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Acid Hydrolase Activity in the Resorbing Carrageenan Granuloma[1] (38330)

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(Introduced by P. Starr)

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The involvement of lysosomal hydrolases in the *in vivo* degradation of connective tissue has recently become a focal point of attention. These enzymes are implicated in all physiological remodeling processes and their levels are almost invariably elevated when connective tissues undergo resorption (1).

It was believed that the granuloma induced by the subcutaneous injection of carrageenan, a sulfated polygalactose obtained from the sea weed commonly known as Irish moss, would be an excellent model for studying the involvement of acid hydrolases in connective tissue degradation. Such a granuloma, which consists in part of collagen fibers, ground substance, and connective tissue cells, attains it maximum weight in a rat in 7–10 days and is then completely resorbed in 2–4 wk.

In a number of studies over the past two decades, the dynamics of carrageenan granuloma growth and resorption have been investigated. Robertson and Schwartz (2) were the first to use the technique to study the biosynthesis of collagen, and Jackson (3) followed up with a detailed study of the changes in collagen content, as well as the changes in solubility properties of the collagen, during growth and resorption of carrageenan granulomas in guinea pigs. Prodi and Romeo (4) found that hyaluronic acid of the ground substance was produced in considerable amounts during the initial stages of growth, then decreased in concentration while the sulfated mucopolysaccharides increased in amount up to Day 11 when the experiment was terminated.

Although investigators have mainly used the carrageenan technique as a model to study other phenomena, some attention has been given to the catabolic and enzymatic aspects of the granulomatous tissue *per se*. Robert *et al.* (5) have demonstrated an increase in intra- and extracellular catheptic activity in carrageenan granuloma tissue and postulated that the increased production and release of lytic enzymes could be involved in the eventual dissolution of the fibrous network of the granuloma. Acid phosphatase activity has also been shown to increase during carrageenan granuloma growth, and it was suggested that this enzyme is part of a system regulating the formation of sulfated acid mucopolysaccharides (6).

It was the purpose of this investigation to further evaluate the catabolic aspects of the metabolism of carrageenan granulomas, and to study more completely the role of lysosomal acid hydrolases in this process. Therefore, over a 4-wk period of growth and resorption of such granulomas, changes were determined in the activities of five acid hydrolases, which were presumably of lysosomal origin. Also determined were granuloma wet weight, protein content, collagen content, and solubility properties, and finally, content of hexosamine, a component of ground substance mucoproteins and mucopolysaccharides.

Methods. Carrageenan, kindly supplied as SeaKem 7 by Marine Colloids, Inc., Springfield, NJ, was used for granuloma induction after a modification of the procedure of Fukuhara and Tsurufuji (7). The anterior, dorsal surface of male Holtzmann rats, weighing 135–145 g, was shaved and 3 ml of air were injected subdermally. Residual air was removed 24 hr later and 3 ml of a warm, sterile 2% (w/v) carrageenan solution were injected into the air-formed pocket. Five to nine animals were randomly selected for granuloma harvesting, either

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every second day after carrageenan injection, or daily from Days 7–14. The dissected granulomas were stored at −15° until the experiment was terminated at 28 days. The frozen granulomatous tissue was then utilized for collagen fractionation, for hexosamine and protein determinations, and for acid hydrolase analyses.

One gram of tissue from each granuloma was sequentially extracted with 0.15 *M* NaCl, 1.0 *M* NaCl, and finally with 0.5 *M* citrate buffer of pH 3.6. The extracts and insoluble residues were then analyzed for collagen hydroxyproline using a modification of the procedure of Neuman and Logan (8).

A second gram of tissue was also extracted with 0.15 *M* NaCl, and this material was used for the enzyme assays, all of which were carried out at 37°. Cathepsin activity was determined at pH 3.6 by the method of Anson (9), using hemoglobin as substrate. Solubilized protein was equated to tyrosine absorbance at 280 nm. β-Glucosaminidase was determined with 1.1×10^{-3} *M p*-nitrophenyl-*N*- acetyl- β- D- glucosaminide as substrate (0.2 *M* citrate-phosphate buffer, pH 4.4); β- glucuronidase with 1.2×10^{-3} *M p*- nitrophenyl- β- D- glucuronide (0.2 *M* acetate buffer, pH 4.5); β- galactosidase with 2.5×10^{-3} *M p*- nitrophenyl- β- D- galactopyranoside (0.2 *M* citrate– phosphate buffer, pH 3.5); and acid phosphatase with 1.4×10^{-3} *M p*-nitrophenyl phosphate (0.2 *M* acetate buffer, pH 4.5). Liberated *p*- nitrophenyl was measured at 400 nm at pH 12.

