PRODUCTION OF HYPERIMMUNE SERUM AGAINST COLLAGEN AND ITS USE FOR THE ISOLATION OF SPECIFIC COLLAGEN PEPTIDES ON IMMUNOSORBENT COLUMNS

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1. Introduction

The principle antigenic determinants of soluble collagen are located in the amino- and carboxy terminal regions of the α-chains [1,2]. The sequences are also involved in the formation of intra- and intermolecular crosslinks [2,3]. Cleavage of the polypeptide chain of soluble collagen at methionine residues by the action of CNBr results in the release of these terminal sequences intact [4]. Moreover, evidence has been presented for the release from intact collagen fibres of composite peptides in which these regions are crosslinked to larger peptides from the helical region of the α-chains [5,6].

However, since these peptides contain one or more sequences corresponding to antigenic determinants a more powerful method for isolating crosslinked peptides should be affinity chromatography on immunosorbent columns prepared from anti-collagen antibodies of appropriate specificity. To date this has not been attempted probably due to the reported poor immunogenicity of collagen. Such antisera which have been prepared usually have low titre and cannot be demonstrated by precipitation techniques [1].

This paper describes the production of sheep antisera with a high titre of precipitating antibodies to soluble rat collagen and the use of an anti-collagen immunosorbent in isolating antigenic peptides containing the reducible intermolecular crosslinks.

2. Materials and methods

2.1. *Antigens*

Tail tendons and skin sections were excised from 6-month white rats and thoroughly washed in physiological saline, buffered at pH 7.0 with 0.02 M Tris–HCl. Acid soluble collagen was obtained from the tendons and skin by extraction with 0.5 M acetic acid and purified by repeated precipitation with 5% NaCl. Skin was also extracted with a neutral solution of 8 M urea in order to retain the carboxy-terminal telopeptide reported to be present in calf skin collagen [7] and in rat collagen [8].

2.2. *Antisera*

All three antigens were prepared in concentrated solutions (10 mg/ml) and dialysed against Tris-buffered saline, pH 7.4. The acid-extracted collagens from tail tendon and skin formed precipitates of fibre-like appearance and the precipitate was retained for use in the antigen preparation. The urea-extracted skin collagen remained in solution. Emulsions were prepared of antigen solution and Freund's complete adjuvant (FCA) (v/v) and sheep were immunized at

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15 day intervals with a total of 20 mg of protein (4 ml of emulsion with FCA). The antigen was administered with multi-site injections consisting of 4 subcutaneous and 2 intramuscular for each dose.

2.3. *CNBr digestion*

The cleavage with CNBr was performed under acid conditions as described by Butler [4]. The substrate employed was freshly excised intact rat tail tendons reduced with potassium borotritide [9]. These collagen fibres thereby contain crosslink peptides in which the crosslinks have been stabilised and tritium labelled during borotritide reduction to facilitate their separation and identification. The excess of reagents were removed by chromatography on Sephadex G10 and the peptides lyophilized.

2.4. *Quantitative precipitin assay*

Sera were assayed for collagen-precipitating antibody by the method described by Maurer [10].

2.5. *Immunosorbents*

Immunosorbents were prepared by coupling antigen or antibody to Sepharose 4B (agarose beads) following an activation treatment with CNBr [11]. Antigen was prepared for the coupling reaction by dialysis against 0.02 M Tris-buffered saline pH 7.4 and the precipitated collagen dissolved by heat denaturation at 50°C. Collagen-specific antibody was prepared by affinity chromatography of antisera on collagen–Sepharose immunosorbents.

3. Results

3.1. *Immune response to soluble collagens*

The precipitating antibody titres found in sera taken from sheep injected with the three types of soluble collagen studied are listed in table 1. All sheep except number 5 gave a high titre response after three sets of injections at the most; sheep 6 was particularly industrious, showing the remarkable titre of 7.8 mg/ml after only two injections. The poor response of sheep 5 is surprising in that it received the same antigen as sheep 6. It was, however, a rather weak animal, and may have had a deficient immune system.

