Studies on Thymic Humoral Factor Prepared from Guinea Pigs: The Influence of Dietary Vitamin C (34384)

MICHAEL P. DIETER
(Introduced by P. D. Altland)

National Institute of Arthritis and Metabolic Diseases, LPB, National Institutes of Health, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland 20014

Numerous reports suggest there is a relationship between ascorbic acid and ontogenesis. The vitamic C requirement of young primates is known to be greater than that of adults (1). It has been demonstrated that in certain tissues undergoing rapid proliferation, *e.g.*, in sea urchin eggs (2), and in chick embryos (3) marked changes in content and concentration of vitamin C occur, the highest levels being found in tissues undergoing entomesodermal differentiation. Ascorbic acid is also thought to be necessary for fibroblast maturation (4). In addition, ascorbic acid is needed for the optimal conversion of folic acid to folinic acid (5), and the latter is known to function synergistically with vitamin B_{12} to transfer methyl groups to nucleic acid precursors. One report claims a need for ascorbate in the derepression of nucleic acids via histone inactivation (6). All of these findings indicate that there is functional participation of ascorbic acid in rapidly developing organs.

Previous studies in aves suggested ascorbic acid was functionally involved in lymphatic tissue development or metabolism. The concentration and oxidation state of ascorbic acid in the lymphatic organs varies markedly during normal development (7) or after lympholytic steroid hormone treatment (8). In the thymus the concentration of total ascorbic acid decreases 40% during involution and, perhaps more important, the ratio of oxidized to reduced ascorbic acid rises 3-fold.

Recent investigations demonstrated that a humoral factor extracted from the thymus stimulates the growth and immunologic activity of peripheral lymphoid organs in intact or thymectomized animals (9, 10). In view of the aforementioned alterations in thymus ascorbic acid during age involution or after hormone treatment, the possible relationship of ascorbic acid to thymic humoral factor production or activity was investigated. It is known that vitamin C deficiency stimulates the breakdown of protein and diminishes protein resynthesis in liver, brain, and spleen (11). A deficiency of ascorbic acid during thymic development might also impair protein synthesis in this organ and affect the production of the thymic humoral factor.

A bioassay procedure was developed to measure the activity of thymic extracts prepared from guinea pigs maintained on high and low vitamin C diets. Restoration of the X-irradiation depressed weight and hexose monophosphate shunt enzyme activity of rat lymphatic organs served as the end point for thymic humoral factor assay.

Materials and Methods. Male guinea pigs of the Hartley strain from the National Institutes of Health, 250–300 g, were maintained five to a cage on vitamin C deficient diets[1] and supplemented with high and low levels of vitamin C in the drinking water for 6 weeks, a period sufficient to deplete tissue stores of vitamin C in the hypovitaminotic group (12). The high level of vitamin C was 1.0% and the low level 0.1%. At the termination of the experimental period the animals were sacrificed with chloroform and the heart-blood, thymus, and spleen were removed. A few of these organs were analyzed for vitamin C by the indophenol method (13). Heart-blood was used for microhematocrit and hemoglobin analysis (14). In other animals, the

[1] Ascorbic acid deficient diet (guinea pig) from Nutritional Biochemicals Corporation, Cleveland, Ohio.

thymus and spleen were weighed and homogenized in cold 0.85% sterile saline (15–25% w/v) for spleen and thymus extract preparation (9). The tissue homogenates were spun in a refrigerated centrifuge for 15 min at 700*g* and the supernatant ultracentrifuged for 1 hr at 105,000*g*. One-tenth ml of the supernatant was analyzed for total protein (15) and then adjusted with saline to a final concentration of 10 mg of protein/ml. These tissue extracts were used immediately or stored frozen for up to 30 days before assay.

