by only 1.75 mg. The differences in spleen weight were not significant. Diarrhea was observed in 2 of the 8 urease-injected mice at 1 and 3 hr after the second urease injection and in two additional animals at 36 hr. Mice carrying ascitic tumors given 0.2 unit of urease/mouse/injection on days 6 and 7 after tumor inoculation showed significant ($p<.05$) elevations in ascitic fluid ammonia at sacrifice, on day 5 after the second urease injection (Table II). The cell/ascitic fluid ratio was also elevated ($p<.1$). There was a significant reduction in body weight at 24 and 48 hr after the second urease injection ($p<.05$). The half-times (days) for disappearance of ^{3}H-thymidine from DNA in EAT cells of ascitic fluid were 2.43 ± 1.05 and 3.69 ± 1.71 in the saline- and urease-treated animals, respectively. Two mice bearing EAT at 9 days after inoculation and injected with 0.5 ml of 0.5 *M* NH_4Cl showed 34, 29, 28, and 26% labeling of cells at 2, 8, 24, and 48 hr following injection of ^{3}H-thymidine. Corresponding percentages for two mice injected with 0.5 *M* NaCl solution were 47, 45, 41 and 42. The maximum variation between animals of the same treatment was 7% for the pair receiving NH_4Cl and 3% for those given NaCl. Baserga and Weibel (1) observed 50.2% of the cells labeled following ^{3}H-thymidine injection at day 7 after EAT inoculation.

Normal mice (28–34 g) had colonic temperatures of 38.0 to 38.2°; whereas ascitic tumor-bearing mice showed a significant drop in colonic temperatures 7 to 9 days after tumor inoculation. Actual temperatures (°) in ascitic tumor mice weighing 36.3 ± 0.7 g were 36.4 ± 0.2 [8] at day 7; 35.7 [1]; and 35.3 [1]; and below 35.0 [5] on day 8. The number in brackets is the number of animals examined.

Discussion. These results show that injections of urease can cause cell death and inhibition of EAT growth. Urease injections have previously been shown to reduce hematocrit, hemoglobin, and circulating leukocytes in rabbits and DNA synthesis in the ileum, colon, and spleen of mice (5, 39). Urease immunization has been shown to protect animals against whole-body irradiation, which is known to cause disturbances in cellular kinetics (17, 31). Hypothermia has been observed in other groups of normal mice injected with 0.4 unit of urease (our unpublished data). The increases in liver and spleen weight which accompanied the growth of the tumor (Table I) have been observed by others (20, 26, 27). The fact that urease injections caused suppression of tumor growth, a de-

crease in appetite, body weight loss, and decreased colon temperature shows a generalized toxicity due to ammonia released from urea by the enzyme. Loss of appetite and fasting are known to slow tumor growth (1). However the evidence from these and other studies shows that inanition was not the prime factor responsible for decreased tumor growth. In our experience, mice inoculated with EAT ip and given 0.05 mg of selenium/25 g of body weight as Na_2SeO_4 showed marked losses of body weight; but the number of tumors formed and tumor growth rate were not influenced.

Inhibition of tumor growth has been studied with asparaginase and glutaminase which inhibit EAT growth in mice (7, 25). Because of the quantities of asparaginase often used it has been suggested that small amounts of glutaminase may have been present as a contaminant causing release of ammonia, cell death, and other side effects (16). El-Asmar *et al.* (12), who also studied the effect of glutaminase on tumor growth in mice suggested that ammonia released from glutamine may contribute to the inhibitory effect on tumor growth. They have demonstrated a 20% diminution in tumor growth following administration of 12 μmoles of $(NH_4)_2CO_3$ twice daily for 3 days.

The present studies employed crystalline jack bean urease which has been shown to contain one immunologic component and the quantity of protein per unit of activity is approximately 11 μg (32). There is no question that hyperammonemia was produced (5, 39). Immunizing dosages have been shown to release significant amounts of ammonia, which proved toxic to the formed elements of blood (5) and release of ammonia *in vivo* and *in vitro* has been found to change DNA synthesis (39).

