Collagen metabolism in adjuvant-induced arthritis in the rat

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The adjuvant arthritis of the rat is a systemic disease afflicting the musculoskeletal structures, accompanied by a number of visceral manifestations (Pearson, 1956; Pearson and Wood, 1959). It has been receiving considerable attention in studies of the factors that may influence the development and course of the rheumatic diseases in man (Leader, Gorham, and Wagner, 1967). This experimental arthritis often serves for testing the efficacy of anti-inflammatory and immunosuppressive agents (Currey and Ziff, 1966; Ward and Cloud, 1966; Winter and Nuss, 1966; Rosenthale and Nagra, 1967; Winder, Lembke, and Stephens, 1969). Our present knowledge of the pathogenesis of the adjuvant arthritis indicates that this experimental disease is a generalized immunological cell-mediated response to an antigen present in mycobacteria.

The systemic character of the disease and its common features with human disease instigated our studies of the metabolic turnover of collagen in rats with adjuvant arthritis. We were interested in learning the reactions shown by this essential protein component of connective tissue during various phases of the arthritic process. Up to the present, only the metabolic turnover of the matrix mucopolysaccharides occurring directly within the inflammatory focus located in the rat limb, has been described (Gietka, 1969).

Material and methods

Our experiments were carried out on male Wistar rats, initially weighing 80 g. each. The experimental group received 0.1 ml. Freund's adjuvant per animal, injected intradermally into the dorsum of the tail root. The adjuvant contained 10 mg. heat-killed *Mycobacterium tuberculosis*, H₃₇R_v strain, per millilitre sterile paraffin oil. Both the experimental and control animals received a standard diet; starting 12 hrs before and continuing during the urine collection in metabolic cages, the animals received only water. The whole experiment was divided into three parts, as follows:

(1) A subgroup of the controls and a subgroup of the experimental animals with adjuvant arthritis received, on

the 25th day after the injection of Freund's adjuvant, 15 μ Ci of ¹⁴C-proline per 100 g. body weight (uniformly labelled ¹⁴C-proline, specific radio activity 98.1 μ Ci/mMol).

(2) Other subgroups of the controls and of the arthritic animals received the same doses of labelled proline on the 46th day after the injection of the Freund's adjuvant. In both these experiments urine was collected after 12 hrs, and 24-hr urine collections were then obtained between the 4th and 5th days after the administration of the labelled proline. At the same intervals, viz. 12 and 120 hrs after proline injection, the animals were killed and their skins were removed. The skin was shaved, liberated from subcutaneous fat, homogenized, and extracted with 0.45 M solution of sodium chloride (pH 7.4) at 5°C. for 24 hrs. After centrifugation at 25,000 G. the extraction was twice repeated. The combined supernatants containing neutral salt soluble collagen (NSC) were dialysed against running water for 48 hrs. The fibrils thus obtained were briefly centrifuged and then hydrolysed with 6 N HCl for 20 hrs at 105°C. The residue of the homogenate, consisting of insoluble collagen (ISC), was likewise hydrolysed. In all hydrolysates of both the NSC and ISC, the hydroxyproline contents were determined by the method of Stegemann (1958), and the radioactivity of ¹⁴C-hydroxyproline was determined by the method of Juva and Prockop (1966) modified by Nordwig, Kobler, and Pfab (1967). Urine samples were likewise hydrolysed; the hydroxyproline contents were determined by the method of Prockop and Udenfriend (1960), and the radioactivity of ¹⁴C-hydroxyproline by the modified method of Juva and Prockop (1966). The radioactivity was measured with a scintillation counter Mark I (Nuclear Chicago). The counting efficiency of the liquid scintillation counter was 80 per cent.

(3) The remaining subgroups of animals with adjuvant arthritis and controls received 25 μ Ci of ¹⁴C-proline per 100 g. body weight intraperitoneally on the 32nd day after the administration of Freund's adjuvant. At intervals of 25, 28, and 30 days after the injection of the labelled proline, 24-hr urine was collected from these animals and processed as described above.

Results

The metabolism of collagen in rats with adjuvant arthritis was investigated in the manner described for extended periods of up to 62 days, after the

administration of Freund's adjuvant. In our animals, arthritis began at about 18 days after administration.

