Radioimmunoassay of soluble and insoluble collagenases in fibrotic liver

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We have postulated that an insufficient activity of collagenase relative to increased collagen synthesis may be the cause of the increased collagen accumulation in fibrotic tissues. In the present study, ^{125}I -collagenase and rabbit anti-collagenase immunoglobulin G were used to develop a sensitive radioimmunoassay that detects 0.1 nM (3 ng) of collagenase protein in tissue samples. The assay also can detect collagenase protein that is associated with extracellular-matrix collagen fibrils. Good correlation with an assay of enzyme activity validates the radioimmunoassay for quantification of collagenase. The assay was used to measure amounts of collagenase in relation to fibrotic processes in livers of mice with schistosomiasis. Results indicate that the amounts of collagenase relative to synthesized collagens were significantly lower, and this may contribute to the progressive fibrosis. The occurrence of a maximum amount of collagenase at 7 weeks after infection with *Schistosoma mansoni* cercariae in concanavalin A-treated animals, as compared with 8 weeks in controls, could account for the large remission of fibrosis in mice so treated. The results emphasize the possible importance of collagenase in controlling or limiting fibrosis.

Collagen content of a tissue is determined by a balance between processes of synthesis and degradation (Harkness *et al.*, 1954; Bornstein & Traub, 1979). Excessive accumulation of collagen in fibrotic livers may represent a pathophysiological process in which degradation owing to collagenase activity does not keep pace with increased collagen synthesis, so that collagen accumulates in an increased amount. Efficient collagenolysis (Gross & Lapiere, 1962) is required in tissues during the periods of rapid growth and remodelling accompanied by biosynthesis of collagen, and in turnover of mature cross-linked fibrils. The presence of collagenase (EC 3.4.24.7) in the livers of rats made fibrotic by administration of CCl_4 has been demonstrated by immunohistochemical methods by using anti-(active-form collagenase) antibody (Montfort & Perez-Tamayo, 1978). In that study, antibody reacted only with livers in the reversible stages of fibrosis, suggesting that collagenase may be involved in reversing fibrosis and may be absent when the process has become

irreversible. We previously have shown that collagenase is present in fibrotic livers of mice with schistosomiasis (Takahashi *et al.*, 1980), and that most of the immunoreactive collagenase was associated with regions of granulomatous tissues undergoing various degrees of remodelling and degradation (Biempica *et al.*, 1983).

Collagen accumulation of livers of mice with schistosomiasis results from a T-cell-mediated inflammatory host response against a soluble egg antigen, with subsequent granuloma formation around eggs in portal tracts (Warren *et al.*, 1967); the egg granulomas release fibroblast-stimulating factor (Wyler *et al.*, 1978). Although the human host lymphocyte response against schistosomal stimulants later becomes impaired, the response against non-schistosomal stimulants remains intact (Colley *et al.*, 1977). Con A has been shown to stimulate mononuclear cells, which in turn modulate target cells to increase collagenase production *in vitro* (Dayer *et al.*, 1977; Deshmukh-Phadke *et al.*, 1978). Administration of Con A to mice infected with schistosomes decreased liver collagen content by 50% compared with untreated infected animals (Takahashi & Kobayashi, 1982). That decrease was not caused by decreased collagen synthesis or enhanced collagenase production alone, but by a change in phase of syn-

Abbreviations used: Con A, concanavalin A; PBS, phosphate-buffered saline (1.5 mM- KH_2PO_4 /8.2 mM- Na_2HPO_4 /2.7 mM-KCl/137 mM-NaCl); RIA, radioimmunoassay; SDS, sodium dodecyl sulphate; IgG, immunoglobulin G.

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thesis and degradation, and by greater solubility of the liver collagen in granulomas. However, neither the insufficient production of collagenase activity nor the amounts of the enzyme protein in these tissues have been established with certainty. The present study was designed to correlate the amounts of collagenase detected by RIA and the activity measured by enzyme assay, and then to evaluate whether, in schistosomiasis, the amounts of collagenase relative to increased collagenase synthesis are low enough to account for the observed accumulation of collagen.

