EVIDENCE FOR TWO ANTIGENIC DETERMINANTS IN THE *C*-TERMINAL REGION OF RAT SKIN COLLAGEN

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1. Introduction

The ability of the antigenic determinants of collagen to react with antibodies does not depend on the intact triplehelical structure. This property qualifies collagen for a comprehensive immunochemical investigation. Recent success in the chemical characterization of peptides obtained by cleavage with cyanogen bromide [1, 2] or with collagenase [3] has favoured such studies. Thus, Pontz et al. [4] were able to localize two different antigenic determinants on the *C*-terminal and on a middle region, respectively, of the calf skin collagen molecule. Michaeli et al. [5] assigned an antigenic determinant to the *N*-terminal region of guinea-pig collagen. In these studies, rabbit antibodies have been employed and it may be predicted that both calf skin and guinea-pig collagen should differ in their sequence from rabbit collagen at least at the antibody-recognition sites. A consistent application of immunological methods should highly facilitate the localization and elucidation of structural variations among different collagens and might also lead to a better understanding of the phenomenon of immunological discrimination. For these reasons some of the antigenic determinants of rat skin collagen were characterized by the same methods as already applied to calf collagen [4].

2. Experimental part

Neutral salt-soluble collagen was isolated and purified from the skin of lathyritic rats as described earlier [6]. Some of this material was treated with pronase (0.1 M $CaCl_2$, pH 7.5, enzyme substrate ratio 1:10 (w/w), 20 hr, 18°) [7]. The α1 and α2-chains were separated by chromatography on CM-cellulose [6, 8]. The α-chains were cleaved with cyanogen bromide and the resulting peptides (CB-peptides) purified by ion-exchange chromatography and gel filtration [1, 2]. A peptide 1500 Å in length, α2(1500), covering the middle region of the α2-chain was obtained by treating the native molecule with collagenase [3]. The designation of the peptides used in this study and their localization within the α-chains are given in fig. 1.

Nine rabbits were immunized with native rat skin collagen incorporated into complete Freund's adjuvant following a previously applied schedule [9]. Prior to further studies, the antisera were exhaustively absorbed on a rabbit collagen immunoadsorbent [10]. The absorbed antisera were then characterized by passive hemagglutination [11] using tanned human red cells which were either coated with rat collagen, with the single α-chains or with the different CB-peptides. In the hemagglutination-inhibition tests, an equal amount of inhibitor was added to different dilutions of the antisera prior to the addition of the coated cells and the reduction of the original titer was recorded.

3. Results and discussion

Rat skin collagen provoked antibodies in the rabbit which could easily be detected by passive hemagglutination. Beside a strong reaction with a rat collagen, all sera exhibited a lower but considerable ag-

glutinating activity for rabbit collagen. This suggests the occurrence of antibodies which react with collagen independently of its species origin as previously described for rabbit antisera to calf collagen [11]. In order to obtain antibody solutions which detect structural features strictly specific to rat collagen, it was necessary to remove these kinds of antibodies on a rabbit collagen immuno-adsorbent. This treatment did not impair the agglutinating activity for rat skin collagen.

The localization of the antigenic determinants specific for rat skin collagen was feasible by two different experiments:

1) tanned red cells were coated with the different CB-peptides and the ability of absorbed antisera to agglutinate such cells was assessed.

2) in inhibition tests, the CB-peptides were mixed with the antisera prior to the addition of coated red cells and the reduction of the agglutination titer was recorded.

The absorbed antisera revealed a similar agglutinating activity for the two *C*-terminal peptides α1-CB6 and α2-CB5 which was comparable to the activity of both α-chains or the whole molecule (table 1). Practically no reaction was found with the large peptides from the middle region of the molecule. In these experiments, the small peptides α1-CB1, α1-CB2, α1-CB4, α1-CB5, α2-CB1 and α2-CB2 could not be included due to a less appropriate coating behaviour for tanned red cells.

In more informative inhibition studies, evidence was provided that the agglutination pattern observed is the result of a simultaneous occurrence of two antibody populations which differ in their specificity. The agglutination of α1-CB6-coated red cells (fig. 2A) could be effectively inhibited by the α1-chain and α1-CB6. A significant, although lower activity, was found for the α2-chain and α2-CB5. All the other cyanogen bromide peptides were inactive. The agglutination reaction with α2-CB5-coated red cells (fig. 2) [13] is only inhibited by the α2-chain and α2-CB5. The assumption of a second antibody fraction which reacts only with α2-CB5 but not with α1-CB6 would be the most likely explanation. This implicates at least two antigenic determinants in the *C*-terminal region of rat skin collagen. One of these is present on both polypeptide chains although the differences in the inhibiting capacity may suggest no complete correspondence in structure. The other one is confined to the α2-chain. This interpretation is supported by absorption of the antisera with α1-chains insolubilized by cross-linking with glutaraldehyde [12]. Such treatment completely removed the antibodies reacting with α1-CB6, but had no serious effect on the agglutinating activity for α2-CB5.