All sera except that from sheep 5 gave strong lines

Table 1

Immune response to injection of soluble collagen

Day	Sheep							
	1	2	3	4	5	6	7	8
16	0.4	–	–	–	0.3	1.2	–	–
21	0.9	–	–	–	–	–	–	–
25	–	–	–	–	–	7.8	–	–
29	–	–	–	–	0.6	7.3*	0.3	1.7
41	–	–	–	–	0.3		1.4	2.2*
43	1.7	1.1	0.6	0.4	–		–	
47	–	–	–	–	–		2.4*	
50	2.4	2.5	2.1	1.5	–			
69	2.3*	2.3*	1.9*	1.5*	0.4*			

† Each antiserum was titrated against the immunising antigen.
* On this day the sheep was killed and bled out.

Specific precipitating antibody† titre in mg/ml. Sheep 1 to 4 injected with acid extracted rat tail tendon tropocollagen, sheep 5 and 6 injected with acid extracted rat skin tropocollagen and sheep 7 and 8 injected with urea extracted rat skin collagen.

in gel diffusion tests against their homologous antigens, and the antisera raised against skin collagens cross-reacted with each other and with tendon collagen (fig. 1a). However, the antisera raised against tail tendon collagen did not cross-react with skin collagen (fig. 1b), and this suggests that the precipitating antibody response to the tendon collagen was restricted to the determinants in the non-helical telopeptide sequences of the α-chains since tissue differences are most often manifest in the telopeptide region [8,12].

The antisera were shown to be specific for collagen by demonstrating the action of collagenase, which both prevented formation of immune precipitation and dissolved pre-formed precipitated immune complex. Also amino acid analysis of a thoroughly washed immune precipitate showed the presence of both hydroxyproline and hydroxylysine.

3.2. *Affinity chromatography*

In the course of preparation of a satisfactory immunosorbent it was found essential that the anti-collagen immunoglobulin was further purified by affinity chromatography on Sepharose–collagen immunosorbent. Although the immunosorbents prepared from gamma globulin concentrates showed