Experimental

Materials

A kit of radioiodination reagent was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Na^{125}I was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). Goat anti-(rabbit-IgG) antibodies were purchased from Miles Biochemicals (Elkhart, IN, U.S.A.). CNBr-activated Sepharose 4B and Con A-conjugated Sepharose 4B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Female CF-1 mice (9 weeks old; 20–25g) that had been given a subcutaneous injection of 50 cercariae of a Puerto Rican strain of *Schistosoma mansoni* were obtained from Dr. A. Mahmoud, Case Western Reserve University School of Medicine, Cleveland, OH, U.S.A. Active-form collagenase was isolated in our laboratory from the media of cultures of granuloma explants obtained from the livers of mice with schistosomiasis as follows: media were precipitated with $(\text{NH}_4)_2\text{SO}_4$, activated with trypsin, and subsequently chromatographed on Sephadex G-200 and G-100 columns, an ion-exchange column and on a Sephadex G-100 column successively as described (Takahashi & Simpson, 1981). Finally, the preparation was further purified on a Con A-Sepharose 4B affinity column. The purified collagenase was free of other proteinases, including the EDTA-sensitive neutral proteinase that contaminates many other preparations. The collagenase had a specific activity of 33 units/mg of protein, determined by the collagen-fibril assay at 35°C. It had $\text{pI} 6.2$ and apparent M_r 60 000. On electrophoresis in polyacrylamide gels, with and without SDS, and an isoelectric-focusing gel, the enzyme migrated as a homogeneous protein band (Takahashi & Simpson, 1981). Substrate and rabbit anti-collagenase IgG that inactivates enzyme activity (by 80%) were prepared in this laboratory (Biempica *et al.*, 1983).

Administration of reagents

All reagents were sterilized by use of a 0.2 μm Millipore filter. Mice at 5–6 weeks after infection

with *S. mansoni* cercariae were injected intraperitoneally daily with Con A (200 μg) in 0.1 ml of PBS; control mice were given 0.1 ml of PBS. Animals were killed at 7, 8 or 9 weeks after infection. Six mice were used to study each time-point or dosage.

Preparation of inactive-form collagenases

The inactive-form collagenases were prepared from the same culture medium as above. Proteinase inhibitors (1 mM-di-isopropyl phosphorofluoridate/5 mM-EDTA) were added to the medium, which was then saturated with $(\text{NH}_4)_2\text{SO}_4$. The precipitate formed was collected by centrifugation (27 000g for 10 min), dissolved in 50 mM-Tris/HCl/0.2 M-NaCl (Tris/NaCl) buffer (pH 7.5) and chromatographed on a Sephadex G-200 column (2.5 cm $\times$ 93 cm). The collagenases were eluted at 1.8 times void volume. The fractions containing inactive-form collagenases, detected by immunoprecipitation with anti-collagenase IgG, were dialysed against 10 mM-Tris/HCl buffer (pH 8.1), and were chromatographed on a DEAE-Sephacel column (1.4 cm $\times$ 5 cm), pre-equilibrated and eluted with the same buffer. The bound inactive-form collagenases were eluted with the same buffer containing 40 mM-NaCl, and were dialysed against Tris/NaCl buffer. The collagenases were further purified on a column (0.6 cm $\times$ 5 cm) of Sepharose 4B conjugated with anti-collagenase IgG, pre-equilibrated and eluted with Tris/NaCl buffer. The bound collagenases were eluted with the same buffer containing 3 M-KSCN, and subsequently KSCN was removed by extensive dialysis against Tris/NaCl buffer. Some properties of the isolated inactive-form collagenases were as follows. On electrophoresis in SDS/polyacrylamide gel, the enzymes migrated as doublets, with apparent M_r 69 000 and 71 000; collagenases did not cross-react with anti-(EDTA-sensitive neutral proteinase) IgG; collagenases become bound to a column of Sepharose 4B conjugated with Con A; collagenases were not activated by tryptic digestion in the absence of added crude enzyme preparations, but were autoactivated simultaneously, the apparent M_r decreasing to 60 000 and 55 000. It should be noted that antibody raised against the active-form collagenase with M_r 60 000 (the antibody used in these studies) reacts with four collagenases (see Fig. 6) of different M_r , suggesting that immunologically these have structural similarities.

