Multiple antigenic determinants on type III collagen

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Eight monoclonal antibodies have been produced against human pepsin-soluble type III collagen. All antibodies were shown to be highly specific for type III collagen and did not cross-react with a range of other collagen types or connective-tissue proteins. Examination of type III collagen from other species showed that these antibodies had a wide range of species specificities, indicating that several distinct epitopes were being recognized. The location of the epitopes was investigated by using reactivity of the antibodies to CNBr fragments and to sequential fragments formed by tryptic digestion of renaturing type III collagen. These data also indicated that several distinct epitopes were recognized and that they were located over the length of the type III collagen.

INTRODUCTION

The helical domains of the interstitial collagens have generally been regarded as being particularly poor immunogens (Timpl, 1976), and this has been used to advantage in the development of a variety of collagen-based biomaterials (Ramshaw *et al.*, 1990). Nevertheless, antibodies against these collagens can be raised in animals (Timpl, 1976), and an immunological response to collagen implants does occur in a small number of patients (DeLustro *et al.*, 1987).

In some species, such as rabbit, the epitopes which lead to generation of antibodies are located on the individual α -chains (central determinants) and can therefore be mapped by examining CNBr fragments (Pontz *et al.*, 1970). In other species, including mouse and man, epitopes which elicit antibody responses are dependent on the intact triple-helical structure of the molecule (conformational determinants) (Linsenmayer *et al.*, 1983; Ellingsworth *et al.*, 1986; Ramshaw & Werkmeister, 1988). Murine monoclonal antibodies (MAbs) therefore provide a way to characterize these conformation-dependent epitopes on collagen which may be immunologically important in man.

Previously, a small number of MAbs developed against the pepsin-soluble helical domains of mammalian collagens have been reported (Mayne, 1988). These include four reports (Sundar-Raj *et al.*, 1982; Sakakibara *et al.*, 1986; Macarak *et al.*, 1986; Keene *et al.*, 1987) which describe five MAbs specific for type III collagen. However, for only a single antibody (Keene *et al.*, 1987) has an approximate location of the epitope been reported. Thus it is not clear whether one or several epitopes are being recognized on type III collagen. In the present study, a range of MAbs to human, pepsin-soluble type III collagen has been prepared and characterized, and the approximate locations of the determinants which they recognize have been examined.

EXPERIMENTAL

Collagen preparation

Human placental type III collagen was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), whereas other type III collagens were prepared from skin by pepsin digestion. Samples of calf and sheep skins were obtained from the abattoir, and samples of rat skin were obtained from laboratory animals. Skin samples were frozen in liquid N₂ for mechanical removal of hair

and then powdered before digestion at 4 °C for 48 h with pepsin (EC 3.4.23.1) (1 mg/ml in 0.1 M-acetic acid), adjusted to pH 2.5 with HCl. All collagens, including the human type III collagen, were purified by fractional NaCl precipitation at pH 2.5 and pH 7.4, followed by rapid (NH₄)₂SO₄ precipitation (Trelstad *et al.*, 1976). Further purification, if required, was by precipitation of refolded type III collagen after denaturation in 4 M-guanidinium chloride (ChandraRajan, 1978). Samples of human collagens, fibronectin, laminin and bovine elastin were as previously described (Werkmeister *et al.*, 1990). For CNBr digestion of type III collagen, samples were treated with CNBr [20 mg/ml in 70 % (w/v) formic acid] for 4 h (Scott & Veis, 1976).

Electrophoresis and electroblotting

Collagen purity was examined by SDS/PAGE (Laemmli, 1970) using 5 % (w/v) running gels with 3.5 % (w/v) stacking gels. To distinguish α 1(III) from α 1(I) chains, either an interrupted electrophoresis system (Sykes *et al.*, 1976) or gels including 3.6 M-urea (Hayashi & Nagai, 1979) were used. For separation of CNBr fragments, 12.5 % (w/v) running gels were used. Electroblotting on to nitrocellulose after SDS/PAGE was as previously described (Towbin *et al.*, 1979; Ramshaw & Werkmeister, 1988), except that, after transfer of CNBr frag-

Table 1. Species specificity of anti-(type III collagen) MAbs

Reactivity was assessed by e.l.i.s.a. and by immunoblotting after non-denaturing PAGE: ++, strong reactivity; +, moderate reactivity; –, no reactivity.