Only animals with fully developed polyarthritis were included in the experiment. The growth rate of the animals with adjuvant arthritis being slower than that of the control animals of the same age ('age controls'), we used at each sampling interval an additional control group of animals, in which the individual body weight approximated to that of the arthritic rats ('weight controls'). These weight controls, however, were younger than the former. Because weight controls did not show any substantial difference against age controls, we present only the results with age controls (hereafter termed 'controls'). At each experimental interval, four rats with adjuvant arthritis and four controls were killed and processed.

GROUPS 1 AND 2

Collagen synthesis in rats with adjuvant arthritis

Collagen synthesis was checked by determining both the specific and total radioactivities of ^{14}C -hydroxyproline of the neutral salt soluble collagen (NSC), at intervals of 12 and 120 hrs after the administration of ^{14}C -proline, on the 25th or 46th day, respectively, after the injection of Freund's adjuvant. In neither experiment were differences found between the contents of the soluble collagen in the experimental groups and in the age controls (Table I). In both experiments, the specific and total radioactivities of ^{14}C -hydroxyproline in the animals with

adjuvant arthritis were lower than in the control group.

Catabolism of soluble collagen in rats with adjuvant arthritis

A possible explanation of the decreased specific and total radioactivities of NSC in arthritic rats was obtained by determining the urinary excretion of ^{14}C -hydroxyproline during the first 12 hrs after the injection of ^{14}C -proline (Table II). In this period the excretion of hydroxyproline indicates the catabolism of soluble collagen. In both groups, the animals with adjuvant arthritis always excreted more hydroxyproline (160 to 170 per cent. of that excreted by the control group).

The specific radioactivity of urinary ^{14}C -hydroxyproline in the arthritic rats was lower than that in the controls. This may have been due to dilution, because the arthritic rats excreted much more hydroxyproline. In both experiments, however, the total radioactivity of urinary ^{14}C -hydroxyproline in the arthritic rats was raised, especially in the second experiment (46 days after the application of Freund's adjuvant).

Conversion of soluble to insoluble collagen in rats with adjuvant arthritis

This was investigated by determining the radioactivity of ^{14}C -hydroxyproline in insoluble collagen at intervals of 12 and 120 hrs after the injection of ^{14}C -proline. Again, two sets of experiments were performed, the labelled proline being administered on

Table I Yield of neutral salt-soluble collagen and specific and total activity of ^{14}C -hydroxyproline of neutral salt soluble collagen in controls and in animals with adjuvant-induced arthritis

Days after injection of adjuvant	Hours after administration of ^{14}C -proline	Group	Neutral salt soluble collagen (mean and range)		
			Content (mg./100 mg. collagen)	Specific activity (dpm/ μg . hydroxyproline)	Total activity (dpm/100 mg. collagen)
25	12	C	2.95	13.69	5,409
			2.33-3.78	10.44-16.37	4,912-6,019
		A	2.61	11.70	4,529
			2.45-3.27	10.56-13.66	4,312-4,779
	120	C	3.10	1.83	760
			3.04-3.20	1.68-2.12	705-864
		A	2.71	1.03	381
			2.00-3.35	0.83-1.22	222-522
46	12	C	2.71	16.12	4,978
			1.97-3.06	7.66-20.11	3,141-6,638
		A	2.68	10.67	3,855
			2.36-2.90	8.89-12.34	2,821-4,498
	120	C	3.74	2.96	1,496
			3.14-4.12	2.34-3.69	1,205-2,041
		A	3.21	2.17	936
			3.11-3.32	1.83-2.89	764-1,272

C = control; A = adjuvant arthritis.

Table II *Urinary excretion of hydroxyproline and specific and total activity of urinary ^{14}C -hydroxyproline during first 12 hrs after ^{14}C -proline administration in controls and in animals with adjuvant-induced arthritis*

Days after injection of adjuvant	Group	Urinary hydroxyproline (mean and range)		
		Excretion ($\mu\text{g.}/12\text{ hrs}$)	Specific activity ($\text{dpm}/\mu\text{g. hydroxyproline}$)	Total activity (dpm)
25	C	189.1	16.42	3,148
		141.7–237.6	14.00–20.53	2,906–3,323
	A	342.5	10.87	3,935
		269.5–444.7	8.82–12.60	3,921–3,949
46	C	239.5	14.42	3,481
		192.3–289.1	10.74–25.71	2,672–5,779
	A	413.1	13.67	5,484
		321.5–516.0	11.35–17.00	4,475–6,030

C = control; A = adjuvant arthritis.