The extracts used for the enzyme analyses were also employed for the determination of protein, using the biuret reagent, and for the determination of hexosamine, using a modification of the procedure of Rondle and Morgan (10). Unknown hexosamine concentrations were equated to a glucosamine–galactosamine standard.

Results. Table I summarizes the changes in carrageenan granuloma wet weight, hexosamine, and protein[3]. These three variables seem closely related since the totals increase to maxima on Day 8, and decrease steadily thereafter. Hexosamine concentration also peaks at Day 8, while protein concentration peaks at Day 24. After 28 days granuloma resorption was essentially complete, as can be seen from these data.

The changes in granuloma collagen are given in Table II. Total collagen per granuloma reaches a maximum value on Day 11, which is just a day earlier than its concentration peaks. The concentration of insoluble collagen achieves its highest value on Day 12, while the soluble collagens reach maximum concentrations on Days 18–20. Thus, the soluble forms of collagen are present in highest concentrations during the period of rapid resorption of the granuloma.

In Table III are given the total activites of the acid hydrolases during the course of the experiment[3]. It is interesting to note that max-

[3] To conserve space, data are not reported for Days 7, 9, 11, and 13. Inclusion of these data would not affect the interpretation of the results as they fit in nicely with remaining data, and maximum values were not obtained on these days.

TABLE I. Granuloma Wet Weight, Hexosamine, and Protein.

Days after carrageenan injection	Wet weight (g)	Hexosamine		Protein	
		Total (mg)	Concn. (mg/g)	Total (mg)	Concn. (mg/g)
2	1.27	1.19	0.94	42	33.4
4	7.20	6.91	0.96	233	32.4
6	9.10	9.56	1.05	274	30.1
8	9.87	12.14	1.23	307	31.1
10	8.20	8.61	1.05	271	33.0
12	5.49	5.00	0.91	188	34.3
14	4.64	4.41	0.95	166	35.8
16	3.98	3.62	0.91	146	36.8
18	3.29	2.96	0.90	122	37.0
20	2.68	1.74	0.65	104	38.8
22	2.36	1.77	0.75	96	40.8
24	1.84	1.75	0.95	83	44.9
26	0.99	.89	0.90	43	43.0
28	0.62	.38	0.61	24	39.2

TABLE II. Collagen Content of Granulomas.

Days after carrageenan injection	Total collagen (mg)	Collagen concentrations (mg/g wet weight)				
		0.15 *M* NaCl	1.0 *M* NaCl	Total salt soluble	0.5 *M* Citrate	Insoluble
2	7	0.69	0.58	1.27	0.15	4.3
4	19	1.08	0.21	1.29	0.04	1.3
6	58	1.05	0.33	1.38	0.08	4.9
7	60	0.82	0.28	1.10	0.09	5.0
8	82	1.28	0.43	1.71	0.10	6.8
9	77	0.97	0.61	1.58	0.11	7.1
10	77	1.33	0.53	1.86	0.12	7.4
11	112	1.36	0.78	2.14	0.16	11.6
12	88	1.38	0.85	2.23	0.16	13.7
13	73	1.38	0.93	2.31	0.18	12.7
14	68	1.38	1.04	2.42	0.21	12.0
16	58	1.36	1.17	2.53	0.22	11.7
18	47	1.49	0.90	2.39	0.22	11.6
20	36	1.18	1.55	2.73	0.37	10.5
22	28	1.41	1.15	2.56	0.30	8.8
24	19	1.18	1.16	2.34	0.28	7.9
26	9	0.92	0.92	1.84	0.18	7.3
28	7	0.74	0.59	1.33	0.14	5.5

imum values are achieved at different times for the different enzymes. Cathepsin peaks at Day 8, the same time as granuloma wet weight and total protein reach maxima. β-Galactosidase also appears to peak at Day 8, while the other enzymes reach maximum values from Days 12–18.

Finally, Table IV and Fig. 1 summarize the specific enzyme activities[3]. In all cases maximum values are obtained well after resorption of the granuloma has commenced. Also, with the exception of acid phosphatase, the specific activities continue to increase after total activities have begun to decrease, indicating a conservation of the enzymes during the period of rapid tissue degradation.

Discussion. With the exception of acid phos-

TABLE III. Total Activity of Acid Hydrolases.