Antibody		Species reactivity			
		Man	Sheep	Calf	Rat
1.	4D3-C4/Col3 IgG _{2b} , κ	++	++	++	–
2.	4F3-C7/Col3 IgM, κ	++	++	++	++
3.	1E7-D7/Col3 IgG ₁ , κ	++	–	–	++
4.	5F6-D8/Col3 IgG _{2b} , κ	++	–	–	++
5.	3D2-C7/Col3 IgG ₁ , κ	++	+	–	–
6.	3B9-E9/Col3 IgG ₃ , κ	+	+	–	–
7.	2G8-B1/Col3 IgM, κ	++	++	++	–
8.	4F7-E11/Col3 IgG ₁ , κ	++	++	++	+

Abbreviation used: MAb, monoclonal antibody.

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ments, the nitrocellulose was incubated at 4 °C for 48 h after blocking and washing before immunostaining. Non-denaturing PAGE in lactic acid, pH 3.1, using 3% (w/v) gels and subsequent immunoblotting, were as described by Ramshaw & Werkmeister (1988).

Antibody preparation and characterization

Female SJL/J mice, 12 weeks old, were immunized twice intraperitoneally with 200 µg of human type III collagen as previously described (Werkmeister *et al.*, 1990). At 3 weeks after the second immunization, mice were boosted with 100 µg of collagen intravenously and hybridomas were then prepared by using NS-1 cells (Werkmeister *et al.*, 1985, 1990). Antibodies with specificity for human type III collagen were identified by an e.l.i.s.a. (Werkmeister *et al.*, 1990), using rabbit anti-mouse Ig coupled to alkaline phosphatase (EC 3.1.3.1) (Sigma). Specificity to collagen was confirmed by e.l.i.s.a. after digestion of the immunogen with bacterial collagenase (EC 3.4.24.3) as previously described (Werkmeister *et al.*, 1990).

Collagen type specificity of the antibodies, and their reactivity against a panel of type III collagens from various species, were determined by an alkaline phosphatase e.l.i.s.a., by a competitive e.l.i.s.a. performed under non-equilibrium conditions (Rennard *et al.*, 1980) and by immunoblotting after non-denaturing PAGE (Ramshaw & Werkmeister, 1988). Antibody subclasses were determined by e.l.i.s.a. with subclass-specific goat anti-mouse antibodies (Nordic Immunology, Tilburg, The Netherlands) and rabbit anti-goat Ig coupled to horseradish peroxidase (EC 1.11.1.7) (Nordic Immunology).

For epitope mapping, a series of type III collagen fragments of increasing size was prepared as described by Bachinger *et al.* (1980). Type III collagen, 0.5 mg/ml, was denatured at 45 °C for 1 h and then cooled to 25 °C to allow renaturation. Samples were taken at 1 min intervals and digested with 4% (w/w) trypsin (EC 3.4.21.4) [Worthington; tosylphenylalanylchloromethane ('TPCK')-treated] for 5 min; digestion was terminated by adding excess phenylmethanesulphonyl fluoride. This sequential series of samples, increasing in size from the C-terminal of type III collagen, was examined by e.l.i.s.a. using the different MABs (Keene *et al.*, 1987).

RESULTS AND DISCUSSION

Preliminary studies on serum response to type III collagen in various strains of mice (Balb/c, SJL/J, DBA/2, C57BL/6) confirmed that type III collagen was a very poor immunogen. The best response, although still very low, was obtained in SJL/J mice, and so this strain was used for further studies. Subsequently, from three fusions, eight MABs were derived and characterized (Table 1).