In limited preliminary studies, we have observed no changes in blood ammonia or urea in mice at 0.5, 1, 2, 3, and 6 hr after 0.3 unit of *E. coli* glutaminase[4] has been injected/mouse. This evidence suggests that ammonia concentrations in tissue fluid do not play a significant role in the case of glutaminase. However, the percentage of labeled EAT cells in ascitic fluid of mice injected with 0.5 ml of 0.5 *M* NH_4Cl was significantly reduced. In our experience, urease injections produce persistent hyperammonemia for 6 to 12 hr; whereas single injections of ammonium salts are cleared from the blood in less than 1 hr (5). The evidence brought forth from these experiments shows that ammonia can alter ^{3}H-thymidine incorporation into DNA of EAT cells *in vivo* and this data has been verified *in vitro* in our unpublished experiments with media containing 25 and 50 m*M* NH_4Cl.

The toxicity of ammonia in plant and animal cells has been thoroughly documented (22, 30), but the responsible mechanism is still unclear. Mechanisms proposed include depletion of α-ketoglutarate and ATP (24), alteration in the breakdown of pyridine nucleotides (19) or their reoxidation (33) or general derangement of the electron transport system (18). A depletion of reduced pyridine nucleotides has been demonstrated *in vivo* with a nonstoichiometric increase in the oxidized pyridine nucleotides (4). The data show that ammonia causes profound changes in intermediary metabolism (30). Although ammonia can be utilized in biosynthetic reactions such as glutamine, protein, and nucleic acid synthesis (10, 11, 15, 28), these pathways depend upon factors formed by pathways of intermediary carbohydrate metabolism. Our experimental evidence shows that ammonia alters concentrations of these substrates (30).

The number of EAT cells inoculated per animal was large in the studies described herein. Further work is needed to establish if urease injections could completely suppress development of the tumor. In the studies with mice given tumor cells sc and 0.2 unit of urease, the average tumor size was reduced only 24%. Two urease-treated animals of this group had palpable tumors 2 days after tumor cell inoculation but this was not observed in any of the non-urease-treated animals before 5 days. In other work, we have observed stimulation of DNA synthesis by small dosages of urease (39). This evidence suggests that urea hydrolysis induced by urease may enhance tumor formation under

[4] Sigma Chemical Co., St. Louis, MO.

certain conditions. Although crystalline jack bean urease is a potent antigen, studies in our laboratory with 5000 animals of nine species, including man, have not revealed undesired reactions under the conditions employed with the exception of those due to excess dosage and subsequent ammonia intoxication (30).

Summary. Mature male Swiss Webster mice bearing solid and ascitic forms of Ehrlich ascites tumor (EAT) were injected with 0.1 to 0.4 unit of crystalline jack bean urease per injection. Mice given 12×10^6 EAT cells sc and injected with 0.4 unit of enzyme at 12 and 24 hr thereafter had tumors at 3 weeks which weighed 40% of those in mice given 0.85% NaCl instead of urease. Liver, spleen, and brain weight changed significantly in mice bearing solid EAT and this change was reduced significantly in urease-treated animals. Animals given 0.4 unit of urease as described above showed a significant reduction in body weight compared to non-urease-treated animals. Mice bearing ascitic EAT injected with 0.2 unit of urease on days 6 and 7 after EAT inoculation and killed 5 days thereafter showed significant elevations of ascitic fluid ammonia and cell to ascitic fluid volume ratio. The latter urease-treated animals showed a significant decrease in body weight at 24 and 48 hr after the second urease injection. The half-time (days) for disappearance of ^{3}H-thymidine from DNA in EAT cells growing intraperitoneally were 2.93 ± 1.05 and 3.69 ± 1.71 in urease- and saline-treated animals, respectively. Mice given 0.2 unit of urease at 12 hr before and at 12, 24, and 36 hr after EAT cells were given ip showed a significant reduction in the number of animals with tumors at 21 days and a persistent depression in body weight. Tumor-bearing animals injected with urease showed a depression in colonic temperature and feed intake.

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