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Demonstration of a Unique Antigenic Specificity for the Collagen α1(II) Chain from Cartilaginous Tissue

E. HAHN,* R. TIMPL AND E. J. MILLER

Max Planck Institut für Biochemie, Martinsried bei München, Germany, and Institute for Dental Research, University of Alabama Medical Center, Birmingham, Alabama 35294, *U.S.A.*

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Summary. The rabbit antibody response to native collagen (chain composition $[\alpha 1(II)]_3$) from cartilaginous tissue, has been examined by agglutination assays, gel diffusion, haemagglutination–inhibition studies, and immunoadsorption. The results show that the rabbit antibody response to the cartilage-type collagen is characterized by considerable reactivity to both helical $[\alpha 1(II)]_3$ as well as α1(II) chains. This is in contrast to the rat antibody response to the same antigens where titres are generated to largely helical antigenic determinants. Similarly to the rat response, rabbit antibodies to $[\alpha 1(II)]_3$ exhibit no strong cross-reaction with the genetically distinct $[\alpha 1(I)]_2\alpha 2$ collagen or its component chains. Strong cross-reactions were, however, observed between bovine and chick α1(II) chains. One of the major antigenic sites on $[\alpha 1(II)]_3$ collagen appears to reside in the sequence represented by CB-11, a peptide derived from the helical portion of the $[\alpha 1(II)]_3$ molecule after cyanogen bromide cleavage. The data, however, are compatible with the presence of other antigenic determinants which are probably located in the amino- and carboxy-terminal portions of the molecule.

INTRODUCTION

Although the collagen molecule is a relatively poor immunogen, the availability of increasingly more sensitive methods for detecting specific antibodies has considerably facilitated studies on the antigenicity of this important structural macromolecule. When pursued in conjunction with more recent information on the molecular structure of collagen, these studies have been successful in elucidating the locations of the major antigenic determinants of the collagen molecule. In general, the results show that when rabbits are immunized with $[\alpha 1(I)]_2\alpha 2$ collagen molecules derived from the dermis of several species, the response is characterized by antibodies to determinants at the extremities of the molecule, i.e. to sites at the amino- and carboxy-termini of the component α1(I) and α2 chains (Michaeli, Martin, Kettman, Benjamini, Leung and Blatt, 1969; Timpl, Fietzek, Furthmayr, Meigel and Kühn, 1970; Pontz, Meigel, Rauterberg and Kühn, 1970; Lindsley, Mannik and Bornstein, 1971). On the other hand, the rat response to immunization with $[\alpha 1(I)]_2\alpha 2$ molecules is characterized by antibodies to more centrally

* Present address: Medizinische Universitätsklinik, Marburg, West Germany.

Correspondence: Dr Rupert Timpl, Max Planck Institut für Biochemie, 8033 Martinsried bei München, West Germany.

located and clearly helical portions of the molecule which do not cross-react with α chains (Hahn and Timpl, 1973). Moreover, we have recently demonstrated that $[\alpha 1(II)]_3$ collagen molecules derived from cartilaginous tissues elicit a specific antibody response in the rat and that there is very little, if any, cross-reactivity between antibodies produced to $[\alpha 1(I)]_2\alpha 2$ and $[\alpha 1(II)]_3$ molecules (Hahn, Timpl and Miller, 1974).

In view of these findings, the present studies were designed to examine the characteristics of the rabbit response to $[\alpha 1(II)]_3$ molecules.

MATERIALS AND METHODS

Collagens

Native collagen molecules with the chain composition, $[\alpha 1(II)]_3$ and α1(II) chains were obtained by neutral-salt extraction of lathyritic chick sternal cartilages (Miller, 1971a) or by limited pepsin digestion of insoluble chick sternal cartilage collagen and bovine nasal cartilage collagen (Miller, 1972).* Native molecules with the chain composition, $[\alpha 1(I)]_2\alpha 2$, were obtained following dilute acetic acid extraction of lathyritic chick bones (Miller, Martin, Piez and Powers, 1967) or calf skin (Pontz *et al.*, 1970). Chick α1(I) and α2 chains were obtained by extraction with 6 M guanidine hydrochloride from lathyritic chick bones and subsequently purified by carboxymethyl cellulose chromatography (Stolz, Timpl and Kühn, 1972). Two of the larger cyanogen bromide peptide fragments (CB-10 and CB-11) were prepared and purified from both chick and bovine αI(II) chains as previously described (Miller, 1971b; Miller and Lunde, 1973). These peptides contain 316 and 272 amino acid residues, respectively, and thus account for approximately 60 per cent of the entire α1(II) chain.