All the MABs were specific for collagen, since digestion of the antigen with bacterial collagenase led to complete or partial loss of reactivity. Also, none of the MABs showed any cross-reactivity by e.l.i.s.a. with other selected non-collagenous connective-tissue proteins (Fig. 1). The MABs were all highly specific for type III collagen and were shown by e.l.i.s.a. (Fig. 1) and by non-denaturing PAGE followed by immunoblotting to be unreactive with collagen types I and V.

The MABs showed a range of species specificities (Table 1), as determined by both e.l.i.s.a. and non-denaturing PAGE and immunoblotting, and belonged to various antibody sub-types. Some, for example 4D3-C4/Col3 (#1) and 4F3-C7/Col3 (#2), showed a very broad range of species reactivities, whereas others, for example 3D2-C7/Col3 (#5), were more selective. Certain of these MABs have been shown to be useful for immunohistology of skin sections (Werkmeister & Ramshaw, 1988, 1989). This

confirmed the species reactivity of these MABs, with the differential species reactivity of certain MABs being of use in studying the performance of collagen-based biomaterials in trials in heterologous hosts (Werkmeister *et al.*, 1989).

The differential species specificities indicated that several different epitopes were being recognized. This was confirmed by examination of immunoblots of CNBr fragments after SDS/PAGE separation (Fig. 2). It has previously been shown that reactivity of MABs to heterotrimeric collagens cannot be detected by blotting after SDS/PAGE, since the reactivity of murine MABs is dependent on the intact triple-helical structure of the collagen and the component chains are at different locations on the gel (Ramshaw & Werkmeister, 1988). However, since type III

Fig. 1. Specificity of anti-(type III collagen) antibodies

Competition e.l.i.s.a. of the specificity of MAB 1E7-D7/Col3 (#3), using antibody and various concentrations of other collagens or connective-tissue proteins as inhibitors, was carried out. Human collagen types I and V, fibronectin, laminin and elastin (○) (the bars indicate the range of observations) and human type III collagen (●) were used. All the other MABs gave similar results.

Fig. 2. Antibody reactivity to type III collagen CNBr fragments

The Figure shows the separation of CNBr fragments of type III collagen by SDS/PAGE, followed by electroblotting on to nitrocellulose. Blots were immunostained with MABs #1-#8 as given in Table 1, as shown by the labels to the lanes. Lane S shows a CNBr digest of a human type I/type III collagen mixture stained for protein with Amido Black. Type III collagen CNBr fragments which react with antibody are labelled as described in the text.

Fig. 3. Restoration of antibody reactivity to fragments derived from renaturing type III collagen

Human type III collagen was heat-denatured at 45 °C for 1 h and then allowed to renature at 25 °C. After various renaturation times, aliquots were digested with trypsin and then examined for their ability to react with MAbs #1–#8 (Table 1). Key to symbols: ▲, MAb #1, 4D3-C4/Col3; ■, MAb #3, 1E7-D7/Col3; ▽, #4, 5F6-D8/Col3; ○, MAb #5, 3D2-C7/Col3; ●, MAb #6, 3B9-E9/Col3; □, MAb #7, 2G8-B1/Col3; △, MAb #8, 4F7-E11/Col3.

collagen is a homotrimer, immunoblotting after SDS/PAGE can be used as refolding of material in single bands can lead to native helical conformation. In the present studies it was necessary to incubate the electroblot for 48 h at 4 °C in order to achieve adequate refolding of the fragments into helical conformation before immunostaining. A range of different fragments were shown to be detected by the MAbs, indicating that the epitopes were distributed throughout the molecule. Two of the MAbs, namely 4D3-C4/Col3 (#1, lane 1) and 4F3-C7/Col3 (#2, lane 2) did not give any immunostaining. This suggested that the epitopes which these MAbs recognized were either parts of small CNBr fragments which were not efficiently transferred to the immunoblot or did not readily renature to give a helical conformation, or that the epitopes contained a methionine residue which was lost during the CNBr digestion.