Preparation of liver fractions

Livers of mice were perfused and homogenized. The supernatants of liver homogenates (8 ml/g of liver) were obtained after centrifugation and the sediments containing collagenase were solubilized by urea as described by Takahashi & Kobayashi (1982). In brief, the centrifugations were done at

27000g for 1 h, at 4°C, and extractions were done each for 24 h, at 4°C with constant rocking. The precipitates were washed twice with 1 ml of cold PBS; wash solutions were combined with the supernatants and the collagen-associated insoluble collagenase was extracted twice with 4 ml of 4M-urea; both extracts were combined before assay. The residues were extracted with 2 × 2 ml of 8M-urea; each extract was diluted to 4M-urea before assay.

Preparation of collagenase fractions

The inactive-form collagenase in the liver fractions above, and that material after activation by trypsin (Takahashi *et al.*, 1980), were sequentially chromatographed on a Sephacryl S-200 column (2.5 cm × 93 cm), pre-equilibrated and eluted with 50 mM-Tris/HCl/0.2 M-NaCl (Tris/NaCl) buffer (pH 7.5). Fractions were followed by RIA. Both inactive- and active-form collagenases were respectively eluted at 1.8 times the void volume. The two were concentrated to their original volume by use of a Diaflo apparatus. As controls, the eluates at 1 and 3 times the void volume were also concentrated. The collagenase in the 4M-urea extract was also eluted at the same void volume from the same kind of columns as above; however, the column was pre-equilibrated with the buffer containing 4M-urea. The control eluates were treated similarly. The collagenase fractions were then chromatographed on a Sepharose 4B column conjugated with rabbit anti-collagenase IgG, pre-equilibrated and eluted with Tris/NaCl buffer. Bound collagenase was eluted with 3M-KSCN in the same buffer. The eluates were dialysed against Tris/NaCl buffer and passed over a Protein A-conjugated Sepharose 4B column, pre-equilibrated with the same buffer.

Enzyme assays

Collagenase activity was determined with a substrate of guinea-pig skin type I collagen labelled with either [¹⁴C]glycine or [¹⁴C]acetic anhydride as described previously (Takahashi & Kobayashi, 1982; Takahashi & Simpser, 1981). Enzymic activity was estimated in terms of radioactivity appearing in the supernatant, and expressed as units: 1 unit of collagenase is the amount that degrades 1 µg of native collagen/min at 35°C.

Iodination of collagenase antigen

The purified collagenase antigen (54 µg/100 µl) was labelled with Na¹²⁵I (1.0 mCi) by using lactoperoxidase (van Vunakis, 1980). The yield of ¹²⁵I-labelled collagenase antigen was 21 × 10⁶ c.p.m./51.3 µg of protein (95%). ¹²⁵I-labelled collagenase exhibited full enzymic activity.