All materials were stored as lyophilized protein and prior to use were dissolved in 0·05 per cent acetic acid. Antigens which had been isolated in native, triple-helical conformation were used in this state for immunization. In several assays, random coil α chains were used and were prepared by denaturation of the native collagens by warming to 50° for 30 minutes in 0·05 per cent acetic acid.

Antisera

Adult rabbits (2·5–3 kg) were immunized with chick $[\alpha 1(II)]_3$ solubilized with pepsin (five animals) and bovine ($[\alpha 1(II)]_3$ solubilized with pepsin (five animals). The antigens were dissolved in 0·05 per cent acetic acid. The first injections (5 mg of antigen) were administered subcutaneously after mixing the acid solutions with an equal volume of Freund's complete adjuvant. An intraperitoneal booster (10 mg of antigen) was administered without adjuvant 28 days later. Blood was collected by cardiac puncture on day 20 (primary response) and day 59 (secondary response) after the first administration of antigen.

Serological methods

For passive haemagglutination, tanned red cells were coated with random coil α chains and cyanogen bromide peptides (Timpl, Beil, Furthmayr, Meigel and Pontz, 1971) or with native collagens (Beil, Timpl and Furthmayr, 1973). Titrations were

* In describing the results, α1(II) chains derived from neutral salt-extracted cartilage collagen will be referred to simply as α1(II). The α1(II) chains derived from pepsin-solubilized cartilage collagen are designated as α1(II)p. Unless otherwise indicated, $[\alpha 1(II)]_3$ refers to native, helical, cartilage-type collagen derived from either chick or bovine tissues by limited cleavage and solubilization with pepsin.

performed on glass plates at room temperature or in a microtitre system (Cooke Engineering Incorporated, Alexandria, Virginia). For the haemagglutination inhibition assay, the antigens were dialysed at 4° versus phosphate-buffered saline, pH 7·2. Constant amounts of inhibitor solution were added to serial dilutions of antibodies prior to the addition of coated red cells. The titre reduction was recorded relative to control antibody solutions to which buffer alone had been added.

Immunodiffusion was performed in 0·6 per cent agarose gels prepared in phosphate buffer, pH 7·3, ionic strength, 0·15. Immunoelectrophoresis was performed as described by Scheidegger (1955).

Immunoadsorption

Collagen α chains were conjugated to diazotized *p*-aminobenzylcellulose, and antibodies bound to these adsorbents were displaced by elution with 1·0 M acetic acid (Beil *et al.*, 1973). Antibodies in the eluant were quantified by optical density measurements assuming an extinction coefficient for a 1·0 per cent solution of rabbit IgG at pH 7 and 280 nm of 15·00. In order to estimate antibody concentration in the original antisera, the optical density measurements were corrected for nonspecific protein adsorption as measured after passing an equal volume of normal rabbit serum through the adsorbent.

RESULTS

THE REACTION OF ANTIBODIES AGAINST $[\alpha 1(II)]_3$ COLLAGEN WITH COLLAGEN α CHAINS

The haemagglutination titres for antisera to pepsin-solubilized $[\alpha 1(II)]_3$ collagen versus α1(II) and α1(II)p chains as well as a mixture of α1(I) and α2 chains are listed in Table 1. These data were obtained with secondary response sera. Primary response sera

TABLE 1

AGGLUTINATION TITRE OF ANTISERA TO PEPSIN-SOLUBILIZED $[\alpha 1(II)]_3$ MOLECULES FOR VARIOUS COLLAGENS BEFORE AND AFTER ADSORPTION WITH α1(I) AND α2 CHAINS. RESULTS ARE EXPRESSED AS AVERAGE TITRES $\log_2) \pm$ s.d.

