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A Quantitative Study of Muramidase Distribution in Normal and Nitrogen Mustard-Treated Rats*

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A relationship between serum muramidase (lysozyme) activity and granulocyte kinetics has been described (1–5). Increased turnover of polymorphonuclear leukocytes and monocytes usually is associated with increased serum muramidase activity. Conditions associated with reduced numbers of granulocytes, such as aplastic anemia and acute lymphocytic leukemia, frequently have reduced serum muramidase activity(3,4). Nitrogen mustard-induced granulocytopenia in rabbits results in reduction in serum muramidase activity whereas rapid granulocyte destruction in response to an injection of antigranulocyte serum causes a transitory increase in serum muramidase activity(6). These clinical observations and experiments all are in accord with the concept that most of the serum muramidase is derived from the degradation of granulocytes and monocytes, and that a direct quantitative relationship exists between the turnover of these cells and serum enzyme activity. These studies suggest the possibility that tissue muramidase may be mostly of leukocyte origin. In order to test this hypothesis, the distribution of muramidase in serum and tissues was studied in rats treated with intravenous nitrogen mustard and correlated with changes in circulating leukocytes and tissue histology. The total animal muramidase pool was also estimated.

METHODS

All studies were performed on male Sprague–Dawley rats weighing 200–300 g. They were maintained on a standard lab chow and water *ad lib.*

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Total rat muramidase activities were determined by two different techniques, each carried out on 24–36 rats. The first technique involved death by cervical dislocation after which the entire animal was passed through a meat grinder. Total rat muramidase activity was calculated from the results of muramidase assay of aliquots (of homogenates) of this material. The second method consisted of cervical dislocation death followed by individual organ muramidase assay with summation of the results for each animal in order to determine total rat enzyme activity. Total marrow mass and serum volume were estimated on the basis of published standard values(7,8).

The effect of severe granulocytopenia on organ muramidase activity was determined in a group of 36 rats. Freshly prepared nitrogen mustard in a dose of 0.1 mg/kg was administered to 30 of the rats. At 24 h intervals thereafter, and for the next 8 days two or more animals were killed by means of rapid exsanguination while under ether narcosis. Muramidase activity was determined for each organ or tissue removed. The remaining rats served as controls. Each control animal was given saline in place of nitrogen mustard and then killed for tissue muramidase activity after rapid exsanguination. The combined daily results for each organ in the nitrogen mustard group was expressed as percentage of the combined results for each organ in the control group. Total and differential leukocyte counts and histologic sections of tissue were performed by routine methods.

The technique for the extraction of muramidase was a modification of the method described by Cohn and Hirsch(9). Each organ was rinsed in cold normal saline and weighed. Aliquots from representative portions of each organ were placed in 0.40 M sucrose. For bone marrow determinations, identical lengths of femur were flushed repeatedly with known volumes of sucrose solution. The organs were homogenized and pH shocked with acetic and hydrochloric acids to pH 2 and then returned to pH 5.5–6.0 with sodium hydroxide solution. After brief centrifugation, the supernatant factions were assayed for enzyme activity. Serum and urine samples were assayed directly. All assays were performed on lysoplates using the technique of Osserman and Lawlor(10). Further purified amorphous muramidase for standards was obtained by bentonite extraction and dialysis(11).

Using lysoplate assay technique, colinear straight lines were obtained when the logarithm of purified rat organ muramidase or human monocytic leukemic urinary muramidase activity was plotted against the diameter of lytic zones in agarose gels containing *Micrococcus lysodeikticus,* a substrate of muramidase.

Standards were obtained from muramidase purified from urine of patients with monocytic leukemia. This was compared on a weight-for-weight basis with muramidase of similar purity of rat tissue origin and the enzymes from the two species were shown to have equivalent activity at all concentrations. Therefore, the more plentiful human muramidase was substituted for standards for all the studies. Egg white muramidase was several times less active on a weight-for-weight basis than either of the mammalian enzymes.

RESULTS

Table 1 shows the muramidase concentration and total content of various rat organs. It can be seen that lung, kidney, spleen, and bone marrow are the most active. Large and small intestine, adrenal, salivary gland, and liver are in the intermediate range. Heart, muscle, fat, and brain have appreciably smaller enzyme concentrations. The average total white cell count in these animals was 16,850/mm^3, and the absolute granulocyte count was 5280/mm^3. Total muramidase distribution by organ is shown in Fig. 1. Gut, kidney, bone marrow, and lung account for about 75% of the total. It also is apparent that serum muramidase is, quantitatively speaking, an insignificant part of the total animal muramidase pool.

