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Production and specificity of antibodies against the aminoterminal region in type III collagen

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Summary. A cross-linked fragment (peptide T1X) with a molecular weight of 13,000 could be isolated from a tryptic digest of insoluble type III collagen of calf skin. Peptide T1X was conjugated on to bovine serum albumin by glutaraldehyde and used for immunization of rabbits. The antisera reacted in passive haemagglutination and radioimmune assay with peptide T1X, type III collagen and its constituent α1(III) chain. Little or no reaction was observed with type I collagen and α1(I) chain. While rabbit antisera to neutral salt-soluble type III collagen also showed a strong binding for ^{125}I-labelled peptide T1X much less reaction was observed with antisera to type I collagen. The antigenicity of type III collagen was largely destroyed by pepsin treatment suggesting that it resided in non-helical segments. A fragment of peptide T1X produced by digestion with collagenase retained antigenic activity. The data indicated that the aminoterminal region of type III collagen contains strong antigenic determinants located in a non-helical sequence of about sixteen amino acids.

Antibodies to these antigenic determinants were purified and rendered specific for type III collagen by immunoadsorption. The antibodies stained in indirect immunofluorescence tests particularly those regions in various connective tissues which are rich in reticulin fibres. Different staining patterns were observed with antibodies to type I collagen.

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INTRODUCTION

Most of the previous immunochemical studies on collagens (Furthmayr and Timpl, 1975) have been restricted to type I collagen which is the prominent fibrillar component in various connective tissues such as skin, bone and tendon. The recent discovery of additional types of collagen (Miller, 1973; Kefalides, 1973; Chung and Miller, 1974; Epstein, 1974) has raised the question whether it is possible to distinguish such types by antibodies. It could be shown that antisera to type I collagen exhibited only little cross-reaction with type II collagen from hyaline cartilage and *vice versa* (Hahn, Timpl and Miller, 1974, 1975; Nowack, Hahn and Timpl, 1975). Purified antibodies against both types of collagen were used to study tissue distribution of collagen by immunofluorescence (Wick, Nowack, Hahn, Timpl and Miller, unpublished results; von der Mark, von der Mark and Gay, 1976). Biochemical studies have shown that type III collagen occurs together with type I collagen in most connective tissues but it seems enriched in blood vessels, uterus and skin (Chung and Miller, 1974; Epstein, 1974). Antibodies which are specific for type III collagen have so far not been characterized by immunochemical methods.

Trypsin digestion of denatured insoluble collagen from calf skin liberates a peptide T1X which is particularly rich in tyrosine (Becker, Furthmayr and Timpl, 1975). Antibodies against tyrosine-containing antigenic determinants in type I collagen, however,

failed to react with peptide T1X. More recent studies demonstrated that this fragment originates from the aminoterminal cross-linking region of type III collagen and that it consists of two identical peptide chains connected to each other in a small non-helical segment (Becker, Fietzek, Nowack and Timpl, in preparation). Since rabbits usually show a strong antibody response against non-helical regions in type I collagen (Furthmayr and Timpl, 1975) it was of interest to characterize the immunological properties of peptide T1X. In the present study we have prepared antibodies to this peptide and these antibodies showed a high specificity for type III collagen.

MATERIALS AND METHODS

Preparation of proteins and peptides

Type I collagen was extracted from 6-week-old calf skin with citrate buffer, pH 3·6, and purified by standard methods (Pontz, Meigel, Rauterberg and Kühn, 1970). Neutral salt-soluble type III collagen was extracted from foetal bovine skin and purified by salt precipitation and chromatography on DEAE-cellulose (Timpl, Glanville, Nowack, Wiedemann, Fietzek and Kühn, 1975). All collagens were dissolved in 0·05 per cent acetic acid and stored at −20°. α1(III) chains were prepared by reduction and *S*-carboxymethylation of type III collagen in 8 M urea (Timpl *et al.*, 1975). α1(I) chains were obtained from type I collagen by chromatography on CM-cellulose (Piez, Eigner and Lewis, 1963). For serological studies lyophilized chains were dissolved in phosphate-buffered saline, pH 7·2, by heating for 30 min at 50°.