	Before adsorption					After adsorption	
Antisera to:*	Chick α1(II)	Chick α1(II)p	Bovine α1(II)p	Chick α1(I)+α2	Bovine α1(I)+α2	α1(II)p†	α1(I)+α2
Chick $[\alpha 1(II)]_3$	6·5±2·1	7·5±1·2	5·7±0·5	2·7±0·5	1·9±0·4	7·5±1·5	<1·0
Bovine $[\alpha 1(II)]_3$	n.t.‡	8·1±1·9	9·3±1·6	2·7±0·5	2·5±0·9	7·5±0·6	<1·0

* Five antisera in each experiment.
† Red cells coated with collagen corresponding to the species origin of the collagen used for immunization.
‡ n.t. = Not tested.

gave comparable titres (not shown) indicating an early onset of the antibody response. All subsequent studies were performed with secondary response sera.

The data show that antisera to pepsin-solubilized $[\alpha 1(II)]_3$ molecules possess relatively low titres for α1(I) and α2 chains and that the cross-reacting antibodies could be effectively eliminated from the antisera by immunoadsorption without appreciably decreasing the titres versus α1(II)p chains. Moreover, the antibody response does not appear to be species-specific, since comparably high titres were observed for antisera to both chick and bovine collagens regardless of the origin of the α1(II)p chains coated on red cells in the agglutination assay.

These results were corroborated in gel diffusion studies (Fig. 1) in which a single precipitin line could be observed for antisera to both chicks and cattle $[\alpha 1(II)]_3$ when tested with $\alpha 1(II)p$ chains from either species. In contrast to the agglutination assay, however, the gel diffusion test did not demonstrate a reaction of antibodies with chick $\alpha 1(II)$ chains.

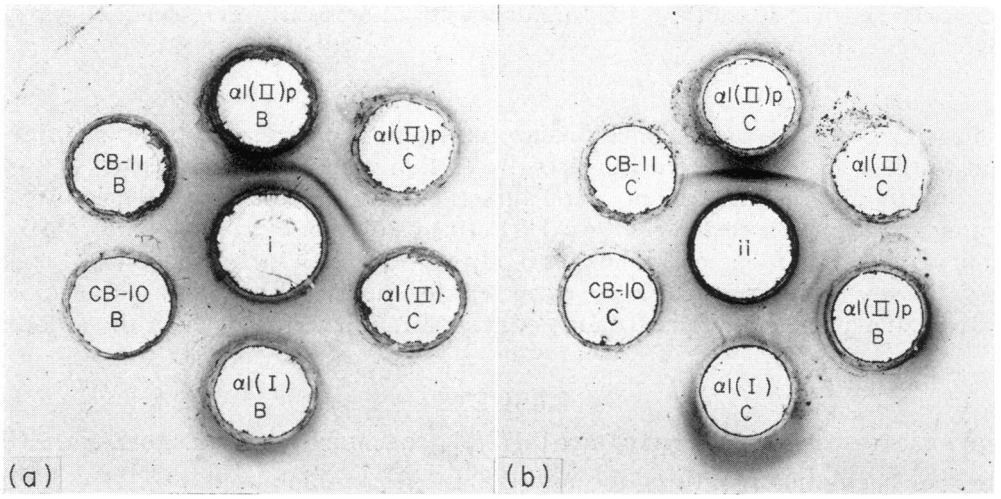

FIG. 1. Immunodiffusion reaction of (a) antiserum number 597 against bovine $[\alpha 1(II)]_3$ collagen (i) and (b) antiserum number 606 against chick $[\alpha 1(II)]_3$ collagen (ii) with α chains and cyanogen bromide peptides. The antigens were either of bovine (B) or chick (C) origin and used at a concentration of 10 μmol/l. The pattern was photographed after staining with Amido Black.