The peptides α1-CB6 and α2-CB5 consist of about 200 and 320 amino acid residues, respectively [1, 2]. The antigenic determinants, however, must be located at the COOH-terminal end, since pronase treatment of the native collagen molecule under conditions maintaining the triplehelical structure, produced serologically inactive α-chains (fig. 2). As estimated by

Table 1

Agglutinating activity of rat skin collagen antibodies for tanned red cells coated either with rat skin collagen, with the corresponding α-chains or with cyanogen bromide peptides*.

Antiserum no.	Collagen	Reciprocal titer for red cells coated with:					
		α1-chain	α2-chain	α1-CB6	α2-CB5	α2-CB4	α1-CB3, α1-CB7 α1-CB8 or α2-CB3
286	64	64	32	64	256	< 2	<2
288	256	512	128	512	512	< 8	<2
289	128	256	64	256	256	< 2	<2
291	512	512	256	512	256	< 8	<8
292	512	1024	256	512	128	< 8	<8
293	512	1024	256	2048	2048	16	<8

* The antisera were titrated after an exhaustive absorption on a rabbit collagen immunoadsorbent.

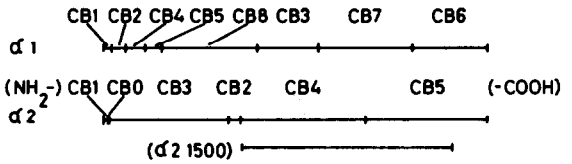

Fig. 1. Order of the α1- and α2-chain cyanogen bromide peptides of rat skin collagen. Taken for α1 from [14, 15] and for α2 from [16].

electron microscopy [13] this enzyme should not remove more than about 15 amino acids from the *C*-terminal end. In accordance, the peptide α2(1500) which covers two thirds of the *N*-terminal part of α2-CB5 (see fig. 1) was found to be serologically inactive. An antigenic determinant has also been located in the *C*-terminal region of calf skin collagen [4], but the corresponding antibodies did not react with rat skin collagen [11]. The sequences concerned must, therefore, differ from each other as well as from sequences located in the same position on rabbit collagen.

The immune response of rabbits to rat skin collagen is more complex than depicted in this study. Some of the antisera contain additional antibodies directed to *N*-terminal sequences. Furthermore, the antibodies which can be removed on a rabbit collagen immunoadsorbent virtually react with all of the cyanogen bromide peptides and should, therefore, be considered as mainly directed to typical amino acid sequences of the helical part of the collagen molecule.

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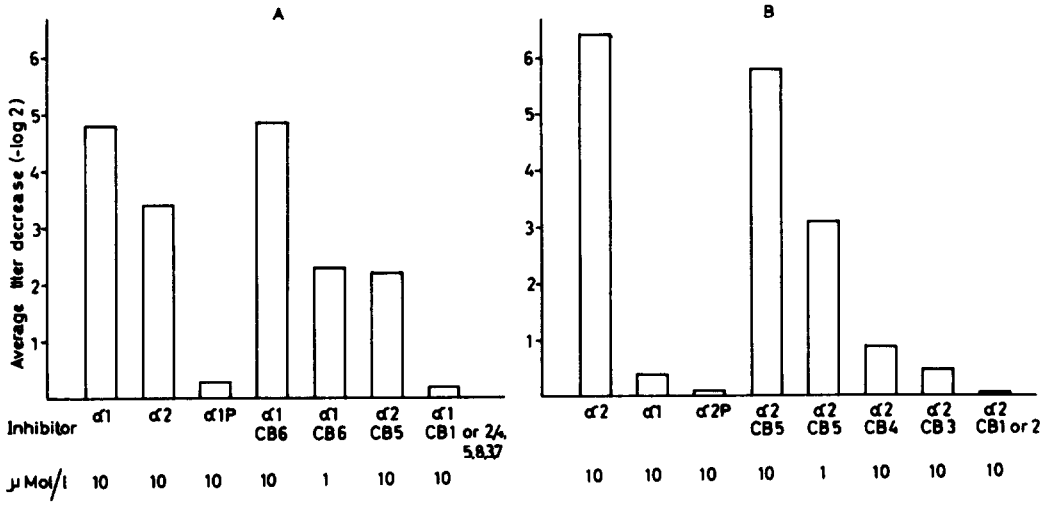

Fig. 2. Inhibiting capacity of α-chains (α1, α2), α-chains from pronase-treated collagen (α1P, α2P) and of cyanogen bromide peptides (α1-CB1–α1-CB8, α2-CB1–α2-CB5) for rat skin collagen antibodies.
A) Titration with α1-CB6-coated red cells;
B) titration with α2-CB5-coated red cells.

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