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Immunogenicity and Specificity of Collagen

XII. DEMONSTRATION BY IMMUNOFLUORESCENCE AND HAEMAGGLUTINATION OF ANTIBODIES WITH DIFFERENT SPECIFICITY TO HUMAN COLLAGEN

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Summary. A comparative investigation, using haemagglutination, haemagglutination-inhibition and immunofluorescence, was performed for demonstration of antibodies with different types of collagen specificity. In rabbit antisera to acid-soluble human collagen a fraction of species-specific antibodies to human collagen and a fraction of general, non-species-specific collagen antibodies could be demonstrated in good accordance by both techniques. Calf, rabbit, rat and guinea-pig collagen were used as heterologous control preparations.

INTRODUCTION

Investigations on specificity of collagen and its antibodies have found growing interest in recent years. In previous investigations on calf collagen we were able to demonstrate two types of collagen antibodies; (i) antibodies showing a general, non-species-specific collagen specificity and (ii) antibodies having species-specific properties (Steffen, Timpl and Wolff, 1967, 1968). The second type could be subdivided into antibodies reacting with protease-stable or protease-labile determinants on collagen. The same types of antibodies could be demonstrated in a recent investigation of human collagen (Steffen, Dichtl and Brunner, 1970). Haemagglutination and haemagglutination inhibition were used in all these studies.

Rothbard and Watson (1961) described species-specific antibodies to rat collagen, as demonstrated by immunofluorescence, Davison, Levine, Drake, Rubin and Bump (1967) observed by complement-fixation species-specific antibodies to telopeptides of human, rat, calf and carp collagen, and Rothbard and Watson (1965, 1967) published further observations on species-specificity of antibodies to human collagen, using immunofluorescence and complement-fixation.

Antibodies to human collagen are not only of experimental interest, since non-species-specific collagen-autoantibodies could be observed in a high percentage of severe cases of rheumatoid arthritis and in a lower percentage in cases with a mild and chronic course of disease (Steffen, Schuster, Tausch, Timpl and Pecker, 1968; Steffen, Carmann, Schuster, Tausch, Bösch and Freilinger, 1971). In experimentally induced antisera to human collagen species-specific and general, non-species-specific collagen antibody fractions could be demonstrated by our standard haemagglutination and haemagglutination-inhibition schedule (Steffen *et al.*, 1968, 1970).

In the light of the other investigations, describing only species-specific antibodies, it was of interest to perform a comparative investigation of the appearance of different antibodies to human collagen by haemagglutination and immunofluorescence, using antisera containing a mixture of non-species-specific and species-specific collagen antibodies.

MATERIALS AND METHODS

Antigens

Preparation of human acid soluble collagen from juvenile dura mater was performed as already described (Steffen *et al.*, 1970). The preparation had a nitrogen/hydroxyproline ratio of 1,3 and contained 13·2 per cent hydroxyproline. Acid soluble collagen from calf tendon, rabbit, rat and guinea-pig skin was prepared as previously described (Steffen and Timpl, 1962).

Antisera and serological technique

Immunization of rabbits to obtain antihuman collagen antisera and performance of haemagglutination and haemagglutination-inhibition were accomplished as earlier described (Steffen *et al.*, 1968). Preparation of collagen-immunoadsorbent and isolation of antibody fractions were performed as described by Timpl, Wolff, Furthmayr and Steffen (1968).

Immunofluorescence technique

As reagent for the sandwich-technique, a conjugate of FITC-coupled goat antirabbit γ-globulin (Hyland Laboratories) was used. The reagent gave gel precipitation with rabbit IgG, purified on DEAE-cellulose, to a titre of 1:8 and was used in a dilution of 1:10 for the further investigations. The molar F/P ratio according to The and Feltkamp (1970) was 2·7.

Specimens of spleen of man, rat and guinea-pig were used as antigenic substrate for demonstration of collagen antibodies. Human tissue obtained at autopsy and fresh animal tissues were snap-frozen. Sections were cut at 4 μ in a cryostat at $-20°$. No fixative was employed since preliminary investigations using various fixatives showed no difference from unfixed sections.