The specificity of the immune response to $[\alpha 1(II)]_3$ collagen was further investigated by haemagglutination–inhibition studies. The results with antisera to chick $[\alpha 1(II)]_3$ showed that appreciable inhibitory activity was present only for the chick antigen and the lowest degree of inhibitory activity was observed for chick $\alpha 1(I)$ and $\alpha 2$ chains (Fig. 2a). Pretreatment of $[\alpha 1(I)]_2\alpha 2$ molecules with pepsin to simulate the conditions under which $[\alpha 1(II)]_3$ molecules were obtained failed to enhance the inhibitory activity of $\alpha 1(I)$ and $\alpha 2$ chains. Similar results were observed for antisera to bovine $[\alpha 1(II)]_3$ (Fig. 2b), although in this instance chick $\alpha 1(II)p$ chains proved to be an effective inhibitor. In agreement with the gel diffusion studies cited above, chick $\alpha 1(II)$ chains proved to be rather ineffective inhibitors with the antisera to chick $[\alpha 1(II)]_3$.

QUANTIFICATION OF THE ANTIBODY RESPONSE TO $[\alpha 1(II)]_3$ COLLAGEN

Quantitative immunoadsorption of a pool of antisera to bovine $[\alpha 1(II)]_3$ collagen on bovine $\alpha 1(II)p$ chains attached to diazotized *p*-aminobenzylcellulose revealed an antibody concentration of 290 μg/ml in the original antiserum. It was further noted that after displacement, approximately 85 per cent of these purified antibodies could be again bound by a second passage over the same adsorbent. Characterization of these antibodies by immunoelectrophoresis indicated their identity as IgG.

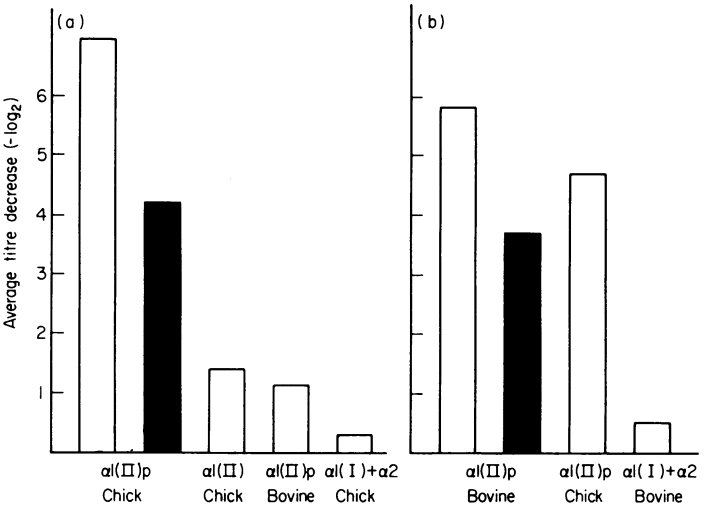

FIG. 2. Haemagglutination–inhibition of (a) antisera to chick $[\alpha 1(II)]_3$ collagen and (b) antisera to bovine $[\alpha 1(II)]_3$ collagen by collagen α chains. Red cells were coated with α1(II)p chains of chick or bovine origin. Inhibitors were used at a concentration of 10 μmol/l (□) or 1 μmol/l (■). Average results from five antisera in each group.

REACTION OF ANTISERA WITH CYANOGEN BROMIDE PEPTIDES FROM THE α1(II) CHAIN

In general, agglutination titres versus CB-10, a large fragment from the helical portion of the α1(II) chain (Miller, Woodall and Vail, 1973) were low (Table 2). On the other hand, agglutination titres versus CB-11, another large fragment likewise derived from the helical portion of α1(II) (Miller *et al.*, 1973) were rather high in about half the antisera. No precipitin line for either of the peptides was observed in the gel diffusion studies (Fig. 1).

TABLE 2

AGGLUTINATION TITRE OF ANTISERA TO $[\alpha 1(II)]_3$ COLLAGEN WITH CNBr PEPTIDES*

Antiserum to:	Number	Reciprocal titre for red cells coated with:†		
		α1(II)p	CB-10	CB-11
$[\alpha 1(II)]_3$ chick	602	128	8	8
	603	512	4	256
	604	256	<4	256
	605	256	<4	4
	606	256	16	8
$[\alpha 1(II)]_3$ bovine	597	256	16	64
	598	128	16	128
	599	256	4	256
	600	512	8	256
	601	256	<4	<4

* Each antiserum was preabsorbed on α1(I) and α2 chains.
† Antigens from the chick or bovine species corresponding to the antiserum used.