Radioimmunoassay

The following RIA was performed in duplicate or triplicate samples. The composition of standard buffer used was PBS containing 0.25% bovine serum albumin, 10 mM-EDTA and 0.03% Triton X-100 (pH 7.2). The binding of ¹²⁵I-labelled collagenase to anti-collagenase IgG was measured as follows: various amounts of the first antibody (0.1–500 µg/ml) were treated with ¹²⁵I-collagenase (12300 c.p.m./30 ng in 0.1 ml) in the presence of a reaction mixture containing 5% normal rabbit serum (0.1 ml) and standard buffer (0.2 ml) for 48 h at 4°C. That was followed by reaction with the second antibody (0.1 ml) for 24 h at 4°C. The immune complex formed was precipitated by centrifugation at 27000g for 5 min, and the ¹²⁵I radioactivity in the precipitate was counted in a LKB 1280 Ultragamma counter. The amount of ¹²⁵I-collagenase bound to antibody was 94 ± 2% (Fig. 1). The recovery of ¹²⁵I-labelled antigen in the precipitates with 10% trichloroacetic acid/0.25% albumin was 99 ± 1%, and the non-specific binding of ¹²⁵I-labelled antigen to normal rabbit serum was 6.4 ± 2%. Each RIA includes: 'total counts' tubes containing ¹²⁵I-collagenase alone, tubes in which normal rabbit serum replaced first antibody, and tubes in which RIA buffer replaced the test sample. The concentration of anti-collagenase IgG was 2.4 µg; that yields a bound-to-free molar ratio (B/F) of approx. 1.0.

Other methods

Protein contents were determined with bovine serum albumin (Bradford, 1976). Determination of *M_r* by gel filtration and by SDS/polyacrylamide-gel electrophoresis, estimation of the rate of collagen

Fig. 1. Binding of ¹²⁵I-collagenase to rabbit anti-collagenase IgG

Values for percentage ¹²⁵I binding (ordinate), after a subtraction for binding occurring in the presence of normal rabbit serum alone (6.4 ± 2%), are plotted against the amount of anti-collagenase IgG added. Details are given in the text.

synthesis and liver collagen content were as previously described (Takahashi *et al.*, 1980; Takahashi & Simpser, 1981). Sepharose 4B conjugated with rabbit anti-collagenase IgG antibody was prepared (Cuatrecasas *et al.*, 1968); the bound IgG was 9.5 mg/ml of Sepharose 4B.

Results

Specific binding of 125 I-collagenase to anti-collagenase IgG

The specific binding of 125 I-collagenase (30 ng) to anti-collagenase IgG (2.4 μ g) at 4°C reached a stable maximum at about 40 h (Fig. 2), and was inhibited in the presence of a 50-fold excess of unlabelled antigen. The mean amount of 125 I-labelled collagenase bound to rabbit anti-collagenase IgG antibody was approx. 381300 c.p.m./ μ g. The EDTA-sensitive neutral proteinase or 0.1 M-urea had no effect on binding of 125 I-collagenase to antibody. Binding of 125 I-collagenase to anti-collagenase IgG at 4°C was saturated at 0.1 μ M (Fig. 3). A Scatchard (1949) plot of these data (Fig. 3, insert) was linear over the free 125 I-collagenase concentration range of 0.1–1.3 nM (5–60 ng) in the presence of the amount of antibody that yielded a bound/free (B/F) ratio of approx. 1.0. From this plot the equilibrium constant for 125 I-collagenase binding-site dissociation ($K_{125\text{I-collagenase}} = 1/\text{slope}$) was calculated to be 0.9 nM.

Fig. 2. Specific binding of 125 I-collagenase to anti-collagenase IgG at 4°C

Symbols represent: ○, total binding of 125 I-collagenase (30 ng) to anti-collagenase IgG (2.4 μ g); ●, binding in the presence of a 50-fold (1.5 μ g) excess of unlabelled collagenase; △, binding in the presence of 1.5 μ g of EDTA-sensitive neutral proteinases; ▼, binding in the presence of 0.1 M-urea. Details are given in the text.

Competition for 125 I-collagenase binding to anti-collagenase IgG by unlabelled agents

The competition curve for RIA was established by the following procedures: 125 I-labelled antigen (41000 c.p.m./100 ng) and various amounts of unlabelled antigen (0.1–500 ng) were competitively bound in the presence of anti-collagenase IgG (2.4 μ g) that yields a B/F ratio of approx. 1.0; this was followed by reaction with the second antibody. Two controls were included: one without competing antigen and another without competing antigen and the first antibody. Results are shown in Fig. 4. It appears that 125 I-labelled collagenase and unlabelled active and inactive-form collagenases have similar, but not identical, binding to the antibody.