Bioassay procedure. Seven-week-old male, Sprague-Dawley, pathogen-free rats were exposed to whole body *x*-irradiation using a 250 kVP Westinghouse Quandrocondex irradiator at 200 kVCP and 15 mA with 0.25 mm Cu and 0.55 mm Al filtration. The rats were irradiated two at a time in Plexiglas restraining holders. The exposure used was 300 R delivered at 139 R/min from tubes 54 cm above and below the animals. Starting 6-hr postirradiation the rats were injected ip with 0.5 ml of tissue extract, containing 5.0 mg of protein, and again 24 and 48 hr later. A typical experiment consisted of 20 animals divided into four groups: (i) nonirradiated rats, (ii) irradiated rats injected with spleen extract prepared from guinea pigs maintained on high vitamin C levels, (iii) irradiated rats injected with thymus extract prepared from guinea pigs maintained on high vitamin C levels, and (iv) irradiated rats injected with thymus extract prepared from guinea pigs maintained on low vitamin C levels. On the third day, after three injections (total protein injected, 15 mg) and 72 hr postirradiation, the rats were killed with chloroform. Body weights were recorded and the superficial cervical lymph nodes, thymus, and spleen were removed, weighed, and used for enzyme assay.

Enzyme assays. The cervical lymph nodes were analyzed immediately for enzyme activity. The thymus and spleen were frozen in dry ice and assayed 24 hr later. Tissues were homogenized in 0.05 *M* Tris buffer (pH 7.4 and 4% w/v) using glass tubes, Teflon pestles and a Lapine cone-drive stirrer. The homogenates were centrifuged for 20 min at 0° and 21,000*g*. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities of the supernatant were measured according to the method outlined previously (16), and are expressed in mμmoles substrate transformed/mg of protein/min (mUnits).

Results. The rate of gain and final body weight of the guinea pigs maintained on different levels of vitamin C were equivalent. Visible pathological signs of scurvy, *e.g.*, lethargy, tenderness and edema of joints, or hemorrhage, were absent. The hematocrit and hemoglobin concentration (gm/100 ml) in hypovitaminotic guinea pigs were slightly but significantly lower than in guinea pigs maintained on high levels of vitamin C (decrease in hematocrit from 43.4 ± 0.6 SEM to 40.7 ± 0.6, and in hemoglobin from 12.7 ± 0.4 to 11.0 ± 0.3, $p<0.05$, 23 *df*).

The concentration of total vitamin C (ascorbate + dehydroascorbate) in the thymus and spleen was significantly lower in the hypovitaminotic guinea pigs (Table I). The ratio of oxidized to reduced ascorbic acid was markedly higher in the thymus of hypovita-

TABLE I. Comparison of the Tissue Concentration of Vitamin C in Organs of Guinea Pigs Maintained on Vitamin C-Deficient Diets.[a]

Supplement in drinking water	Thymus			Spleen		
	AsA[b]	DHA	AA	AsA	DHA	AA
Vitamin C, 1.0%	50.6 ± 5.3	16.8 ± 2.1	67.4 ± 5.5	34.9 ± 1.0	22.5 ± 7.7	57.3 ± 11.4
0.1%	9.9 ± 2.8[c]	16.1 ± 2.6	26.1 ± 3.8[c]	16.1 ± 1.5[c]	15.0 ± 1.6	31.3 ± 3.0[c]

[a] Values represent the mean ± SEM of four animals per group.

[b] AsA, DHA, AA indicate ascorbate, dehydroascorbate, and total vitamin C.

[c] Indicates means are significantly different from those in the high vitamin C group at $p<0.05$, Student's *t* test.

minotic guinea pigs (5-fold increase) and perhaps has some physiological significance, as suggested by subsequent data.

Table II illustrates changes in the lymphatic organ weights of irradiated animals following tissue extract administration. Spleen extract was injected into irradiated rats as an added control and it was found this treatment did not accelerate the regeneration of lymphatic tissue weight. The most radiosensitive organ was the thymus (88% wt loss), followed in order by the lymph nodes (69% loss) and spleen (63% loss). Thymus extract injections prepared from guinea pigs maintained on high levels of vitamin C caused a percentage increase in lymphatic organ weight that was inversely proportional to the loss incurred following irradiation. Thymus extract prepared from guinea pigs maintained on a low viatmin C supplement did not accelerate the regeneration of lymphatic organ weights in irradiated rats. Indeed, there was about a 30% further loss of weight in the lymph nodes and spleen.