Direct assay of totally homogenized rats revealed 45.0 mg of rat kidney muramidase (RKM) equivalents/300-g male rat. This represented an average of 150-μg RKM equivalents per gram of rat tissue. Total animal muramidase calculated on the basis of the sum of individual organ and tissue activities was 46.5 mg RKM equivalents, a figure in good agreement with that obtained by direct total animal assay.

Rats treated with nitrogen mustard demonstrated three types of change in muramidase activity (Fig. 2 and Table 2). The most frequent change was a progressive decrease in organ muramidase activity during the 3 to 4 postinjection

TABLE 1
MURAMIDASE CONCENTRATION AND TOTAL CONTENT OF VARIOUS RAT ORGANS[a]

Organ	Av wt (gr)	Muramidase[b] Av μg/g tissue ± 1 SD	Mg/organ
Kidney	5.0	1860 ± 385	9.3
Lung	2.5	2420 ± 640	6.1
Spleen	1.0	1390 ± 260	1.3
Bone marrow	10.0	617 ± 96	8.0
Small intestine	26.0	356 ± 77	7.4
Adrenal gland	—	286 ± 88	—
Large intestine	13.0	167 ± 43	3.7
Salivary gland	—	150 ± 38	—
Liver	17.0	137 ± 21	2.4
Heart	3.0	96 ± 47	0.3
Fat	30.0	74 ± 12	2.3
Brain	4.0	72 ± 21	0.3
Serum	12.0	48 ± 3.1	0.5
Testes	—	19 ± 7	—
Miscellaneous[c]	—	—	4.9
		Total	46.5

[a] The number of animals in each group ranged from 24 to 36 except for serum which was assayed in 192 rats.

[b] Rat kidney muramidase equivalents.

[c] Includes muscle, skin, and blood vessels.

days followed by rapid recovery. This general pattern, with a few minor exceptions, was characteristic of spleen, kidney, intestine, fat, brain, heart, liver, and adrenal. Serum muramidase activity increased during the first 12 h, but then fell sharply during the next 3 days. Absolute granulocyte counts dropped to 12% of normal during Days 4 and 5 after nitrogen mustard, after which there was rapid recovery. Histologic examination of splenic tissue demonstrated marked hypocellularity on Days 2 through 5 after treatment. Kidney muramidase activity tended to fall more slowly, but the ultimate decrease was comparable to that of most other organs. No alterations in microscopic structure were noted. There was

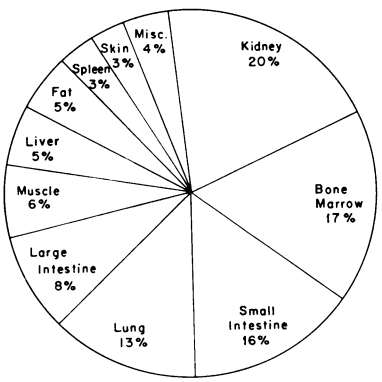

FIG. 1. Percentage distribution of rat muramidase by organ. These values were calculated on the basis of average organ weights and muramidase concentrations. The miscellaneous category includes blood vessels, brain, heart, and serum.