Sections were covered with antisera under investigation and control sera in various dilutions, incubated in a moist chamber at 37° for 30 minutes, drained, rinsed and washed in Coons's buffer. These sections were covered afterwards with a drop of conjugate, stained for 30 minutes in a moist chamber, were washed again and mounted in buffered glycerol for microscopical examination by a Reichert Zetopan fluorescence microscope (exciter filter Schott UG-1/1,5; transmission between 300 and 400 mμ, maximum at 350 mμ; dark field condenser and barrier filter Sp2, transmission over 440 mμ).

Absorption: Since antisera even to highly purified acid soluble collagen still show by passive haemagglutination antibodies to serum proteins of the antigen donor (Steffen *et al.*, 1970), antisera for immunofluorescence were absorbed with human serum proteins cross-linked by glutaraldehyde (Avrameas and Ternynck, 1969) and were tested subsequently by passive haemagglutination against serum protein antigens. After absorption antibodies to serum proteins could no longer be observed.

RESULTS

Three antisera to soluble human collagen were defined in their content of collagen antibody fractions by passive haemagglutination and haemagglutination inhibition (Fig. 1). Serum 93 contained a major part of the general, non-species-specific collagen antibody fraction (A-specificity) and a minor part of the species-specific fraction (S-specificity). This identification results from lower differences in haemagglutination between homologous collagen and collagens of different species, from lower differences in inhibiting

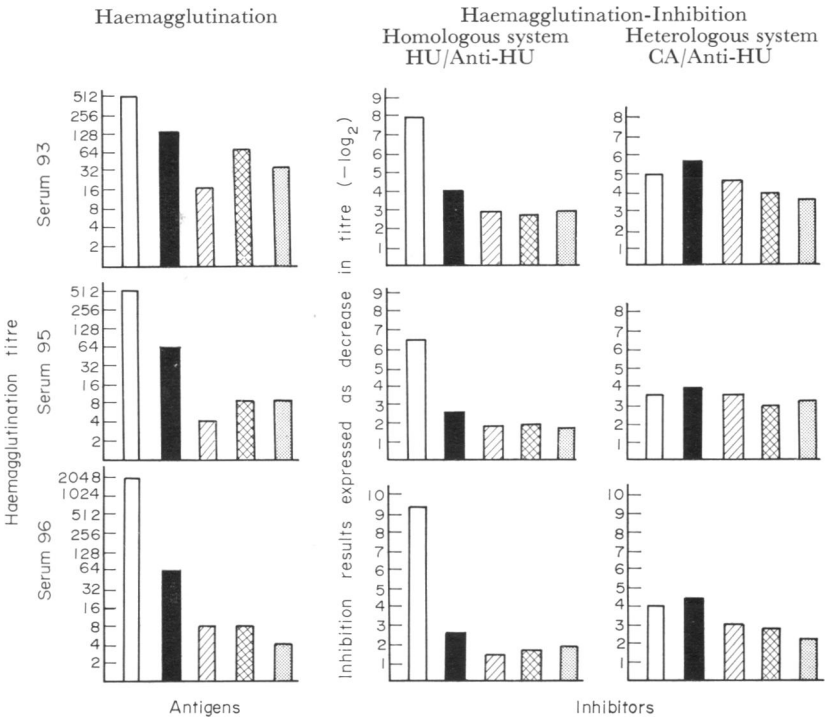

FIG. 1. Results of haemagglutination and haemagglutination-inhibition of rabbit antisera to human collagen. Average results of at least four different inhibition experiments with each antigen are recorded. Abbreviations of collagen antigens: open columns, human; closed columns, calf; hatched columns, rabbit; cross-hatched columns, rat; stippled columns, guinea-pig.

activity of collagens of different species in the homologous inhibiting system and from similar inhibiting activity of these collagens in the heterologous inhibiting system. Classification of this serum was therefore A/S.

