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Immunogenicity and Specificity of Collagen

VI. SEPARATION OF ANTIBODY FRACTIONS WITH RESTRICTED SPECIFICITY FROM ANTI-COLLAGEN SERA USING AN IMMUNOADSORBENT TECHNIQUE

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Summary. Rabbit antisera to calf collagen were investigated using columns of denatured rabbit collagen insolubilized by coupling to diazotized *p*-aminobenzyl cellulose. Only antibodies with general collagen specificity were bound onto the immunoadsorbent. Species specific antibodies passed through the column and were isolated in the effluent. Column-bound antibodies could be desorbed by elution with collagen peptides. The separated antibody fractions exhibited in haemagglutination–inhibition experiments with calf and rabbit collagen and collagen peptides a characteristic behaviour allowing the differentiation between them. The results support the assumption drawn from serological experiments that most antisera contain mixtures of antibodies with different specificities.

INTRODUCTION

In the preceding part of this investigation (Steffen, Timpl and Wolff, 1968) we have shown by haemagglutination–inhibition tests that calf collagen can induce in rabbits at least three types of antibodies with different specificities. The serological results suggested that most antisera contain mixtures of these different antibodies. In order to support this interpretation and also to obtain antibody solutions with restricted specificity, we have developed a technique for the separation of the anti-collagen antibodies according to their specificities.

MATERIALS AND METHODS

Antigens, antisera and the passive haemagglutination method

These were described in the preceding paper (Steffen *et al.*, 1968).

Enzymes

Crystallized chymotrypsin was purchased from Boehringer, Mannheim, and crystallized pepsin from Armour, Eastbourne.

Collagen peptides

Denatured calf collagen was digested with trypsin or collagenase as described previously (Steffen and Timpl, 1965). Additionally, peptides obtained after trypsin treatment were

digested further with chymotrypsin (enzyme–substrate ratio 1:100, pH 8·0, 4 hours, 37°) followed immediately by pepsin (enzyme–substrate ratio 1:100, pH 2·5, 4 hours, 37°). After neutralization the enzymes were inactivated by heating to 100° for 5 minutes. During the sequential enzyme action the average chain length of the peptides dropped from about sixteen to fourteen amino acid residues as determined by a ninhydrin method (Steffen and Timpl, 1965).

Preparation of the immunoadsorbent

Denatured acid-soluble collagen (parent gelatin) obtained from rabbit skin was coupled to diazotized *p*-aminobenzyl cellulose (Serva, Heidelberg) according to a procedure described elsewhere (Timpl, Furthmayr, Steffen and Doleschel, 1967). The immunoadsorbent contained 5–6 mg bound collagen per gram corresponding to 0·07 per cent hydroxyproline detected by the method of Stegemann (1958) after hydrolysis of the immunoadsorbent with 6 N HCl.

Isolation of the antibody fractions

A column 1·6×8–10 cm was prepared from about 6 g immunoadsorbent and cooled with running tap water. After application of 10 ml anti-calf collagen serum, washing was performed with 150–200 ml isotonic phosphate buffer, pH 7·2, at a flow rate of 50–70 ml/hr. The effluent was sampled with a fraction collector and tested against erythrocytes coated with denatured rabbit collagen. Serum effluents which still showed a positive reaction were passed a second time through either a new column, or the same column regenerated by peptide elution (see below). When the serological reaction became negative, the effluent samples containing the serum proteins were pooled, mixed with an equal volume saturated ammonium sulphate solution and the precipitate dissolved in a small volume of saline or normal rabbit serum. After dialysis overnight at 4° against saline, the volume was adjusted to the applied serum volume.

From the washed column (the last fractions proved to be without any detectable protein content) the adsorbed antibodies were eluted with 5–10 ml of a solution of peptides obtained after trypsin digestion of calf collagen, containing 2·5–4 mg N/ml. Further washing was performed with 100 ml phosphate buffer, pH 7·2. The pooled fractions containing the peptides and the desorbed antibodies (examined by extinction measurement at 215 mμ) were precipitated with ammonium sulphate as above. After standing for 3 hours at 4°, the precipitate was collected by centrifugation (30 minutes, 4°, 2000 ***g***), dissolved in 1–1·5 ml normal rabbit serum and dialysed overnight at 4° against saline. It should be noted that precipitation of antibody by ammonium sulphate is only complete, when 5–10 per cent of the peptides are also precipitable. If one uses peptide solutions which do not show this characteristic, as for instance those obtained after collagenase treatment, another concentration procedure may be preferable. All the antibody solutions were stored at −30° until used.