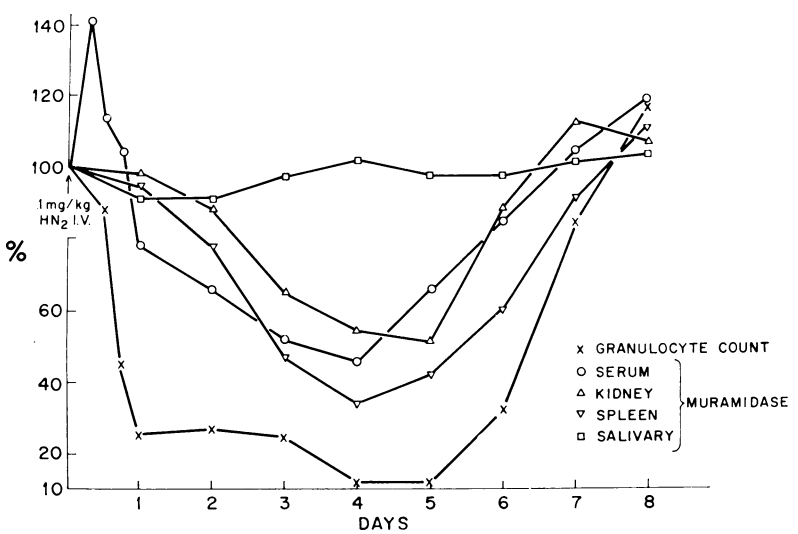

FIG. 2. Change in total granulocyte count and muramidase concentration in various organs of rats after the administration of nitrogen mustard (HN_2). The values are shown as an average in percentage of pretreatment organ muramidase concentration.

both gross and microscopic evidence of adrenal hypertrophy with most change noted in the zona fasciculata.

The second pattern was exemplified by the lungs which showed considerable increase in muramidase activity during the entire experimental period (Table 2). Hematoxylin and eosin-stained sections of lung revealed an acute inflammatory exudate made up of large numbers of bacteria and polymorphonuclear leukocytes.

Salivary gland typified the third type of change (Fig. 2, Table 2). There was no significant change in either muramidase activity or histologic appearance after treatment with nitrogen mustard.

Change in total body muramidase activity with time after treatment with nitrogen mustard is demonstrated in Fig. 3. Each point on the figure was derived from a summation of all individual organ and tissue activities for that particular day. Although the serum muramidase represents only about 1% of total body activity,

TABLE 2

MURAMIDASE ACTIVITY IN RATS TREATED WITH NITROGEN MUSTARD[a]

Organ or tissue	% Organ muramidase at daily intervals after HN_2							
	1	2	3	4	5	6	7	8
Fat	64	58	61	42	71	91	102	—
Brain	73	79	71	74	94	92	90	—
Lung	87	113	115	126	107	109	108	98
Heart	81	79	79	61	96	103	100	—
Spleen	95	77	48	33	41	60	92	111
Liver	83	77	70	58	58	80	90	—
Adrenal	88	81	77	79	85	94	110	—
Small intestine	83	75	73	55	65	60	84	107
Large intestine	68	64	70	62	77	91	105	128
Salivary gland	92	85	97	101	96	93	103	105
Kidney	99	87	64	54	49	86	112	109
Serum	78	66	53	49	66	84	103	118

[a] Each assay represents the average value from two or more rats.

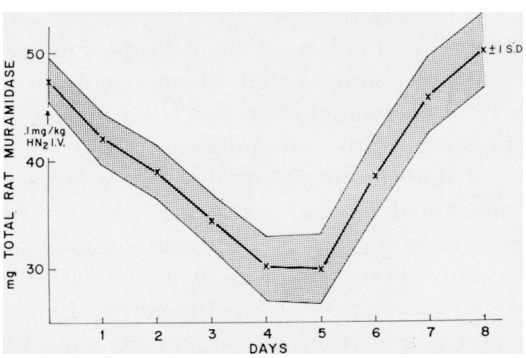

FIG. 3. Change in total rat muramidase content after the administration of nitrogen mustard (HN_2). A total of 24 rats was used in the study. Each point represents the average of two or more animals.

change in serum activity closely parallels changes in total body activity (Figs. 2 and 3).

DISCUSSION

In general, the distribution of tissue muramidase in the rat reported here is in reasonable agreement with the results of Suu(12), Speece(13), and Perri(14). The magnitude of the tissue enzyme concentrations, however, are different in this study due to differences in extraction techniques and the use of rat kidney muramidase standardization of the reported tissue enzyme concentrations, rather than egg white muramidase. By both lysoplate and turbidimetric methods it has been shown that egg white muramidase and mammalian muramidase have very different activities. Furthermore, in both types of assay procedure, differences in activity were noted in a fixed ratio but varied with the initial concentration. The reasons for this are not clear but cast some doubt on the quantitative validity of previous mammalian studies which used egg white muramidase as standards of assay. In the population of rats studied, the total white cell counts and differential cell counts agreed well with known literature values(15) suggesting the presence of normal leukokinetics. It is also clear that the muramidase which was assayed within tissues could not represent serum contamination since micrograms of enzyme per gram of tissue exceeded serum levels in almost all organs. Thus, serum contamination would serve to lower average muramidase content per gram of tissue.