Sera 95 and 96 contained a major part of antibodies with S-specificity and a minor part of antibodies with A-specificity. This identification results from the more pronounced differences in haemagglutination between homologous collagen and collagens of different species, from distinct differences between the inhibiting activity of homologous and heterologous collagens in the homologous system (identifying the S-specific fraction) and from a still present and similar inhibiting activity of these collagens in the heterologous system (identifying the residue of the A-specific fraction). Classification of these sera was

therefore S/A. For detailed information about this classification schedule see Steffen *et al.* (1968).

In contrast to rabbit, rat and guinea-pig collagen, calf collagen showed a haemagglutinating and inhibiting activity closer to human collagen. This indicates that the species-specific determinants of calf and human collagen possess similar structures (Steffen *et al.*, 1970).

TABLE 1

COMPARISON OF THE RESULTS OBTAINED BY IMMUNOFLUORESCENCE (IF) AND PASSIVE HAEMAGGLUTINATION (HGGL) WITH ANTISERA TO HUMAN COLLAGEN, ISOLATED ANTIBODY FRACTIONS AND NORMAL RABBIT SERA

		Antigens investigated					
		Hu coll		Rat coll		G-p coll	
Serum dilution		HGGL	IF	HGGL	IF	HGGL	IF
95	1 : 4		Pos.		Pos.		Pos.
	1 : 16		Pos.		±		±
	1 : 32		Pos.		Neg.		Neg.
	1 : 64		Pos.		Neg.		Neg.
	1 : 256		±		Neg.		Neg.
		1 : 512*		1 : 8*		1 : 8*	
96	1 : 4		Pos.		Pos.		Pos.
	1 : 8		Pos.		±		±
	1 : 16		Pos.		Neg.		Neg.
	1 : 32†		Pos.		Neg.		Neg.
		1 : 2048*		1 : 8*		1 : 4*	
S-specific	1 : 4		Pos.		Neg.		Neg.
fraction	1 : 8		Pos.		Neg.		Neg.
of 93	1 : 64†		Pos.		Neg.		Neg.
		1 : 512*					
Rabbit	1 : 2		Neg.		Neg.		Neg.
normal	1 : 4		Neg.		Neg.		Neg
serum							

* Final dilution of antiserum giving positive haemagglutination.
† Final dilution tested.

Serum 95 (S/A) and 96 (S/A) were investigated by immunofluorescence technique on spleen samples of man, rat and guinea-pig. Antiserum dilutions induced specific staining of collagen of human spleen, and the last (±) positive staining was observed with a dilution 1:256 (Table 1). Results of positive staining of collagen in human spleen sections by antiserum dilutions 1:8 and 1:64 are shown in Fig. 2, Nos 2 and 3.

Dilution 1:4 of serum 95 gave positive staining with collagen of human, as well as rat and guinea-pig tissue (Fig. 2, Nos 4–6). A change in this reactivity appeared at an antiserum dilution 1:16 (Table 1). Dilution 1:32 gave positive staining of human spleen sections only and negative reactions with rat and guinea-pig tissues (Fig. 2, Nos 7–9).

Serum 96 was investigated in a similar way. Dilution 1:4 still reacted positively with collagen of human, rat and guinea-pig spleen (Fig. 3, Nos 1–3); a final titre of A-specificity appeared at dilution 1:8, and dilution 1:16 showed only positive staining of human tissue (Fig. 3, Nos 4–6).

An immunoadsorbent technique (Timpl *et al.*, 1968) makes it possible to isolate the A-specific fraction from the S-specific fractions. Serum 93 (A/S), which contained a major

A-specific fraction was applied to a column, prepared from denatured acid-soluble rat skin collagen coupled to diazotized *p*-aminobenzyl cellulose. The A-specific fraction was bound to the immunoadsorbent and only species specific collagen antibodies passed through the column and appeared in the effluent, as shown by haemagglutination.