RESULTS

Haemagglutination–inhibition tests have suggested the simultaneous existence of antibody fractions differing in specificity in most rabbit antisera against calf collagen. Definite proof for the independence of these fractions from each other was provided by the experiments described below. All antisera possessing antibody fractions of different

specificities contained antibodies reacting with calf and rabbit collagen, which we designated as antibodies with general specificity, type A. Additionally the sera contained species specific antibodies reacting only with calf collagen which were directed either against pepsin-stable or pepsin-labile determinants (type S or P). It was attempted to remove and isolate the A-specific antibody fraction by treating the sera with an immunoadsorbent prepared from denatured rabbit collagen.

The type of specificity of the thirteen antisera used in this study and the respective titres of their specific antibody fractions as determined in the whole serum (Steffen *et al.*, 1968) are given in Table 1. All the sera contained A-specific antibodies which showed a

TABLE 1

FRACTIONATION OF ANTI-CALF COLLAGEN ANTISERA WITH MIXED SPECIFICITY ON A RABBIT COLLAGEN IMMUNOADSORBENT

Serum No.	Specificity type	Titre of the antibody fraction in the whole serum*		Antibody fraction A-1, bound and eluted after a first passage through the column titre with:†		Antibody fraction A-2, bound and eluted after a second passage through the column titre with:†		Antibody fraction P or S, not bound onto the column titre with:	
		A-specific	P- or S-specific	CS	RS	CS	RS	CS	RS
114	A,P	128	64	128	64	4	2	128	2
116	A,P	1024	64	512	512	32	32	128	2
216	A,P	128	64	64	32			NT‡	NT
118	P,A	128	512	64	64	4	4	1024	<2
119	P,A	128	512	128	128	8	4	512	<2
206	P,A	32	1024	32	32			1024	<2
207	P,A	8	256	16	8			512	<2
226	P,A	16	256	16	8			256	<2
227	P,A	64	1024	64	64			1024	4
235	P,A	128	256	64	64	4	4	128	2
229	A,S	512	128	512	512			64	4
239	A,S	256	16	128	128	16	16	4	<2
275	S,A	64	512	32	32			512	<2

Results as reciprocal titres using erythrocytes coated with denatured collagen from calf (CS) or rabbit skin (RS).

* The titres were determined by passive haemagglutination inhibition experiments as described in the preceding paper (Steffen *et al.*, 1968).

† Titre of the concentrated solutions after correction to the applied serum volume.

‡ Not tested.

complete cross-reaction with rabbit collagen. After a first passage of the sera through the immunoadsorbent, the serum effluents of seven sera showed no reaction with rabbit collagen, indicating the complete removal of A-specific antibodies. The other sera showed a strongly diminished reaction with rabbit collagen which was not detectable after a second passage through a new or regenerated immunoadsorbent.

The serum effluents which contained the P- or S-specific fractions showed after concentration to the original volume a high titre with calf collagen which was with one exception (serum No. 239) practically the same as that determined for the respective fraction in the whole serum (Table 1). The reaction with rabbit collagen was either negative or below a titre of 1:8.

The column-bound antibodies were desorbed by elution with collagen peptides and designated as antibody fraction A-1 when obtained after the first or A-2 when obtained after the second serum passage through the immunoadsorbent. The antibody eluates showed equal agglutination titres with collagen from calf and rabbit, in accordance with

our definition of the A-specificity (Steffen *et al.*, 1968). The titre of A-1 was always much higher than A-2, indicating that most antibodies were removed by the first passage. The titres of A-1 were not significantly different from the A-specific titres in the whole sera (Table 1).