Validation that the collagenase protein measured in the liver homogenates by RIA was identical with the collagenase used as antigen was accomplished as follows. Firstly, the amount of collagenase measured in the supernatant of the liver homogenate, before and after activation, correlated well with the amount measured in the fractions at 1.8 times the void volume, where the collagenase antigen was eluted from a gel-filtration column. Secondly, the amount of collagenase measured in the 4M-urea extract of the liver sediment correlated well with the amount measured in the fractions at 1.8 times the void volume eluted from a gel-filtration column (Figs. 4 and 5). Thirdly, no collagenase protein was detected in the other fractions (Fig. 4). Fourthly,

Fig. 3. 125 I-collagenase bound to anti-collagenase IgG at 4°C as a function of the concentration of free 125 I-collagenase at equilibrium

Symbols represent: ○, total binding; ●, binding in the presence of a 50-fold excess of unlabelled collagenase. Insert: Scatchard plot of data: abscissa, concentration of bound 125 I-collagenase; ordinate, ratio of bound to free (B/F) 125 I-collagenase.

Fig. 4. Competition for binding of ^{125}I -collagenase to anti-collagenase IgG by unlabelled antigen collagenase and collagenase in various fractions of the liver

Ordinate: percentage of control binding = $100 \times \text{c.p.m. bound in presence of competing agent} / \text{c.p.m. bound in the absence of competing agent}$. Abscissa: upper scale (μl) applies to all competing agents, and lower scale (ng) applies only to the antigen collagenase and to inactive-form collagenase. Competing agents are: $\blacktriangledown$, purified EDTA-sensitive neutral proteinase ($100 \mu\text{g/ml}$); fractions obtained from the chromatography of the liver supernatants on a Sephacryl S-200 column (∇) at 1 void volume, ($\square$) at 3 times the void volume, (Δ) at 1.8 times the void volume (contained inactive-form collagenase), and ($\circ$) at 1.8 times the void volume (contained active-form collagenase); fractions obtained from the chromatography of 4M-urea extract of the liver sediments on a Sephacryl S-200 column: ($\blacktriangle$) at 1 void volume, ($\blacksquare$) at 3 times the void volume, and ($\bullet$) at 1.8 times the void volume. The liver homogenates were made from 1 g of liver/8 ml, and the amount of collagenase was expressed in terms of the collagenase protein per g of liver. Details are given in the text. $\diamond$, Unlabelled antigen active-form collagenase; $\circ$, partially purified inactive-form collagenase.

Fig. 5. Competition for binding of ^{125}I -collagenase to anti-collagenase IgG antibody by unlabelled antigen collagenase and collagenase in the livers of mice with schistosomiasis

Ordinate: percentage of control binding = $100 \times \text{c.p.m. bound in presence of competing agent} / \text{c.p.m. bound in the absence of competing agent}$. Abscissa: upper scale (μl) applies to all competing agents, and lower scale (ng) applies only to the antigen collagenase. Competing agents are as follows: open symbols indicate the collagenase in the supernatants of the liver homogenates; solid symbols are compatible with open symbols, except that the continuous lines are for 4M-urea extract and broken lines are for 8M-urea extract of the sediments of the liver homogenates; $\circ$, livers of mice with schistosomiasis that had been treated with Con A at 5–6 weeks after infection, and were killed at 8 weeks after infection; Δ , infected control liver at 8 weeks after infection; $\square$, age- and body-weight-matched normal control liver; ($\bullet$), unlabelled antigen collagenase. Other conditions were the same as in Fig. 4, and details are given in the text.

the SDS/polyacrylamide-gel-electrophoretic patterns of activated collagenase eluted from a rabbit anti-collagenase-IgG-conjugated Sepharose 4B column exhibited an apparent M_r of 60 000, similar to that of the collagenase antigen (Fig. 6).