These effluents were pooled and mixed with an equal volume of saturated ammonium sulphate solution. The precipitate was dissolved in a small volume of saline, dialysed,

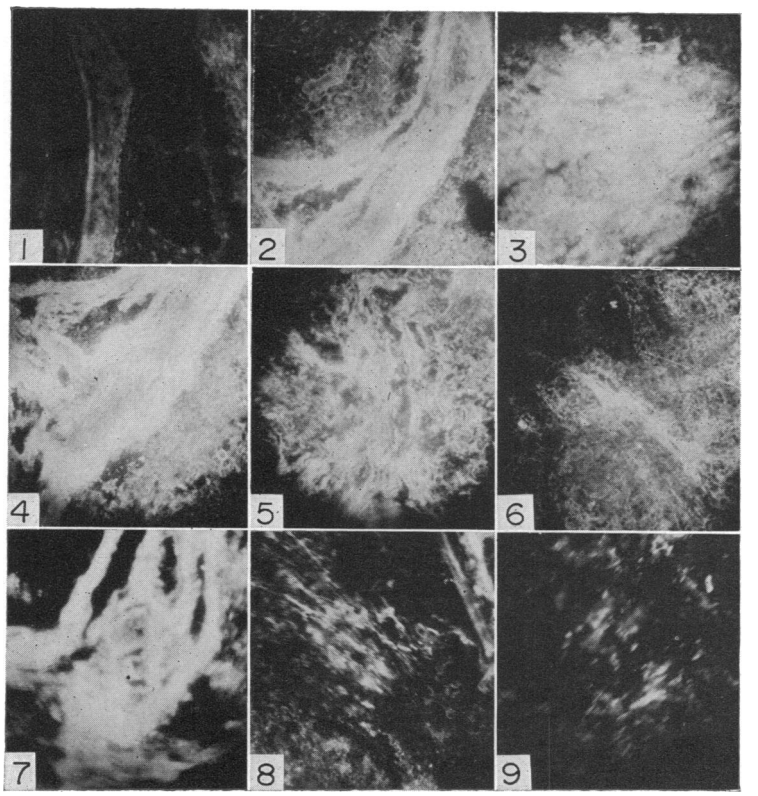

FIG. 2. Investigation on fixation of normal rabbit serum and of rabbit antiserum to human collagen on collagen of human, rat and guinea-pig spleen performed with immunofluorescence sandwich technique. No. 1, Normal rabbit serum 1 : 4, human spleen. Nos 2 and 3, Antiserum 95 dilutions 1 : 8 and 1 : 64, human spleen. No. 4, Antiserum 95 dilution 1 : 4, human spleen. No. 5, Same dilution, rat spleen. No. 6, Same dilution, guinea-pig spleen. No. 7, Antiserum 95 dilution 1 : 32, human spleen. No. 8, Same dilution, rat spleen. No. 9, Same dilution, guinea-pig spleen.

adjusted to a 1:4 dilution of the original serum and was used for investigation by immunofluorescence. While serum 93 reacted positively at this dilution in haemagglutination with human collagen as well as with rat and guinea-pig collagen, the isolated S-specific fraction showed positive staining only with collagen in human spleen sections (Fig. 3, Nos 7–9). Identical reactions appeared in passive haemagglutination.

Controls with normal rabbit serum in dilutions 1:2 and 1:4 showed negative staining (Fig. 2, No. 1), and also reacted negatively in haemagglutination with collagen antigens.

Absorption of antisera by human serum proteins cross-linked with glutaraldehyde removed contamination by antiserum protein antibodies, as demonstrated by negative haemagglutination of cells coated with serum proteins.