The immunoadsorbent was not able to bind anti-collagen antibodies from antibody fractions that showed no reaction with rabbit collagen. This was demonstrated by passing 7–8 ml P-specific antibody fraction of the sera No. 118 or 206 (obtained as listed in Table 1) through the column. The effluents showed no diminished titre with calf collagen. After elution of the column with collagen peptides and tenfold concentration of the eluate in regard to the applied volume, the agglutination tests with calf and rabbit collagen were negative for serum No. 206 and yielded a titre of 1:4 for serum No. 118.

The serological behaviour of the isolated antibody fractions was in accordance with the type of specificity and resembled sera with a restricted specificity (e.g. sera that contain only A-, P- or S-specific antibodies). This was proved by inhibition tests as well as by agglutination. As shown in Fig. 1 the reaction of the P- and S-specific antibody fraction could only be inhibited by calf but not by rabbit collagen. The A-specific fraction, however, was inhibited by both preparations though rabbit collagen showed a ten-fold lower inhibiting capacity.

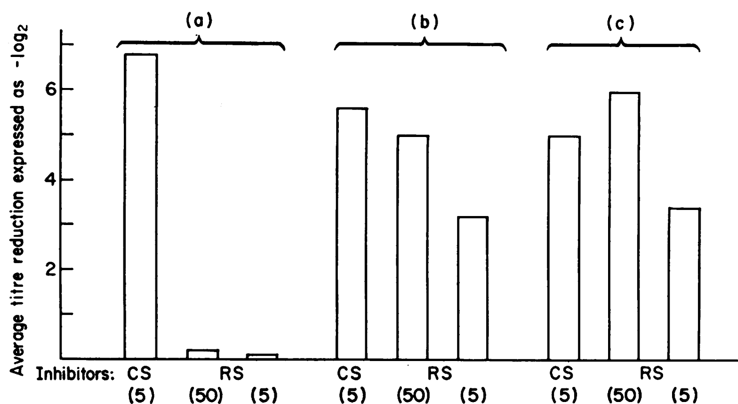

Fig. 1. Results of haemagglutination–inhibition of separated antibody fractions by denatured calf skin collagen (CS) and rabbit skin collagen (RS). Cells coated with: (a) calf collagen; (b) calf collagen; (c) rabbit collagen. Antibody fraction: (a) P and S; (b) A-1; (c) A-1. Concentrations as μg/0·05 ml are given in parentheses.

Further inhibition experiments were performed with collagen peptides. Earlier studies using antisera with restricted specificities (Steffen and Timpl, 1965; Steffen *et al.*, 1968) have shown that P- and S-specific antisera were equally well inhibited by peptides obtained after trypsin (T-peptides) or collagenase (C-peptides) digestion, whereas A-specific antibodies were much better inhibited by T-peptides. The fractions isolated in the present study behaved identically (Fig. 2).

The separated antibody fractions could, furthermore, be differentiated by their reactivity with peptides obtained after a sequential action of trypsin, chymotrypsin and pepsin (TCP-peptides). In comparison to the T-peptides, the activity was only slightly reduced for the A-specific antibody fraction, but strongly diminished for the P- or S-specific

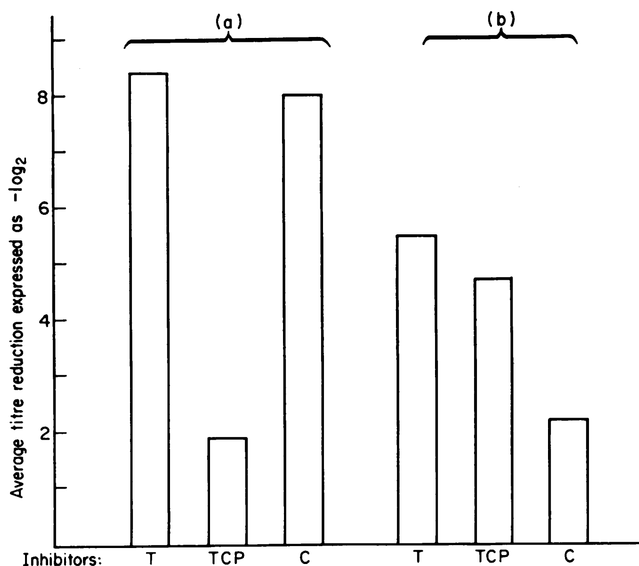

FIG. 2. Results of haemagglutination–inhibition of separated antibody fractions by collagen peptides (cells coated with calf skin collagen). Antibody fraction: (a) P and S; (b) A-1. T = peptides obtained after trypsin treatment of calf skin collagen; TCP = peptides obtained after sequential treatment with trypsin, chymotrypsin and pepsin; C = peptides obtained after collagenase treatment of calf skin collagen. Concentration of the peptide solutions : 50 μg N/0·05 ml.