Tables III and IV summarize the data of three replicate experiments showing the effect of thymus extract injections on hexose monophosphate shunt (HMS) enzyme activity of the lymphatic organs. There was little variation between the replicate experiments. Both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity were significantly lower in the lymphatic organs of the irradiated rats injected with spleen extract than in nonirradiated rats, with the exception of glucose-6-phosphate dehydrogenase in the spleen. Preliminary work showed again, as it did in the weight responses, that spleen extract injections did not restore the HMS enzyme activity of the lymphatic organs after irradiation. Thymus extract injections (prepared from guinea pigs maintained on high vitamin C levels) restored the activity of glucose-6-phosphate dehydrogenase in the thymus and lymph nodes (Table III). The effect of thymus extract prepared from hypovitaminotic guinea pigs on the restoration of glucose-6-phosphate dehydrogenase activity in thymus and spleen was significantly less ($p<0.02$).

Injection of thymus extract prepared from guinea pigs maintained on a high vitamin C supplement restored the activity of 6-phosphogluconate dehydrogenase in all three lymphatic organs to levels found in nonirradiated rats (Table IV). The 6-phosphogluconate activity was significantly less ($p<0.02$) in all of the lymphatic organs of rats injected with thymus extract prepared from hypovitaminotic guinea pigs than in rats injected with extract prepared from guinea pigs maintained on high vitamin C levels.

Discussion. This study shows that guinea pig thymus extract, but not spleen extract, accelerates the regeneration of the spleen and lymph node weight in irradiated rats. Unpublished observations citing increases in the peripheral lymphoid organ weight of irradiated animals following thymus extract administration (17) suggested this procedure might be employed as a useful bioassay to measure thymus extract activity. In addition, it was found that thymus weight in irradiated animals was also increased, in contrast to the

TABLE II. The Effect of Thymus Extract Injections on Lymphatic Organ Weight [mean weight (mg) ± SEM, $N = 20$].

Treatment	Thymus	Lymph nodes	Spleen
None	629 ± 31	98 ± 5	726 ± 29
Irradiated			
+ spleen extract	77 ± 3	30 ± 2	272 ± 12
+ thymus extract[a]	110 ± 4 (+43%)[c]	42 ± 3 (+38%)	321 ± 42 (+18%)
+ thymus extract[b]	74 ± 3 (−4%)	21 ± 1 (−31%)	186 ± 10 (−32%)

[a] Thymus extract prepared from guinea pigs maintained on *high levels* of vitamin C.

[b] Thymus extract prepared from guinea pigs maintained on *low levels* of vitamin C.

[c] Values in parentheses indicate percentage change from irradiated rats injected with spleen extract.

TABLE III. Effect of Thymus Extract Injections on Hexose Monophosphate Shunt Enzyme Activity of Rat Lymphatic Organs (mean ± SEM; sample no. in parentheses).

Treatment	Glucose-6-phosphate dehydrogenase (mUnits)		
	Thymus	Spleen	Lymph nodes
None	29.6 ± 1.2 (17)	35.5 ± 0.9 (17)	43.4 ± 2.7 (15)
Irradiated			
+ spleen extract	21.6 ± 1.2 (10)[c]	37.3 ± 1.3 (10)	32.5 ± 2.1 (15)[c]
+ thymus extract[a]	25.6 ± 0.9 (17)[c]	37.9 ± 0.8 (16)	38.9 ± 1.3 (13)[c]
+ thymus extract[b]	18.4 ± 1.0 (11)[c]	30.3 ± 1.2 (11)[c]	36.3 ± 2.8 (11)

[a] Thymus extract prepared from guinea pigs maintained on *high levels* of vitamin C.
[b] Thymus extract prepared from guinea pigs maintained on *low levels* of vitamin C.
[c] Significantly different ($p < 0.02$, Student's t test) from the mean immediately above.

TABLE IV. Effect of Thymus Extract Injections on Hexose Monophosphate Shunt Enzyme Activity of Rat Lymphatic Organs (mean ± SEM; sample no. in parentheses).