Days after carrageenan injection	Acid phosphatase[a]	Beta-galactosidase[a]	Beta-glucosaminidase[a]	Beta-glucuronidase[a]	Cathepsin[b]
2	25	8	7	12	138
4	97	50	34	77	854
6	116	91	58	103	1198
8	156	181	118	217	1676
10	199	164	429	268	1475
12	234	160	1008	350	1253
14	237	157	1012	329	1226
16	190	145	870	293	1147
18	299	170	880	276	1066
20	195	149	729	233	947
22	149	141	747	213	896
24	112	152	668	233	902
26	50	71	327	140	449
28	29	40	172	94	265

[a] Activity expressed as μmoles *p*-nitrophenol liberated/hr/granuloma.

[b] Activity expressed as μmoles tyrosine liberated/hr/granuloma.

TABLE IV. Specific Activity of Acid Hydrolases.

Days after carrageenan injection	Acid phosphatase[a]	Beta-galactosidase[a]	Beta-glucosaminidase[a]	Beta-glucuronidase[a]	Cathepsin[b]
2	20	7	5	9	108
4	14	7	5	11	119
6	13	10	6	11	133
8	16	18	12	22	170
10	24	20	52	33	180
12	43	29	184	64	228
14	51	34	218	71	264
16	52	39	236	79	311
18	93	52	267	84	324
20	73	55	272	87	353
22	63	60	317	90	380
24	61	83	363	126	490
26	51	72	330	142	454
28	48	65	278	152	428

[a] Activity expressed as μmoles *p*-nitrophenol liberated/hr/g wet weight.
[b] Activity expressed as μmoles tyrosine liberated/hr/g wet weight.

phatase, the enzymes assayed in this study are ones that might be expected to have a significant function in the degradation of structural components in various phases of connective tissue catabolism (11). The fact that they were significantly elevated, in both amount and concentration, during the resorption of the carrageenan granulomas is strong presumptive evidence that they played a major role in this process.

Since the granuloma wet weight began decreasing while the total amount of collagen was still increasing, it is probable that the initial weight loss was due to a decrease in the ground substance content rather than to a degradation of the fibrous network. This idea is supported by the fact that both the hexosamine content and the granuloma wet weight decreased concurrently after reaching maximum values on Day 8, while total collagen increased until Day 11. Once the ground substance is sufficiently disrupted, proteolytic enzymes such as cathepsin may be able to attack the disorganized collagen fibers and bring about complete resorption of the granuloma.

The source of the enzymes was not determined in this study. It is probable, however, that they were derived to a large extent from the lysosomes of the macrophages that invade such

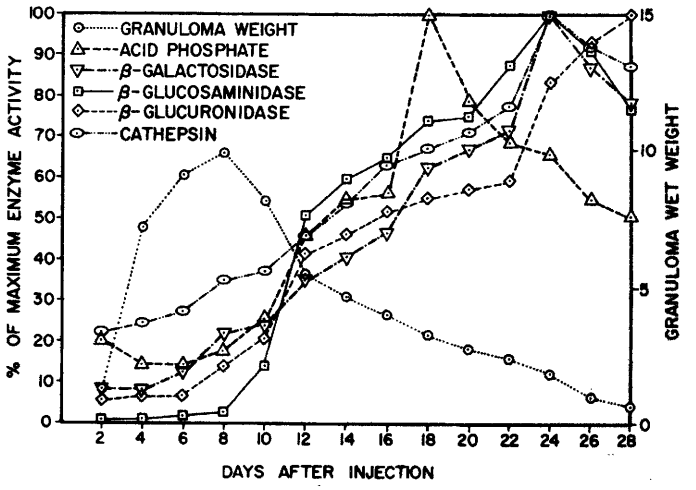

FIG. 1. Specific activity of acid hydrolases and granuloma wet weights.

granulomatous tissue in large numbers after the first several days of growth.

An intriguing problem yet to be resolved is the manner in which the resorption of carrageenan granulomas is triggered. Williams (12) has shown that intradermal carrageenan injections cause the breakdown of collagen fibers without first producing a granuloma, and he suggested that carrageenan itself enhances collagen breakdown. Carrageenan can be shown to be phagocytized by the invading macrophages, and it appears to be toxic in small doses (13). Thus, phagocytic cells may be damaged by the ingested carrageenan, thereby releasing their lysosomal enzymes. Another possibility is simply that phagocytic removal of the stimulus for granuloma formation upsets the metabolic balance in favor of the catabolic aspects of the process.

Summary. The specific activities of the acid hydrolases cathepsin, β-glucuronidase, β-galactosidase, β-glucosaminidase, and acid phosphatase have been shown to increase dramatically during the resorption of carrageenan-induced granulomas. Since these degradative enzymes are of the type normally found in lysosomes, this is strong presumptive evidence that these subcellular particles and their enzymes play a major role in granuloma resorption.

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