The effects of nitrogen mustard in the experimental animal have been well documented(16). Generally, there is a rapid lymphocytopenia which lasts 5 days to 2 weeks. Soon after the administration of nitrogen mustard intravenously, a transient granulocytosis is followed by a profound granulocytopenia with gradual recovery over a 5- to 7-day period if the animal survives. In the present studies, after treatment with nitrogen mustard, a marked but very brief lymphocytosis was noted, after which granulocyte and lymphocyte counts were depressed. It is not surprising that during the early period of granulocytopenia during which time there is a period of considerable injury to mature leukocytes, there is a transient rise in serum muramidase. This is consistent with the findings of Kerby(17) and Fink(6), who showed that *in vivo* leukocyte injury resulted in transient elevations in serum muramidase levels. Urine assays during this period failed to reveal the presence of any muramidase.

These results suggest that serum muramidase levels are a reflection of the degree of active granulocytopoiesis, and that organ muramidase levels may reflect the granulocyte mass within the organ. For example, most animals treated with nitrogen mustard develop the picture of an acute exudative pneumonia with infiltration of numerous polymorphonuclear leukocytes. Lungs assayed for muramidase during this period are richer in muramidase than normal controls at a time when most organs have less than 50% of the normal muramidase content(16). Examination of the relative time course of serum, kidney, and other organ muramidase change versus alterations in granulocyte count provided fur-

ther evidence for this relation. Peripheral blood granulocyte counts fell rapidly and reached very low levels before serum and organ muramidase activities began to fall. With the exception of the salivary gland the decline in organ muramidase had a time sequence change similar to that observed for tissue granulocyte depletion after nitrogen mustard(16). Whether these enzyme activity reductions represented a drop in the numbers of macrophages or granulocytes contained within these tissues or simply a loss of muramidase from the parenchyma of the tissue cannot be stated by simple histologic examination alone. Indirect evidence at the present time, however, strongly favors leukocytes as the major source of muramidase for most tissues of the body. Finally, chronologically, kidney muramidase levels declined most slowly, though eventually reaching very low levels as compared with controls. This delay is consistent with the role of the kidney as an excretory organ. Recovery of renal muramidase levels appeared to be delayed until after the return of serum levels toward normal.

It was interesting to note that serum muramidase began to return to normal values before the reappearance of circulating granulocytes(6). This suggests that proliferating granulocytes in the bone marrow may release muramidase. A preliminary study of quantitative variations of muramidase within bone marrow of rats treated with nitrogen mustard was undertaken. It revealed normal or elevated muramidase levels in marrow for all but the second and third day after treatment when there was a modest reduction in muramidase content. This seems to indicate that ineffective granulopoiesis in the marrow before the release of cells into the circulating pool of granulocytes, accompanied by normal marrow muramidase levels, may explain the early recovery of serum muramidase.

As shown in the results section, salivary gland showed no significant decrease in muramidase content during the course of treatment with nitrogen mustard. In addition, adrenal gland showed only very small decreases in muramidase and in some cases there were modest increases. It has long been known that saliva is a rich source of muramidase(18). The enzyme may either be elaborated by the salivary glands or concentrated from the serum and then excreted. Recently it has been demonstrated that tear muramidase is not derived from blood leukocytes and that lacrimal gland muramidase production probably is entirely independent(19). This study provides strong evidence that the production of muramidase in salivary glands also may be autonomous.

In general, data from the nitrogen mustard studies described suggests that variations in serum and organ muramidase are temporally and causally related to variations in leukokinetics, with the exceptions described above. Serum muramidase is a reasonable mirror of changes in total body muramidase stores in the rat. The rapid disappearance and return of a major portion of the body's total muramidase is consistent with a recent report of a serum $T_{1/2}$ of less than 2 h(20).

SUMMARY

Studies of muramidase distribution in the rat revealed that bone marrow, kidney, spleen, and lung were the richest sources of enzyme followed by salivary gland, intestine, adrenal, and liver. Serum levels were lower than those of most

organs and quantitatively represented but 1% of the total animal lysozyme pool at any one moment. Nitrogen mustard depressed serum and organ muramidase levels in most tissues in proportion to reduction in the number of circulating granulocytes. The exception of salivary gland to this relationship suggests that its production of muramidase is autonomous. Serum muramidase was found to be an accurate mirror of changes in total body muramidase stores.

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