In some studies type III collagen was treated with pepsin under conditions which allowed the removal of non-helical segments. The collagen was dissolved in 0·1 M acetic acid (1 mg/ml) and the pH subsequently adjusted to 2·0 with dilute HCl. Incubation was carried out at a pepsin–collagen ratio of 1:20 for 24 h at 20° (Timpl *et al.*, 1975).

Peptide T1X was isolated from a tryptic digest of insoluble calf skin collagen as described previously (Becker *et al.*, 1975).* A dimeric fragment comprising nineteen amino acids in each chain was isolated from a collagenase digest of T1X by molecular sieve chromatography (Becker, Nowack and Timpl, in preparation). Bovine serum albumin (BSA) was purchased from Armour, Eastbourne, and a monomeric fraction was prepared by chromatography on a Sephadex G-150 column.

Coupling of peptide T1X on to serum albumin

We followed basically the glutaraldehyde conjugation procedure of Olsen and Prockop (1974) for coupling T1X to BSA. Here, 15 mg of monomeric BSA were dissolved in 1 ml 0·1 M sodium phosphate buffer, pH 7·3, and mixed with 5 ml 1·7 M glutaraldehyde in 0·1 M phosphate, pH 7·3, and the solution was stirred for 30 min at room temperature. The solution was then applied to a Sephadex G-25 column (1·5 × 120 cm) which was equilibrated with 0·1 M sodium phosphate, pH 7·3. The column was eluted at a flow rate of 100 ml/h. The protein peak was concentrated to 5 ml by ultrafiltration (Diaflow) and mixed with 7·5 mg of peptide T1X dissolved in 0·5 ml 0·1 M sodium phosphate, pH 7·3. The mixture was stirred for 4 days at 4° and subsequently chromatographed on a Bio-Gel P-150 column (2·5 × 100 cm) equilibrated in 0·1 M Tris–HCl, pH 7·5. Two protein peaks emerged from the column. As judged by electrophoresis on polyacrylamide gels in SDS (Furthmayr and Timpl, 1971) the larger component consisted mainly of oligomeric BSA, while the second peak contained mainly monomeric BSA conjugate. The amount of T1X bound to each fraction of BSA was determined by amino acid analysis assuming eighteen residues of hydroxyproline in peptide T1X (Becker *et al.*, 1975) and fifty-eight residues of leucine and twenty-three residues of arginine in BSA (King and Spencer, 1970). The latter two amino acids are not found in T1X. Oligomeric BSA fractions contained 4·3 moles of peptide T1X while the monomeric BSA fraction contained 1·5 moles peptide T1X per mole BSA.

Antisera

Peptide T1X was dissolved in 0·05 per cent acetic acid (1·25 mg/ml); emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into adult rabbits (2·5 mg per animal). After an intraperitoneal booster (1 mg without adjuvant) on day 29 blood was drawn on day 50. Three rabbits were used for immunization, with the monomeric (nos 724 and 725) and oligomeric (no. 727) T1X–BSA conjugates. The antigens were

* The previously used term peptide 1 is changed in the present study to T1X to account for recent data that this fragment is derived from the aminoterminal region of type III collagen and occurs in a cross-linked form.

dissolved in phosphate-buffered saline, pH 7·2, emulsified with adjuvant and injected subcutaneously (1 mg per animal). A second intraperitoneal injection of 0·5 mg antigen in adjuvant on day 28 was followed by sampling the antisera on day 70. Rabbits were injected subcutaneously with 5 mg type III collagen emulsified in Freund's complete adjuvant. They were boosted on day 26 intraperitoneally with 5 mg collagen without adjuvant, and serum was collected on day 77. Production of antibodies to type I collagen followed the same schedule except that the intraperitoneal booster was given on day 14 and a further subcutaneous booster with 5 mg type I collagen in adjuvant was given fourteen days later. The preparation of a goat antiserum to rabbit immunoglobulin G has been described elsewhere (Lotter and Timpl, 1975).