Amounts of collagenase protein and activity

A regression plot of the data in Table 1 was linear when the amounts of collagenase, 10–72 $\mu\text{g/g}$ of liver as measured by RIA, were plotted against collagenase activity, 0.3–1.7 units/g of liver measured by enzyme assay, and yielded a correlation coefficient r of 0.995 (results not shown). Results indicate that the RIA is a good method for the quantification of the low amounts of colla-

genase in the tissue. It was confirmed by RIA that maximum collagenase protein occurred at 7 weeks after infection in Con A-treated animals ($72 \pm 3.8 \mu\text{g/g}$ of liver) as compared with 8 weeks after infection in infected controls ($68 \pm 4.1 \mu\text{g/g}$ of liver) (Table 1). The amount of collagenase protein in the liver at 8 weeks after infection was similar in both sets of animals (Table 1).

Collagenase and collagen contents

In the following study the collagenase and collagen contents of normal controls were used as baseline values. The amounts of collagenase at 7 weeks after infection in Con A-treated and control animals respectively were about 2-fold and 1.2-fold greater than baseline values. The rate of collagen synthesis in both sets of animals was about 6-fold greater than in normal controls; about three-quarters and half of newly synthesized collagen in

Fig. 6. SDS/polyacrylamide-gel electrophoresis of collagenases

The collagenases eluted from a column of Sepharose 4B conjugated with anti-collagenase IgG were electrophoresed in 0.1% SDS/10% and 12%-polyacrylamide gels. Sample sizes were as follows: 4 μ g of protein for slots 1 and 2; 2 μ g for slots 3, 4, 6 and 8; 9 μ g for slot 5; 1 μ g for slot 7; and 3 μ g for slot 9. The protein bands were detected by staining with silver. Gels are: (a) (10% gels): 1, inactive-form collagenases isolated from media of cultures of granuloma explants obtained from the livers of mice with schistosomiasis; 2, inactive-form collagenases obtained from the supernatant of the liver homogenate before tryptic activation; 3, collagenases obtained from the supernatant of the liver homogenate after tryptic activation with a ratio of trypsin to the supernatant protein of 1:4, at 37°C for 10 min; 4, collagenases obtained from the 4M-urea extraction of the sediment of the liver homogenate; 5 and 6, autoactivated collagenases of slot 2; 7, antigen active-form collagenase isolated from the same media as slot 1 above; (b) (12% gels): 8, antigen active-form collagenase; 9, active-form collagenases of slot 3. Apparent M_r was determined by co-electrophoresis of known protein standards (phosphorylase b, 94000; bovine serum albumin, 68000; ovalbumin, 43000; chymotrypsinogen A, 25000). Other conditions are as described in the text. Abbreviation: BF, buffer front.

Con A-treated animals and in controls respectively was degraded, resulting in increments of collagen accumulation respectively of about 1.4-fold and over 3-fold of the baseline values (Table 2). The amounts of collagenase and the rate of collagen synthesis at 8 weeks after infection in both sets of animals were respectively about 2-fold and 8-fold over baseline values. Three-quarters and over two-thirds of newly synthesized collagen in Con A-treated animals and in infected controls respectively were degraded, resulting in net increments of collagen accumulation respectively of about 2-fold and 2.5-fold of baseline values. Results indicate that amounts of collagenase in these fibrotic tissues may have been about 50% less than the amount needed to keep pace with the rate of increased collagen synthesis.