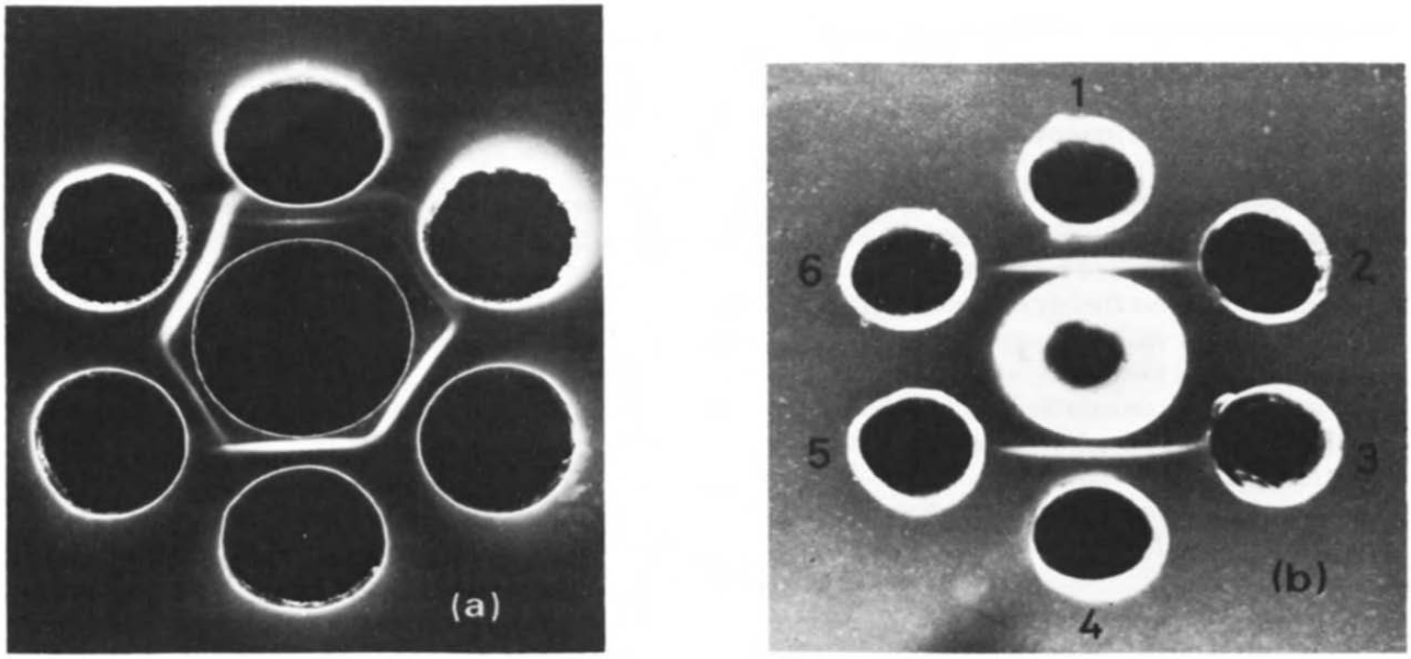

Fig. 1(a). Immunodiffusion patterns of sera from six sheep Centre well contained urea extracted rat skin collagen. Peripheral wells sera from sheep 1–6 listed in table 1; (b) immunodiffusion patterns of tropocollagen extracted from rat skin, bone and tail tendon. Centre well contained anti-(RTF–TC) antiserum. Peripheral well contained rat tail tendon collagen (1 and 4); bone collagen (2 and 5) and skin collagen (3 and 6).

high specificity of adsorption the recoveries of antigen by the usual desorbing reagent were very low, probably due to high avidity of the antisera. In this study immunosorbents were prepared only from sera raised against tail-tendon collagen, chosen because of the indication of restricted specificity mentioned in section 3.1.

Mixtures of peptides obtained by CNBr cleavage

Table 2
Recovery of antigenic peptides from anti-(RTT–TC) immunosorbent columns by elution with 1 M aqueous ammonia

Loaded		Eluted		Adsorbed		Desorbed	
cpm × 10^6	mg	cpm × 10^6	mg	cpm × 10^6	mg	cpm × 10^6	mg
17.0	35.0	9.3	29.9	7.7	5.1	8.1 (104)	5.3 (104)
24.0	51.0	13.5	45.0	10.5	6.0	8.5 (81)	5.8 (96)
9.8	32.6	7.5	30.7	2.3	1.9	2.6 (113)	1.8 (95)
7.0	28.8	5.2	27.5	1.8	1.3	1.6 (123)	1.1 (84)
22.0*		20.5 (93)		1.5 (7)		0.1 (< 1)	

* Sepharose – non-antibody IgG.
Percentage recoveries shown in parentheses.

of thoroughly washed intact rat tail tendons reduced with potassium borotritide were equilibrated in this buffered neutral saline and applied to immunosorbent columns equilibrated in neutral saline. The columns (cooled to 4°C) were washed free of non-absorbed peptides, and then eluted with 1 M aqueous ammonia. After desorption of antigenic peptides the columns were re-equilibrated with neutral saline. The quantities of peptides absorbed and recovered are set out in table 2. Column capacities were high enough to be of preparative value and the recoveries of absorbed peptides were quantitative both by tritium counting (81%—123%) and by quantitative amino acid analysis (84%—104%).

As a check on absorption specificity a column was prepared from gamma-globulin without antibody specificity for collagen. Passage of tritium-labelled collagen peptides, the same peptides as chromatographed on collagen-specific immunosorbents, through the non-specific column showed a very small reduction in counts (7%), and elution with 1 M ammonia produced no detectable radioactivity peak.

3.3. *Characterization of desorbed peptides*

The peptides obtained by affinity chromatography on antibody immunosorbents, and therefore regarded as 'antigenic' peptides, have not yet all been finally identified. However, several interesting parameters have been characterized. The specific radioactivity is much higher (about 8 times) than the non-antigenic peptides, indicating that the antigenic peptide contains a greater proportion of the reducible components than do the non-antigenic peptides.

As expected from crosslinked peptides the amino acid composition did not correspond directly to any single CNBr peptide, but is typically collagen-like with high glycine content and characteristic levels of hydroxylysine and hydroxyproline. However, the tyrosine content is increased indicating enrichment in the proportion of telopeptide derivatives.