Purification of antibodies

Immunoadsorption of various antisera on denatured type I and type III collagen followed previously described procedures (Beil, Timpl and Furthmayr, 1973). Denatured type III collagen was purified by agarose chromatography (Piez, 1968) and the trimeric component was coupled to *p*-aminobenzylcellulose. About 10 mg of peptide T1X were coupled on to 20 ml cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala) (Porath, Axen and Ernback, 1967). Analysis indicated that more than 90 per cent of the peptide was fixed to the Sepharose. Washing of the adsorbent and the procedure for isolating antibodies were the same as described previously (Gollwitzer, Hahn, Nowack and Timpl, 1975). Purified antibodies to type I collagen were those used in previous studies (Wick, Furthymayr and Timpl, 1975; Gay, Balleisen, Remberger, Fietzek, Adelmann and Kühn, 1975). Purified antibodies to the CNBr peptide F-CB1 of bovine fibrinogen (Gollwitzer, Hahn, Lotter, Nowack and Timpl, 1976) were used in the immunofluorescence studies for negative controls. Immunoelectrophoresis in agarose gels was used to characterize the eluted antibodies by a donkey antiserum to rabbit serum purchased from Behringwerke, Marburg. A single immunoglobulin G line was observed with each of the antibody eluates.

Radioimmunoassay with peptide T1X

The chloramine T method (McConahey and Dixon, 1966) was used for labeling 20 μg of peptide T1X with 0·25 mCi of ^{125}I (New England Nuclear Chemicals GmbH, Dreieichenhain). Labelled peptide was separated from free iodine on a Bio-Gel P-2 column (1 × 12 cm) which was equilibrated with phosphate-buffered saline, pH 7·2, containing 0·1 per cent BSA. Radioactivity was measured in a Beckman Gamma 300 Counter. In different experiments it was estimated that 26,000–76,000 c.p.m. were incorporated per 1 ng of peptide T1X. Trichloroacetic acid (10 per cent) precipitated about 90 per cent of the label, indicating that the ^{125}I was peptide-bound.

Radioimmunoassays of different antisera were carried out in 10 per cent normal rabbit serum (Rohde, Nowack, Becker and Timpl, 1976) with 0·05–0·2 ng labelled peptide per sample corresponding to 4000–6000 c.p.m. Goat antiserum to rabbit immunoglobulin G was added to each reaction to precipitate antibody-bound peptide. Only 2–3 per cent of the radioactive peptide was precipitated in the presence of undiluted normal rabbit serum. Antigen-binding capacity (ABC-33) of the antisera was calculated as suggested by Minden and Farr (1973). In the inhibition assay antibodies were preincubated for 6–24 h at 4° with unlabelled antigens prior to the addition of labelled peptide T1X (Lotter and Timpl, 1975). The concentration of inhibitor solutions were determined after acid hydrolysis on the amino acid analyser. Molecular weights assumed were 285,000 for collagen, 95,000 for α chains, 13,000 for peptide T1X and 4,000 for the collagenase fragment.

Other serological methods

Indirect immunofluorescence on frozen tissue sections followed essentially the procedures used previously for anti-collagen antibodies (Wick *et al.*, 1975; Gay *et al.*, 1975). Sections of cartilage were treated with hyaluronidase (von der Mark *et al.*, 1976) prior to the application of antibodies.

Red cells were coated for passive haemagglutination tests with type I or type III collagen using a glutaraldehyde method (Beil *et al.*, 1973). Gel diffusion studies were carried out in 0·6 per cent agarose gels. The radioimmune assay with ^{125}I-labelled collagens was that described by Adelmann, Gentner and Hopper (1973).