The results of the agglutination assay were confirmed and extended in haemagglutination–inhibition studies. For this purpose, antisera numbers 598, 599 and 600 to bovine $[\alpha 1(II)]_3$ were employed with red cells coated with bovine $\alpha 1(II)p$ (Fig. 3a) and CB-11

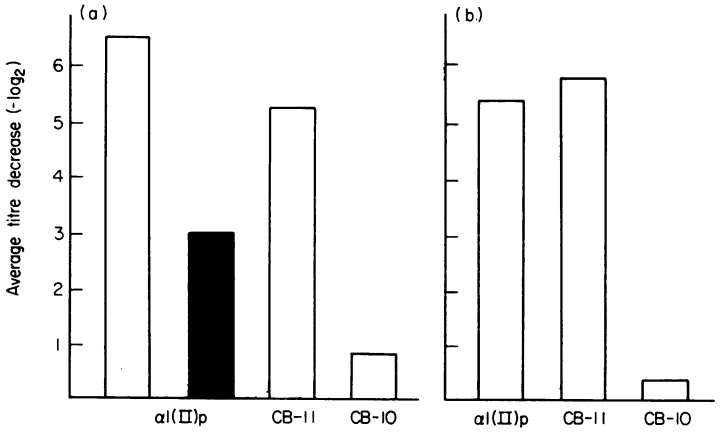

FIG. 3. Average results of haemagglutination studies on antisera numbers 598, 599 and 600 against bovine $[\alpha 1(II)]_3$ collagen by $\alpha 1(II)$ chains and cyanogen bromide peptides. Red cells were coated with either (a) bovine $\alpha 1(II)p$ chains or (b) bovine CB-11. Inhibitors were of bovine origin and were used at a concentration of 10 μmol/l (□) or 1 μmol/l (■).

(Fig. 3b). The results show that CB-11 was an effective inhibitor of the reaction in both systems. The peptide, CB-10, did not exhibit significant inhibitory activity, indicating the unique nature of the antigenic determinants localized in CB-11.

Similar results were observed in an identical series of experiments employing antibodies to chick $[\alpha 1(II)]_3$ and the same antigens but of chick origin. In the latter study, however, it was observed that CB-11 was not an effective inhibitor of the reaction with red cells coated with $\alpha 1(II)p$.

DEMONSTRATION OF ANTIBODIES AGAINST HELICAL ANTIGENIC DETERMINANTS

For these studies, antisera to bovine $[\alpha 1(II)]_3$ collagen were first adsorbed on bovine $\alpha 1(I)$ and $\alpha 2$ chains. Antibodies that were not bound during the adsorption procedure

TABLE 3

DISTRIBUTION OF ANTIBODIES IN ANTISERA TO HELICAL BOVINE $[\alpha 1(II)]_3$ COLLAGEN AFTER IMMUNOADSORPTION ON BOVINE $\alpha 1(II)p$ CHAINS. RESULTS ARE EXPRESSED AS AVERAGE TITRES $(-\log_2) \pm$ S.D. OF FIVE ANTISERA

	Red cells coated with:*		
	$[\alpha 1(II)]_3$	$\alpha 1(II)p$	$[\alpha 1(I)]_2\alpha 2$
Whole antiserum†	$9{\cdot}6 \pm 2{\cdot}6$	$7{\cdot}5 \pm 0{\cdot}6$	$<2{\cdot}0$
Antibodies bound to and eluted from the adsorbent	$9{\cdot}0 \pm 3{\cdot}0$	$8{\cdot}4 \pm 2{\cdot}2$	n.t.‡
Antibodies not bound to the adsorbent	$6{\cdot}6 \pm 3{\cdot}8$	$<2{\cdot}0$	n.t.‡

* All antigens of bovine origin.
† After adsorption on $\alpha 1(I) + \alpha 2$ chains.
‡ n.t. = Not tested.

were subsequently tested and found to exhibit relatively high titres versus native, helical $[\alpha 1(II)]_3$ as well as α1(II)p chains and essentially no titres versus native, helical $[\alpha 1(I)]_2\alpha 2$ (Table 3). Subsequently, these antibodies were adsorbed on to α1(II)p chains. On elution from the adsorbent, it was observed that the bound antibodies exhibited comparably high titres for both helical collagen and denatured chains (Table 3). In four of the antisera, a considerable titre for helical $[\alpha 1(II)]_3$, but not for α1(II)p chains, was observed for antibodies not bound to the adsorbent (Table 3). Evaluation of the specificity of the non-bound antibody fraction by inhibition assay revealed distinct activity for $[\alpha 1(II)]_3$ molecules but not for α1(II)p chains (data not shown).