Discussion

We have postulated that an insufficient amount of collagenase relative to increased collagen synthesis may be the cause of the increased collagen accumulation in fibrotic liver. That is supported by the present study. Not only has good correlation been established between the amounts of enzyme protein and activity, but also the specific activity of the liver collagenase isolated from media of granuloma explant cultures has been shown to be consistent with its activity determined by enzyme assay and the amount of collagenase protein measured by RIA in the liver extracts. These results showed that the amount of collagenase, relative to the rate of collagen synthesis and to the collagen content of the tissue, was significantly lower in fibrotic livers than in normal control livers. The occurrence of a maximum amount of collagenase 7 weeks after infection in Con A-treated animals, compared with 8 weeks in controls, resulting in corresponding net collagen

Table 1. Collagenase protein and activity

Soluble, insoluble and total collagenase protein and activity were measured in the livers of mice infected with 50 *S. mansoni* cercariae, with or without treatment with Con A, and in livers of age-matched normal controls; amounts of enzyme protein and activity were determined at the indicated times after infection. Collagenase protein was measured by RIA using 125 I-labelled antigen collagenase and rabbit anti-collagenase IgG; insoluble collagenase was measured after extraction of enzyme with 4M- and 8M-urea. Collagenase activity was measured by collagen-fibril assay. Assay procedures are given in the text.

Time after infection (weeks)	Samples	Collagenase protein (μ g/g of liver)			Collagenase activity (units/g of liver)		
		Soluble	Insoluble	Total	Soluble	Insoluble	Total
7	Con A-treated	42 $\pm$ 2.4	30 $\pm$ 1.4	72 $\pm$ 3.8	1.00 $\pm$ 0.09	0.70 $\pm$ 0.06	1.70 $\pm$ 0.15
	Infected controls	23 $\pm$ 1.2	15 $\pm$ 0.8	38 $\pm$ 2.0	0.53 $\pm$ 0.05	0.33 $\pm$ 0.05	0.85 $\pm$ 0.10
8	Con A-treated	38 $\pm$ 1.8	30 $\pm$ 1.4	68 $\pm$ 3.2	0.93 $\pm$ 0.07	0.72 $\pm$ 0.07	1.65 $\pm$ 0.14
	Infected controls	39 $\pm$ 2.5	29 $\pm$ 1.6	68 $\pm$ 4.1	0.96 $\pm$ 0.08	0.70 $\pm$ 0.06	1.66 $\pm$ 0.14
9	Con A-treated	33 $\pm$ 2.1	25 $\pm$ 1.3	58 $\pm$ 3.4	0.86 $\pm$ 0.08	0.60 $\pm$ 0.07	1.45 $\pm$ 0.15
	Infected controls	33 $\pm$ 2.0	23 $\pm$ 1.2	56 $\pm$ 3.2	0.83 $\pm$ 0.09	0.60 $\pm$ 0.07	1.43 $\pm$ 0.16
	Normal controls	26 $\pm$ 1.4	10 $\pm$ 0.7	35 $\pm$ 2.1	0.55 $\pm$ 0.06	0.30 $\pm$ 0.04	0.85 $\pm$ 0.10

Table 2. *Amounts of collagenase, rates of collagen synthesis and collagen contents*

The amounts of collagenase, the rates of collagen synthesis and collagen contents in the livers of mice infected with 50 *S. mansoni* cercariae, with or without treatment with Con A, and in livers of age-matched normal controls were measured. The amounts of collagenase are taken from Table 1. Rates of collagen synthesis are expressed as nmol of protein-bound hydroxyproline synthesized/h per g of liver. Collagen contents are expressed as μmol of protein-bound hydroxyproline/g of liver. Assay conditions are given in the text.

Time after infection (weeks)	Samples	Content of collagenase ($\mu\text{g/g}$ of liver)	Rate of collagen synthesis (nmol of hydroxyproline/g per g of liver)	Collagen content (μmol of hydroxyproline/g of liver)
7	Con A-treated	72 ± 3.8	6.1 ± 0.59	2.1 ± 0.02
	Infected controls	38 ± 2.0	6.2 ± 0.61	4.0 ± 0.03
8	Con A-treated	68 ± 3.2	7.8 ± 0.72	3.5 ± 0.03
	Infected controls	68 ± 4.1	8.0 ± 0.78	7.0 ± 0.05
9	Con A-treated	58 ± 3.4	6.5 ± 0.62	5.4 ± 0.04
	Infected controls	56 ± 3.2	6.7 ± 0.70	9.5 ± 1.01
	Normal controls	35 ± 2.1	1.0 ± 0.12	1.0 ± 0.11