RESULTS

Production of antibodies against peptide T1X and cross-reaction with collagen

The amounts of antibody produced in response to

Table 1. Reaction of ^{125}I-labelled peptide T1X with various antisera

Antiserum to:	No.	Antigen-binding capacity	
		ABC-33* (ng/ml)	Percentage of antigen bound by antiserum diluted 1:4
Peptide T1X	629	< 0·6	28†
	630	< 0·6	9†
Peptide T1X-BSA	725	1452·0	100
	727	46·5	99
Type III collagen	717	1452·0	100
	722	6352·5	103
Type I collagen	676	< 0·7	< 1
	680	6·9	83
	681	< 0·7	27
	683	< 0·7	< 1

* Various dilutions (0·1 ml) of the antisera were incubated with 0·055 ng of peptide T1X, except with antisera to T1X where 0·18 ng was used. ABC-33 values were calculated from the amount of antisera required to bind 33 per cent of labelled antigen and represent the antigen binding-capacity of peptide T1X bound per millilitre undiluted antiserum. Each value was corrected for non-specific binding by normal rabbit serum.

† Binding by undiluted antiserum.

Table 2. Reaction of antisera to peptide T1X–BSA conjugate with type I and type III collagen

Antiserum no.	Agglutination titre ($-\log_2$) for collagen		Percentage of ^{125}I-labelled collagen bound by 1:4 diluted antiserum*	
	Type I	Type III	Type I	Type III
724	2	6	10	32
725	< 2	12	5	80
727	5	5	6	91

* 0·1 ml antiserum reacted with 20 ng collagen.

peptide T1X or to conjugates of T1X with BSA was measured by radioimmunoassay. The data in Table 1 show that peptide T1X is a very weak immunogen and significant amount of antigen binding occurred only with undiluted antisera. However, the immunogenicity of peptide T1X was considerably increased when coupled to BSA. With sufficiently high amounts of antisera to T1X–BSA conjugates, all of the labelled peptide was precipitated which indicates that the iodination procedure did not destroy antigenicity. These antisera were also assayed for reactivity with BSA by gel diffusion. Only one of three antisera showed a weak reaction.

The reaction between ^{125}I-labelled peptide T1X and antisera against type I and III collagens was also investigated. As shown in Table 1 antisera to type III collagen reacted as strongly with peptide T1X as did antisera to T1X–BSA itself. However, antisera to type I collagen showed either little or no binding activity. Similarly, antibodies to T1X–BSA conjugate

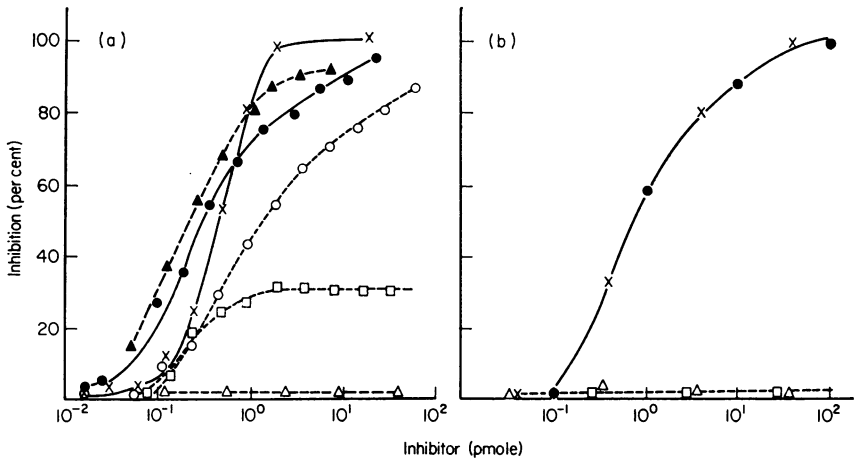

Figure 1. Inhibition of binding of ^{125}I-labelled peptide T1X to antiserum to peptide T1X–BSA conjugate (a) or to antiserum to type III collagen (b). Inhibitors were: peptide T1X (× —×); type III collagen (●—●); pepsin-treated type III collagen (□ - - □); α1(III) chain (○ - - ○) α1(I) chain (Δ - - Δ) and a collagenase fragment of peptide T1X (▲ - - ▲).

reacted better with type III than with type I collagen (Table 2).