Molecular exclusion chromatography on agarose beads (A 1.5 M, Bio-Rad Laboratories) indicates the presence of four components. The largest of the two major components eluted in a position indicating a molecular weight of about 60 000 and contained the highest proportion of the tritium activity. Acid hydrolysis of this peptide and analysis on ion-exchange columns [9] demonstrated that the tritium activity was associated with the crosslinks previously shown to exist between the telopeptide regions and the helical regions of the molecule [2,3]. A higher proportion of the tritium activity was shown to be associated with the tetra-functional crosslink histidino-hydroxymerodesmosine [12,13] than the bifunctional crosslink hydroxylysinonorleucine [9]. Reaction of this 60 000 mol.wt. peptide with sodium periodate followed by borohydride reduction indicated that it was completely cleaved. The intact peptide chromatographed on Sephadex G100 at the void volume, but after periodate treatment the tritium activity was recovered in three broader peaks appearing later in the chromatogram indicating lower molecular weights (fig. 2). Confirmation that the periodate had completely cleaved all the tritium-

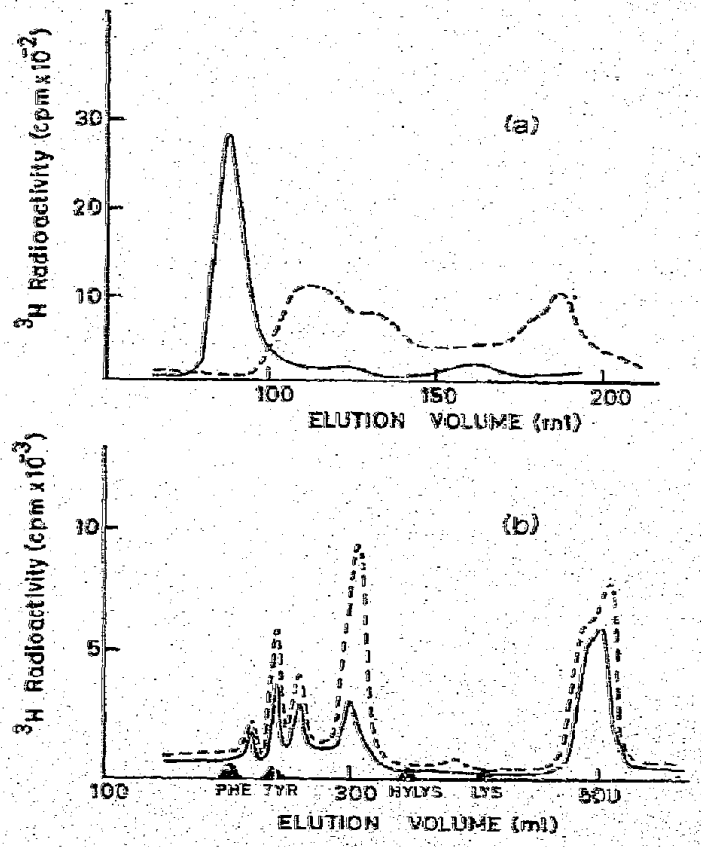

Fig. 2(a). Elution patterns on Sephadex G100 of the 60 000 mol.wt. components desorbed from the immunosorbent columns before, —— and after – – – –, periodate treatment; (b) distribution of the tritium radioactivity of the whole rat tail tendon collagen, ——, and of the 60 000 mol.wt. component, – – – –.

labelled crosslinks was obtained by demonstrating their absence in an acid hydrolysate of the periodate treated material.

4. Discussion

The titres of precipitating anticollagen antibody found in sheep sera are considerably greater than those reported in the rabbit [14,15], or the chicken [16]; and for the first time the antibody has been produced in quantities sufficient to allow the production of antibody immunosorbents for affinity chromatography.

The specificity of these immunosorbents for collagen has been confirmed and the quantitative recoveries of antigenic peptides indicate that the method will be of practical value for isolation of composite (crosslinked) peptides including antigenic determinants in their structure.

The major radioactive components obtained by the immunosorbent technique was demonstrated to be a crosslinked peptide by the presence of characteristic crosslinks derived from allysine in the telopeptide regions, and their cleavage to produce smaller peptides with periodate. The higher proportion of the tetrafunctional histidino-hydroxymerodesmosine than hydroxylysinonorleucine is consistent with the high molecular weight of the peptide. The precise CNBr peptides involved in the crosslinked peptide remain to be established.

Characterization of all the antigenic peptides is still incomplete, but the speedy isolation procedure now available will provide adequate quantities of material for future structural studies.

The induction of an immune response to collagen depends upon correct selection of host animal and injection protocol. We feel the immunosorbent technique has great potential for development by further restriction of the antigen binding specificity of the antiserum, and provides the means for comparison of collagen structures in connective tissue of any origin.

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