accumulation respectively of 1.4- and 3-fold over the baseline values, could account for the large remission of fibrosis. We have shown previously, and also in the present study, that the largest increment in collagen accumulation consistently occurs before 8 weeks, when the amounts of collagenase are low (Takahashi *et al.*, 1980; Takahashi & Kobayashi, 1982). Thus the effect of Con A is to change the time of appearance of maximum activity of collagenase so that it coincides with the period in which collagen accumulation is maximal. This suggests that the presence or absence of sufficient collagenase with increased collagen synthesis, at the time before the formation of collagen cross-links, may have been critical in production of fibrosis.

Previously we showed that the active form appeared to be associated with collagen sedimented from fibrotic liver (Takahashi *et al.*, 1980). That would suggest that the collagenase measured in the sediment by RIA is the active form, whereas the collagenase measured in the supernatant is primarily the inactive form. Elution patterns from a gel-filtration column and mobilities of enzyme protein in SDS/polyacrylamide-gel electrophoresis are very similar for urea-solubilized and for activated collagenase (Fig. 6). That both enzymes are identical, however, requires further investigation. The apparent M_r values of the inactive and active forms were respectively 69 000 and 71 000, and 55 000 and 60 000 (Fig. 6). Those values are in the range of literature values (40 000–84 000). Even though some of the several collagenases studied have immunological cross-reactivity, one has no reason *a priori* to believe that all should have identical M_r . In fact, in previous reports (Nagase *et al.*, 1981, 1983), rabbit synovial fibroblasts were shown to produce collagenases of M_r 57 000, 61 000 and 65 000; these cultures had been treated with phorbol myristate acetate, an agent that stimulates

maximal collagenase production. Noteworthy in that report is the fact that an higher extent of glycosylation of the collagenase molecule was associated with a higher M_r value and with decreased specific activity. Specific activity of the enzyme, on the other hand, can be influenced by the amount of procollagenase activator present during the process of activation; that activator can in fact activate procollagenase without detectable loss of M_r (Vater *et al.*, 1983). In our case, then, whether the observed higher M_r and lower specific activity of granuloma collagenase could be due to a higher degree of glycosylation and a lower amount of activator remains to be studied. In general, both the M_r and the specific activity were not very different from corresponding values reported for other collagenases (McCroskery *et al.*, 1975; Woolley *et al.*, 1978; Nagase *et al.*, 1981). More important is the fact that the preparation that we used was homogeneous by several criteria and free of contaminating proteinases as far as could be determined. One cannot say, however, that the preparation did not contain some inactive form of the enzyme (or, more precisely, non-activated form) that would have near-identical immunoreactivity. That would at worst mean that our RIA method determined some form of the collagenase that was not fully activated. However, that applies to many situations in the literature where one deals with precursor and ultimately modified forms of a protein.

Although general agreement exists that collagenases are released as inactive forms (Vaes, 1971; Harper, 1980), there is no consensus about the mechanism of activation. Proposed mechanisms include proteolysis (Vaes, 1972), autoactivation (Harper & Gross, 1973), autoactivation without loss of M_r (Stricklin *et al.*, 1977), and removal of an inhibitor from a complex with the enzyme (Sellers & Reynolds, 1977; Shinkai & Nagai, 1977;

Murphy *et al.*, 1977; Vater *et al.*, 1978). Ultimately control over expression of collagenase activity would be at the level of biosynthesis of precursor forms of the enzyme, activators and inhibitors, a matter as yet largely unknown. Our own results suggest that increased collagenase production did occur in Con A-treated animals infected with schistosomes as compared with untreated infected controls. However, that does not permit us at this time to conclude that an actual change occurs in biosynthesis of collagenase in this fibrotic condition.

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