TABLE 2
COMPARISON OF ANTIBODY FRACTIONS A-1 AND A-2 IN HAEMAGGLUTINATION–INHIBITION EXPERIMENTS

Antibody fraction	Inhibitors					
	Denatured collagen from:			Collagen peptides after treatment with:		
	Calf (5)	Rabbit (50)	Rabbit (5)	Trypsin (300)	Trypsin chymotrypsin pepsin (300)	Collagenase (300)
A-1	5·5	4·9	2·9	5·6	5·3	1·5
A-2	4·2	3·9	2·1	4·5	3·9	1·6

Average results from corresponding fractions A-1 and A-2 (see Table 1) given as titre-decrease ($-\log_2$). Concentrations in μg/0·05 ml are given in parentheses. Erythrocytes coated with denatured calf collagen.

antibodies (Fig. 2). The same results with TCP-peptides were observed using antisera with a defined restricted specificity (unpublished).

Inhibition results comparing the antibody fractions A-1 and A-2, both A-specific but probably differing in their capacity to bind to the immunoadsorbent, are given in Table 2. A given concentration of collagen or peptides (with the exception of the poorly inhibiting C-peptides) yielded a greater titre-reduction of fraction A-1 than of fraction A-2.

DISCUSSION

As shown in the preceding paper, the antibodies against calf collagen exhibited, owing to their specificities, the following serological characteristics. Antibodies with general collagen specificity (type A) showed the same titre with calf and rabbit collagen and were inhibited by both antigens. Collagen peptides obtained after trypsin treatment were more effective inhibitors than those obtained after collagenase treatment (Steffen and Timpl, 1965). Species specific anti-calf collagen antibodies, type S or P, do not react with rabbit collagen and were not inhibited by it. Both peptide preparations exhibited the same inhibiting activity. The serological studies have suggested that most antisera are mixtures of antibodies differing in specificity.

In this paper, we have described the separation of the antibody fractions of such sera with mixed specificities using a rabbit collagen immunoadsorbent. Antibodies able to bind to the immunoadsorbent showed the characteristic features of A-specific antibodies, whereas the antibodies that were not bound behaved as species specific antibodies. The clear-cut separations obtained confirm the suggested existence in the sera of antibody fractions with different specificities. As discussed earlier, we cannot decide if the antibody fractions obtained are entirely homogeneous in regard to their specificity. They are of a restricted specificity, well defined by the method of separation and the serological systems used.

The two types of species specific anti-calf collagen antibodies behave similarly in most serological tests. They could only be differentiated by their reactivity with pepsin-treated calf collagen. Since we very rarely obtained sera containing a mixture of P- and S-specific antibodies, we have not differentiated or attempted to separate the two types in this study. These antibodies can probably be separated on an immunoadsorbent prepared from pepsin-treated calf collagen.

To inhibit identically the reactions of A-specific antibody fractions with calf collagen-coated erythrocytes, ten times more rabbit collagen than calf collagen was needed. Similar results were obtained when the agglutination of rabbit collagen-coated erythrocytes was inhibited. An explanation for this finding may be that the determinants on rabbit collagen responsible for general collagen specificity are not identical but probably very similar in structure to those on calf collagen. Similar conclusions were drawn for cross-reacting determinants of serum albumin by Weigle (1961) and of ovalbumin by Kaminski (1962). Recently, Rangel (1965) obtained the same evidence from experiments with a cross-reacting antibody fraction of an anti-albumin serum.

The isolation procedure for anti-collagen antibodies using collagen peptides as eluant is highly specific and yields a quantitative recovery. This was shown in earlier experiments using antisera against calf collagen and an immunoadsorbent prepared from denatured calf collagen (Timpl *et al.*, 1967). The variant described in this study may be of some value for further investigations on the antigenic structure of collagen.

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