Characterization of the antigenic determinants in peptide T1X

Antigenic properties of peptide T1X were studied by a radioimmune inhibition assay as shown in Fig. 1. Peptide T1X, native type III collagen, and a collagenase fragment of peptide T1X showed comparable inhibitory activity. Unfolded α1(III)-chains caused also almost complete inhibition but only at 5- to 20-fold higher concentrations when compared to type III collagen. Prior incubation with pepsin reduced the ability of type III collagen to inhibit by 70 per cent (Fig. 1a) or even abolished all activity (Fig. 1b). The α1(I) chain from type I collagen was not an inhibitor even when used at 100-fold higher concentrations than α1(III) chains.

Purification of antibodies by immunoadsorption

Antisera to T1X–BSA conjugate were passed over adsorbents prepared from denatured type I or type III collagen. Results in Table 3 show that little binding occurred with the type I collagen adsorbent whereas the bulk of antibody was bound to and subsequently eluted from type III collagen adsorbent. Similarly, a considerable proportion of the antibodies in antisera to type III collagen were bound to an adsorbent prepared from peptide T1X.

Table 3. Purification by immunoadsorption of antibodies to peptide T1X–BSA conjugate or type III collagen*

Antiserum to:	Immunoadsorbent	Antibody protein recovered (μg/ml)	Binding capacity for ^{125}I-labelled peptide T1X (ng/ml)		Agglutination titres (−log$_2$) of eluted antibodies for collagen	
			Antibodies eluted from adsorbent	Antibodies not bound on adsorbent	Type I	Type III
Peptide T1X-BSA	Type I collagen	< 20	0·4	—	< 2	< 2
	Type III collagen	92	44·5	4·4	< 2	13
Type III collagen	Peptide T1X	296	175·3	6·6	9	12

* Sera from three or four animals were pooled and 20 ml passed over the adsorbent. The amounts of antibody protein eluted from a particular adsorbent was estimated from its absorption at 280 nm and is expressed as μg/ml antiserum. Unspecific adsorption on such columns is in the order of 30–40 μg/ml (Gollwitzer *et al.*, 1975).

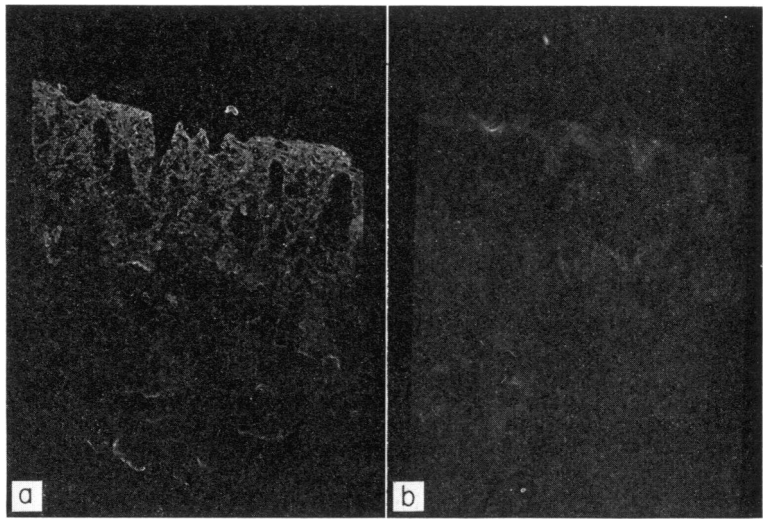

Figure 2. Indirect immunofluorescence produced from the binding of antibodies to peptide T1X to foetal calf skin (a). Control section was exposed to anti-fibrinogen antibodies (b). The epithelium did not stain but showed a weak non-specific fluorescence. Antibody concentration, 15 μg/ml. (Magnification $\times$48.)