Treatment	6-Phosphogluconate dehydrogenase (mUnits)		
	Thymus	Spleen	Lymph nodes
None	20.0 ± 0.9 (15)	19.8 ± 0.9 (15)	25.8 ± 1.2 (15)
Irradiated			
+ spleen extract	15.2 ± 1.4 (10)[c]	16.4 ± 0.8 (10)[c]	18.6 ± 0.8 (15)[c]
+ thymus extract[a]	19.5 ± 1.0 (17)[c]	18.5 ± 0.2 (16)[c]	28.4 ± 1.1 (13)[c]
+ thymus extract[b]	15.9 ± 0.8 (11)[c]	17.1 ± 0.4 (11)[c]	24.7 ± 1.3 (11)[c]

[a] Thymus extract prepared from guinea pigs maintained on *high levels* of vitamin C.
[b] Thymus extract prepared from guinea pigs maintained on *low levels* of vitamin C.
[c] Significantly different ($p < 0.02$, Student's t test) from the mean immediately above.

assays in nonirradiated animals, which employed radioactive glycine and thymidine incorporation into lymphatic tissue protein and nucleic acid as indicators of thymus extract activity (9). The increase of lymph node and spleen weight in irradiated rats injected with thymus extract may have been a consequence of antigenic stimulation, in addition to the direct effect of thymic humoral factor (24). However, the fact that the greatest degree of lymphatic tissue weight regeneration observed in irradiated animals was in the thymus, which is not dependent on antigenic stimulation, and produces neither antibody nor plasma cells (24), suggests thymus extract alone caused the stimulatory effect. Also, as previously illustrated in the results, the acceleration of thymic weight regeneration in irradiated rats was accompanied by enhanced HMS enzyme activity. The lack of a thymic response in nonirradiated animals injected with thymus extract perhaps indicates that antigenic stimulation, together with thymus extract administration, evoked the peripheral lymphoid organ responses.

The weight response in irradiated animals, in conjunction with suitable enzyme measurements, is a sensitive bioassay for thymus extract activity that should prove useful in further investigations. The 6-phosphogluconate dehydrogenase reaction in the lymphatic organs was the most sensitive of the two enzyme tests used. In all instances, after irradiation, or after administration of the two different sources of thymus extract preparation, the changes in 6-phosphogluconate dehydrogenase activity in each of the lymphatic organs were highly significant (see Table IV).

The HMS pathway in lymphatic organs is of considerable importance, accounting for ca. 15% of total glucose oxidation (18). The activities of glucose-6-phosphate dehydro-

genase and 6-phosphogluconate dehydrogenase are higher in the lymphatic organs than in numerous other organs investigated (19). The increase in HMS enzyme activity after thymus extract administration indicates that at least one of the four factors comprising thymic humoral factor (17) stimulates direct glucose oxidation in the lymphatic organs. Hormone stimulation of these reactions would provide NADPH for reductive syntheses (20), provide ribose for incorporation into nucleic acids and nucleotides (21), and thus promote the regeneration of lymphatic tissue weight.

Since thymus extract prepared from hypovitaminotic guinea pigs exhibited significantly less effects, as measured in this bioassay, ascorbic acid appears necessary either for the normal production of thymic humoral factor, or for its optimal activity. Evaluation of the concentration and oxidation state of ascorbic acid in the thymus indicated that not only was there less vitamin present in the tissue, but that the amount remaining was largely in the oxidized form. It is possible that dehydroascorbate altered the configuration of thymic humoral factor during synthesis or inhibited the activity of certain enzymes in the cellular milieu indirectly affecting the synthesis of thymic humoral factor. It is well established that numerous enzymes involved in carbohydrate oxidation and protein synthesis are dependent on functional sulfhydryl groups for catalytic activity (22, 23). Further investigation of the relationship between dehydroascorbate and thymic humoral factor appears warranted.

Summary. The present study describes a bioassay system for evalulating the activity of thymic humoral factor. The X-irradiation depressed weight and hexose monophosphate shunt enzyme activity of rat thymus, spleen, and lymph nodes were partially restored by thymus extract, but not by spleen extract injections. The influence of dietary vitamin C on the maintenance or production of thymic humoral factor was also investigated. Thymus extracts prepared from guinea pigs maintained on high levels of vitamin C accelerated the regeneration of lymphatic tissue weight and restored hexose monophosphate shunt enzyme activity, whereas further loss of tissue weight and depressed enzyme activity occurred in the irradiated rats treated with thymus extract prepared from guinea pigs maintained on low levels of vitamin C. The evidence thus suggests that thymic humoral factor production or activity is dependent in part on vitamin C. The concentration of total ascorbic acid in the thymus of hypovitaminotic guinea pigs had decreased 40% below normal and the ratio of oxidized to reduced ascorbic acid had increased 5-fold, indicating the oxidation state of ascorbic acid is particularly important for the maintenance of thymic humoral factor activity.

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