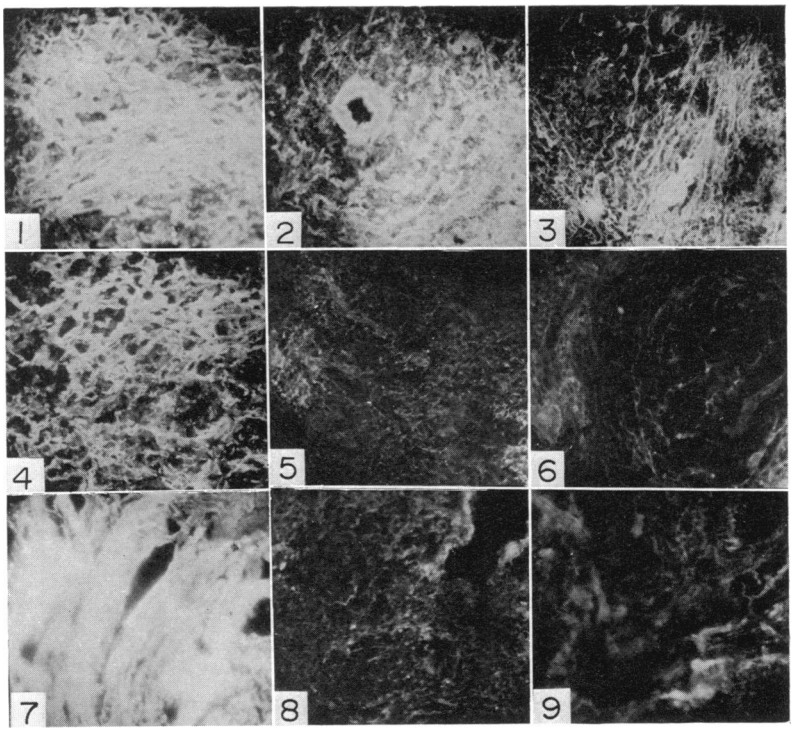

FIG. 3. Investigation on fixation of rabbit antiserum to human collagen and of isolated S-specific collagen antibody fraction on collagen of human, rat and guinea-pig spleen performed with immunofluorescence sandwich technique. No. 1, Antiserum 96 dilution 1 : 4, human spleen. No. 2, Same dilution, rat spleen. No. 3, Same dilution, guinea-pig spleen. No. 4, Antiserum 96 dilution 1 : 16, human spleen. No. 5, Same dilution, rat spleen. No. 6, Same dilution, guinea-pig spleen. No. 7, Isolated species-specific collagen antibody fraction, corresponding to 1 : 4 dilution of original serum 93, human spleen. No. 8, Same antibody preparation, rat spleen. No. 9. Same antibody preparation, guinea-pig spleen.

DISCUSSION

The existence of two types of collagen antibodies, general, non-species-specific collagen antibodies and species-specific antibodies can be regarded as proven by the results of the combination of haemagglutination-inhibition and immunoadsorbent studies (Steffen *et al.*, 1968, Timpl *et al.*, 1968). However, why investigations with immunofluorescence or complement-fixation seemed to reveal only species-specific properties appeared unanswered. Our experience with a large number of collagen antisera shows that antisera with a restricted A- or S-specificity exist, but the majority of antisera consist of mixtures of A- and S-specific fractions in variations A/S or S/A. It might have been possible,

therefore, that those antisera, investigated previously by immunofluorescence or complement-fixation could have been by chance antisera with restricted S-specificity or with a strongly predominant S-fraction.

The results of the studies, presented here, showed that collagen antibodies with A- and S-specificity can be demonstrated and differentiated by immunofluorescence as well as by haemagglutination and haemagglutination-inhibition. Two selected antisera, with the definition S/A according to haemagglutination and haemagglutination inhibition (Nos 95 and 96) were investigated by immunofluorescence, which distinguished two antibody fractions. As shown in Table 1 and Figs 1 and 2 titration of serum 95 with human collagen antigens gave a haemagglutination titre of 1:512 and an immunofluorescence titre of 1:256. Titration of the same serum with rat or guinea-pig collagen gave positive results on haemagglutination up to 1:8 and on immunofluorescence up to 1:16. Heterologous collagen showing positive staining below this titre should, therefore, have reacted with the A-specific fraction, whilst homologous collagen showing positive staining with antiserum dilutions higher than 1:16 would have reacted with the S-specific fraction only. The same can be said for antiserum 96. Dilutions lower than 1:4–1:8 contain reactive A- and S-specific fractions, while in dilutions above this limit only S-specific antibodies are apparently active.