Table III *Yield of insoluble collagen and specific and total activity of ^{14}C -hydroxyproline of insoluble collagen in controls and animals with adjuvant-induced arthritis*

Days after injection of adjuvant	Hours after administration of ^{14}C -proline	Group	Insoluble collagen (mean and range)		
			Content ($\text{mg.}/100\text{ mg. collagen}$)	Specific activity ($\text{dpm}/\mu\text{g. hydroxyproline}$)	Total activity ($\text{dpm}/100\text{ mg. collagen}$)
25	12	C	97.22	0.11	1,434
			96.18–97.87	0.06–0.14	835–1,883
		A	97.15	0.08	1,111
			95.85–97.51	0.07–0.09	987–1,275
	120	C	96.86	0.35	4,586
			96.83–96.92	0.24–0.41	3,130–5,382
46	12	A	97.26	0.16	2,063
			96.61–97.96	0.11–0.21	1,435–2,713
		C	97.35	0.11	1,475
	120		96.62–97.99	0.08–0.13	1,040–1,783
		A	97.31	0.05	660
			97.15–97.60	0.04–0.06	541–773
	120	C	96.22	0.36	4,700
			95.84–96.82	0.32–0.41	4,137–5,270
		A	96.75	0.16	2,041
			96.64–96.85	0.11–0.18	1,451–2,416

C = control; A = adjuvant arthritis.

the 25th and 46th day of the arthritic process. In neither experiment was any difference found in the total mass of insoluble skin collagen between the arthritic and control groups (Table III). Both the specific and the total radioactivities of ^{14}C -hydroxyproline in the insoluble collagen were always lower in the arthritic animals than in the control group. This difference was particularly conspicuous in the second experiment (labelled proline administered on the 46th day of the arthritic process).

If the total radioactivity of insoluble and soluble collagen is expressed as a percentage of the sum of both, 12 hrs after the administration of the labelled proline, 86 per cent. of the registered radioactivity was present in the soluble collagen, and 14 per cent. in the insoluble collagen in the arthritic group.

The percentages in the control group were 77 and 23 per cent. respectively.

After 120 hrs the differences were still more marked: in the arthritic rats, 32 per cent. of the radioactivity was contained in the soluble, and 68 per cent. in the insoluble collagen, whereas the controls showed 24 per cent. in the soluble and 76 per cent. in the insoluble collagen. These findings indicate that the conversion of soluble to insoluble collagen is retarded in animals with adjuvant arthritis.

GROUP 3

Catabolism of insoluble collagen in rats with adjuvant arthritis

In the third part of the experiment, the animals each received 25 μCi ^{14}C -proline on the 32nd day after the

injection of Freund's adjuvant. 24-hr urine was collected on the 25th, 28th, and 30th days after the administration of the radioactive proline. There was no substantial difference in the amounts of hydroxyproline excreted by the individual experimental groups (Table IV). Both groups also exhibited similar values of specific radioactivity, the total radioactivity of urinary ^{14}C -hydroxyproline being slightly lower on average in the arthritic rats.

The conclusion may be drawn that no catabolism of the insoluble collagen took place in the rats with adjuvant arthritis.

Discussion

The isotope technique makes it possible to assess the radioactivity of ^{14}C -hydroxyproline produced by administration of ^{14}C -proline in individual fractions of skin collagen, as well as its urinary excretion. The total metabolic turnover of collagen, its synthesis, the conversion rate of soluble to insoluble collagen, and the catabolism of both the soluble and the insoluble fractions, may be measured. Collagen synthesis can be estimated by determining the radioactivity of ^{14}C -hydroxyproline in soluble collagen within the first hours and days after the injection of ^{14}C -proline. The measurement of radioactivity of ^{14}C -hydroxyproline in soluble and in insoluble collagen yields information about the conversion of the younger soluble forms of collagen to the mature insoluble forms. The radioactivity of urinary ^{14}C -hydroxyproline, determined 12 hrs after the administration of ^{14}C -proline, is an indicator of the catabolism of the soluble collagen synthesized *de novo*. The catabolism of mature insoluble collagen is reflected in the radioactivity of urinary ^{14}C -hydroxyproline excreted 4 wks and more after the administration of ^{14}C -proline (Prockop and Kivirikko, 1968).