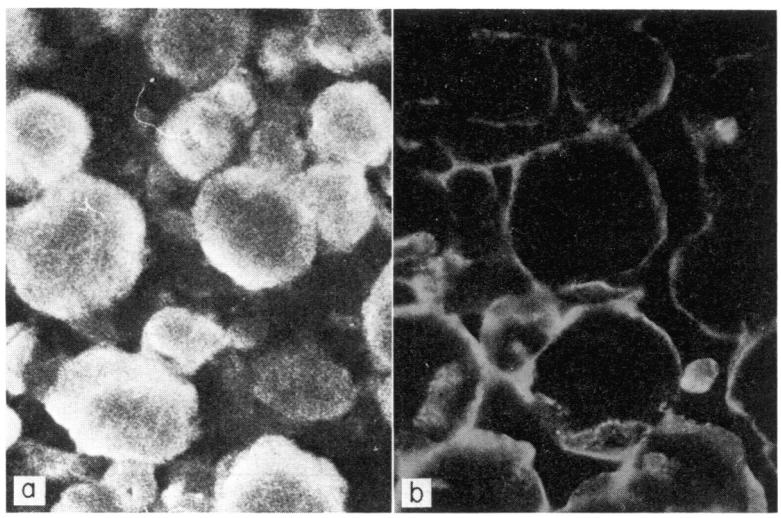

Figure 3. Indirect immunofluorescence tests on cross-sections of human tendon with antibodies to type I collagen (a) or to peptide T1X (b). Note that the staining with anti-peptide T1X was restricted to thin regions around the fibre bundles (endotendineum). Antibody concentration, 30 μg/ml. (Magnification $\times$288.)

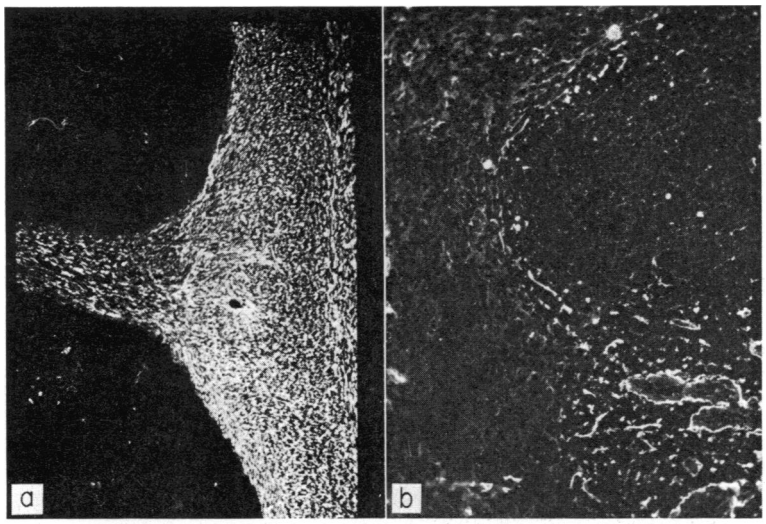

Figure 4. Indirect immunofluorescence reactions with calf spleen using antibodies to type I collagen (a) or to peptide T1X (b). Anti-type I antibodies showed a strong staining of the capsular region and of trabeculae and no reaction with the red and white pulp. Antibodies to peptide T1X reacted with the reticular meshwork of the pulp and with blood vessels (compare Gay *et al.*, 1975). Antibody concentration, 30 μg/ml. (Magnification ×96.)

Studies by passive haemagglutination (Table 3) indicated a high specifity for type III collagen of the antibodies obtained from peptide T1X antisera. Antibodies to type III collagen still showed a distinct cross-reaction with type I collagen.

Immunofluorescence studies localizing type III collagen in tissues

Purified antibodies to peptide T1X or type III collagen (see Table 3) stained various bovine and human connective tissues. Positive reaction could be detected even at antibody concentrations of 1–3 μg/ml. No staining was observed with tissue exposed to normal rabbit serum (diluted 1:20) or with purified antibodies against fibrinogen (compare Fig. 2a, b).