DISCUSSION

The rabbit antibody response to helical or native $[\alpha 1(II)]_3$ collagen shows considerable reactivity for denatured collagen or α1(II) chains. These results are in contrast to the rat antibody response with these antigens where titres are generated largely to helical antigenic determinants (Hahn *et al.*, 1974). In the present studies, antibodies to helical antigenic sites could also be detected, but occurred at lower titres and with less regularity than in the rat response. Similar to the rat response, however, rabbit antibodies to $[\alpha 1(II)]_3$ exhibited no strong cross-reaction with the genetically distinct $[\alpha 1(I)]_2\alpha 2$ collagen or its component chains.

Quantitative immunoadsorption studies revealed antibody levels comparable to that found in rabbits after immunization with bovine $[\alpha 1(I)]_2\alpha 2$ collagen (Timpl *et al.*, 1971; Timpl, Furthmayr, Steffen and Doleschel, 1967), indicating that the cartilage-type collagen is not a better immunogen.

We have further determined that at least one important antigenic site in the $[\alpha 1(II)]_3$ molecule and α1(II)p chains is located in the sequence represented by CB-11. The homologous region on α1(I) chains is α1(I)-CB-8 (Miller *et al.*, 1973), and the latter sequence is not an important site with respect to the rabbit response to $[\alpha 1(I)]_2\alpha 2$ collagen (Timpl *et al.*, 1971). The data indicate, however, the presence of additional antigenic sites in α1(II)p chains. This may be surmised from the nonreactivity of CB-11 in the gel diffusion assay, as well as the apparent inability of CB-11 to inhibit antisera to $[\alpha 1(II)]_3$ collagen in the chick system.

Several considerations indicate that these additional antigenic sites may be located in the terminal portions of the α1(II)p chain. First, antibodies reacting with α1(II)p chains showed a comparable high reactivity for helical, native $[\alpha 1(II)]_3$ collagen (Table 3). This observation closely resembles the findings for terminal antigenic sites on α1(I) and α2 chains (Beil *et al.*, 1973). Secondly, the limited reactivity between antibodies to pepsin-solubilized $[\alpha 1(II)]_3$ and neutral salt-extracted α1(II) chains can only be explained by structural differences. It is known that limited cleavage and solubilization of $[\alpha 1(II)]_3$ collagen with pepsin results in the loss of fifteen to twenty amino acids at the extremities of the molecule (Miller, 1972). Indeed, the pepsin treatment in itself may have created artifactual antigenic sites resembling those produced on $[\alpha 1(I)_2]\alpha 2$ molecules when short, nonhelical regions at the extremities of the molecule are lost during extraction and purification (Stoltz, Timpl, Furthmayr and Kühn, 1973; Becker, Timpl and Kühn, 1972).

The immunological evidence for distinct differences in amino acid sequence of the α1(I) and α1(II) chain is in agreement with recent structural studies on a portion of the helical region of the α1(II) chain (Butler, Miller, Finch and Inagami, 1974). Thus,

rabbit antibodies may be a powerful tool to distinguish genetically distinct types of collagen α chains and to localize intracellular steps in the biosynthesis of cartilage-type collagen. To date, information on the amino acid sequence of antigenic determinants is only available for sites located in the terminal, non-helical portions of $[\alpha 1(I)]_2\alpha 2$ collagen (Becker *et al.*, 1972; Furthmayr and Timpl, 1972; Rauterberg, Timpl and Furthmayr, 1972; Stoltz *et al.*, 1973). The observation of a strong antibody response against a sequential antigenic determinant in the helical sequence of the α1(II) chain (CB-11) should therefore facilitate approaches towards a better structural characterization of immunological activity residing in the triple helical part of the collagen molecule.

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