The development of adjuvant arthritis is accompanied by marked changes in the metabolic turnover of collagen. A local articular inflammatory process is likely to form part of the changes in collagen metabolism. To a great extent these changes reflect a systemic reaction of the whole organism. The development of the adjuvant arthritis is a dynamic process, advancing from an acute to a chronic phase. In the acute phase, on the 25th day after the injection of Freund's adjuvant, the adjuvant-induced disease interacts only with the catabolism of soluble collagen in the organism. Collagen synthesized *de novo* is subject to augmented degradation. On the 46th day after the administration of Freund's adjuvant, when the adjuvant-induced disease has already reached the chronic phase, still more marked changes occur. The degradation of the new collagen is further intensified, and the conversion rate of soluble to insoluble collagen diminishes. In our experiments adjuvant arthritis was not accompanied by increased degradation of insoluble collagen. The chief consequence of adjuvant arthritis is increased degradation of the younger forms of collagen and retarded conversion of the younger forms into the mature insoluble forms.

The articular changes in adjuvant-induced arthritis are accompanied by an increase in the activity of the lysosomal enzymes, beta glucuronidase, alkaline phosphatase, and collagenolytic enzyme (Anderson, 1970). The activity of the last-named enzyme begins to increase between the 25th and the 40th day after the administration of Freund's adjuvant, as does the serum proteolytic activity, casein serving as substrate (Pelczarska, Giędanowski, and Halawa, 1969). It is probable that a release of collagenolytic enzymes may play a role in the catabolism of the collagen synthesized *de novo*, which is less stable and therefore more susceptible to enzymic degradation.

Table IV Urinary excretion of hydroxyproline and specific and total activity of urinary ^{14}C -hydroxyproline in controls and animals with adjuvant-induced arthritis

Days after injection of adjuvant	Days after ^{14}C -proline	Group	Urinary hydroxyproline (mean and range)		
			Excretion ($\mu\text{g.}/24\text{ hrs}$)	Specific activity ($\text{dpm}/\mu\text{g. hydroxyproline}$)	Total activity (dpm)
57	25 (24 hrs)	C	339.5	0.44	151.5
			265.3–397.3	0.27–0.68	98.5–218.3
		A	337.4	0.57	193.6
			261.1–499.5	0.45–0.67	120.0–296.3
60	28 (24 hrs)	C	326.9	0.81	258.0
			287.8–357.5	0.43–1.18	135.3–340.6
		A	371.6	0.61	199.9
			200.0–557.8	0.14–0.98	76.5–390.1
62	30 (24 hrs)	C	420.2	0.63	263.1
			381.3–502.9	0.52–0.69	251.5–275.5
		A	306.8	0.46	139.2
			296.1–326.5	0.36–0.51	117.4–154.2

C = control; A = adjuvant arthritis.

The inhibition of the conversion of soluble to insoluble collagen may be another unfavourable factor, because of the consequent retardation of the formation of more stable forms of collagen which are more resistant to pathogenic influences. The changes in the metabolism of collagen are a manifestation of the systemic damage produced by adjuvant arthritis.

Summary

In rats with adjuvant-induced arthritis, the meta-

bolism of collagen was investigated after an injection of ^{14}C -proline, both the specific and total radioactivity of ^{14}C -hydroxyproline being determined in skin collagen fractions and in urine. It was found that the catabolism of the collagen synthesized *de novo* is intensified in the acute phase of the arthritis, whereas in the chronic phase the catabolic change is accompanied by a retardation in the conversion of soluble to insoluble collagen. Changes in collagen metabolism reflect a mainly systemic reaction to the administration of Freund's adjuvant.

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