In skin antibodies to peptide T1X showed the most intense staining in the subepithelial regions (Fig. 2). A staining throughout the whole dermis has been previously described for antibodies to type I collagen (Wick *et al.*, 1975). However, more distinct differences in staining were observed with antibodies to type I and type III collagen in cross-sections of tendons (Fig. 3) and in spleen (Fig. 4). Antibodies to peptide T1X reacted with a fine fibrous meshwork in the splenic pulp known to be rich in reticulin. Staining by anti-type I antibodies was restricted to the capsula and trabeculae. A similar staining pattern as in spleen was also observed in sections of liver and lymph node. Articular cartilage which can be strongly stained by antibodies to type II collagen (Wick *et al.*, unpublished results) failed to react with antibodies to peptide T1X. Antibodies to type III collagen produced the same staining pattern as antibodies to peptide T1X provided that the antibody was used at a concentration at or below 20 μg/ml. Apparently, at this dilution the antibody fraction which cross-reacts with type I collagen (Table 3) was no longer detected in the indirect immunofluorescence technique.

DISCUSSION

Peptide T1X with a molecular weight of 13,000 can be isolated from insoluble skin collagen (Becker *et al.*, 1975) which consists of about 25 per cent of type III collagen (Epstein, 1974). Recent data (Becker, Fietzek, Nowack and Timpl, in preparation) indicated that peptide T1X is derived from type III collagen and is composed of two identical chains connected to each other by an aldol cross-link similar to that described for the amino terminal cross-linking region in type I collagen (Bornstein and Piez, 1966). Sequence studies showed a non-helical sequence of 16 amino acid residues at the amino-

terminal end which continued with a helical sequence identical to that found at the aminoterminal end of pepsin-treated type III collagen (Fietzek, Allmann, Rauterberg and Wachter, 1976). Peptide T1X has a blocked aminoterminal group and contains four tyrosine residues and one lysine residue per chain. The tyrosine content explains the high efficiency in labelling by ^{125}I while it is likely that the attachment of T1X to BSA by glutaraldehyde occurs via the single amino group in the carboxyterminal position.

Immunization with peptide T1X produced only low antibody titres but much higher titres were obtained using T1X–BSA conjugates. A similar enhancement of antibody response by carrier has also been observed for the collagen-like polymer $(Pro–Gly–Pro)_n$ (Maoz, Fuchs and Sela, 1973). Strong reactions for peptide T1X were found with antisera to neutral salt-soluble type III collagen. Immunoadsorption demonstrated antibody levels in the order of 300 μg/ml which is comparable to those values reported for antisera to other mammalian collagens (Furthmayr and Timpl, 1975).

Results of the inhibition assay provided evidence that the major antigenic determinants in peptide T1X are located within the non-helical sequence. These determinants could be detected on native type III collagen and its unfolded α1(III) chain. Similar observations were reported previously on antibodies to non-helical determinants in type I collagen (Beil *et al.*, 1973). Pepsin treatment considerably reduces antigenicity in type III collagen but did not destroy the triple helical body of the molecule (Timpl *et al.*, 1975). These data agree with the high inhibitory activity observed for the collagenase fragment of peptide T1X which essentially consists of a non-helical sequence.

Antibodies to peptide T1X showed little reaction with soluble type I collagen or insoluble type II collagen in cartilagineous tissue. Thus, we obtained antibodies specific for type III collagen. These antibodies gave a distinct immunofluorescence staining of reticular fibres which could not be stained by anti-type I antibodies. The same results were recently obtained with antibodies prepared against type III collagen and procollagen (Gay *et al.*, 1975; Nowack, Gay, Wick, Becker and Timpl, in preparation). Such antibody reagents may therefore be useful tools in structural studies on components of reticulin which have not yet been sufficiently characterized. It will also be of interest to study whether anti-reticulin antibodies observed in various gastrointestinal diseases (Wright and Alp, 1971; Essen, Savilahti and Pelkonen, 1972) react with type III collagen or procollagen.

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