The CNBr fragments which were detected by the different MAbs were not specifically identified, but were assigned by comparison with separation patterns described by other groups (Cole & Chan, 1981; Light, 1982). However, an alternative assignment of CNBr fragments has been presented (Miller &

Rhodes, 1982) where CB-4 and CB-8 are not separated. By using these alternative data, the locations of certain epitopes would be shifted towards the *N*-terminus, but they would still be distributed over the length of the molecule.

Further data on the location of the epitopes recognized by the different MAbs was obtained by examination of reactivity against type III collagen fragments of increasing size prepared by refolding of heat-denatured type III collagen, using tryptic digestion to remove unfolded α -chain in partially refolded intermediates (Bachinger *et al.*, 1980; Keene *et al.*, 1987). Refolding from the *C*-terminal of the collagen occurs readily, owing to the presence there of two cysteine residues which link the three α -chains of the collagen. These data (Fig. 3) show the relative order, from the *C*-terminal of the protein, of the epitopes recognized by the eight MAbs. The epitope recognized by MAb 4F3-C7/Col3 (#2) was susceptible to tryptic digestion before denaturation, so little regain of reactivity was observed in this experiment. This susceptibility to trypsin suggests that the epitope may be located at the *N*-terminal of the molecule.

Assuming that the rate of renaturation of type III collagen was approximately linear with time (Bachinger *et al.*, 1980), then in combination with the data obtained from immunoblotting of CNBr fragments (Fig. 2) the approximate locations of the various epitopes on type III collagen recognized by the MAbs can be established (Fig. 4). These data indicated that, for one pair of antibodies, namely 1E7-D7/Col3 (#3) and 5F6-D8/Col3 (#4), similar regions of the collagen were recognized. A competitive e.l.i.s.a. was used to distinguish whether these antibodies were directed against separate epitopes (Friguet *et al.*, 1983). This showed that MAbs #3 and #4 could possibly be detecting similar or overlapping epitopes. Although these MAbs were obtained from the same fusion, they are from distinct antibody sub-classes (Table 1), suggesting that each was derived from separate immunoreactive hybrid clones.

Before the present study was undertaken, five other MAbs specific for type III collagen had been described (SundarRaj *et al.*, 1982; Sakakibara *et al.*, 1986; Macarak *et al.*, 1986; Keene *et al.*, 1987). For four of these (SundarRaj *et al.*, 1982; Sakakibara *et al.*, 1986; Macarak *et al.*, 1986), no data on the location of the epitope was available. For the other (Keene *et al.*, 1987), rotary-shadowing and renaturation analysis indicated that the epitope was between residues 250 and 350 of the helical domain (Fig. 4). This location is very similar to that observed for the separate MAbs 1E7-D7/Col3 (#3) and 5F6-D8/Col3 (#4), suggesting that a major immunogenic region of type III collagen may be located in this part of the molecule. Apart from this potentially immunodominant region, the results demonstrated that epitopes were present throughout the entire length of the type III collagen. A similar widespread distribution for central sequential determinants has been reported for bovine type I collagen using rabbit polyclonal antibodies (Timpl, 1976).

For globular proteins it has been suggested that regions of greater chain flexibility may represent the major epitopes on a molecule (Tainer *et al.*, 1984). For the fibrous protein collagen,

Fig. 4. Location of epitopes on type III collagen

The diagram is based on the combination of CNBr-fragment reactivity (Fig. 2) and sequential fragment reactivity (Fig. 3) and shows the possible locations of the epitopes recognized by the eight MAbs of the present study (▼) and the single MAb from a previous study (▼) (Keene *et al.*, 1987). The positions of CNBr cleavage (CNBr ...) and of the resulting fragments are shown.

crystallographic or spectroscopic methods will be unlikely to provide data on specific regions of helical flexibility. However, conformational energy calculations (Nemethy & Scheraga, 1982) or similar approaches, thermal stability of synthetic fragments (Germann & Heidemann, 1988) or susceptibility of the helix to proteolysis (Stark & Kuhn, 1968) may allow reasonable estimates of the flexibility to be obtained. Identification of the exact locations of the epitopes on type III collagen would then enable a correlation with proposed helix flexibility to be examined.

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