Further evidence for differentiation between these two fractions by haemagglutination as well as by immunofluorescence can be deduced from the investigation of antiserum 93 (A/S) and its isolated S-specific fraction. Whilst this serum reacted positively in haemagglutination with rat and guinea-pig collagen up to 1:64 or 1:32, its isolated S-specific fraction, corresponding to a dilution of 1:4 of the original serum, only reacted positively with human collagen by haemagglutination or immunofluorescence.

Non-specific staining was excluded by control tests with normal rabbit serum. Reactions with antibodies to serum proteins were excluded by absorption of antisera with these proteins.

Species specificity of a limited number of rabbit antisera, as observed by Davison *et al.* (1967) could be easily explained by the fact, that complement fixation with heterologous collagens was performed with dilutions which only gave positive complement fixation with the homologous antigen. If these sera were mixtures of S/A, like our antisera 95 and 96, it seems possible that the dilutions still reacting positively with their S-specific fraction and the homologous antigen, were already too highly diluted to show positive reactions of their A-specific fraction with a heterologous antigen. Removal of specific reactivity of these antisera by enzymatic treatment of its collagen antigen, i.e. destruction of the telopeptides, underlines this assumption. Investigations on the susceptibility of collagen determinants to proteolytic treatment showed that terminal sites of the collagen molecule are highly sensitive to proteolytic treatment and react only with one of the two S-specific fractions of collagen antisera (Steffen and Timpl, 1970).

Rothbard and Watson (1965) performed inhibition experiments with complement-fixation and immunofluorescence (*in vivo* fixation of collagen antibodies in renal glomeruli of different species) with antisera to rat, guinea-pig, human and chicken collagen. The antisera were cross-absorbed with these collagens and retested always in a homologous and heterologous system. Absorption with the homologous collagen led always to negative results. Absorption with heterologous collagens was complete only when retested with heterologous collagens. When retested, however, with the homologous collagen, absorption was incomplete and only moderate reduction of titre appeared.

In the light of our findings about A- and S-specific determinants on collagen and A- and S-specific antibody fractions in collagen antisera, these absorption results speak for the existence of both fractions in the investigated antisera. Absorption with homologous collagen removed by its A- and S-specific groupings both antibody fractions and results of renewed testing was therefore negative. Absorption with heterologous collagens removed only the A-specific fraction. When retested with the homologous collagen, the residual S-specific fraction still reacted and only moderate titre reduction appeared. However, when retested with heterologous collagens the tests were negative, since the A-specific fraction was absorbed.

An exception appeared only on cross absorptions of antirat and antimouse collagen antisera by rat and mouse collagen. Here each collagen preparation absorbed all antibodies. This implies close similarity of S-specific groupings of rat and mouse collagen. Both preparations probably absorbed S- and A-specific fractions together.

Additional investigations of various anticollagen sera with collagens of different species by complement-fixation and immunofluorescence showed for antirat collagen sera positive reactions with rat, mouse and guinea-pig, for antimouse collagen antisera positive reactions with rat, mouse and guinea-pig, and for antihuman collagen sera positive reactions with rat, man and chicken. We would explain all these observations not as cross-reactivity of species specific anticollagen sera, but as evidence for the appearance of two antibody fractions, A- and S-specific, in sera which were under investigation. Only in the determination of A-specific reactivity of carp collagen does our experience (Wolff, Wick, Furthmayr, Timpl and Steffen, 1970) differ from that of Rothbard and Watson (1965). For this difference as well as for the less-pronounced demonstration of A-specificity by complement fixation, technical differences of complement fixation and haemagglutination seem to be responsible. As far as haemagglutination and immunofluorescence are concerned our present results show that S-specific human-collagen antibodies as well as A-specific collagen antibodies can be determined by both techniques.

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