

IMMUNOLOGIC CHARACTERIZATION
OF THE MEMBRANE-BOUND COLLAGEN IN NORMAL
HUMAN FIBROBLASTS:
IDENTIFICATION OF A DISTINCT MEMBRANE COLLAGEN*

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Collagen is the major structural protein of connective tissues, comprising the bulk of the protein in the extracellular matrix. Several studies have shown that collagen is also membrane bound in chick (1), rat (2), and mouse L-cell fibroblasts (3). Antibodies to membrane-bound collagen have been shown to evoke complement (C)-dependent cytotoxicity (1). These reports indicate that collagen synthesized by these fibroblasts may be deposited as fibrils in the extracellular matrix and also may be bound to the fibroblast membrane.

Normal human skin fibroblasts have been shown to synthesize at least two genetically distinct types of collagen (4, 5). In this report, we present immunologic evidence that two antigenic types of collagens, type I collagen and a new collagen, type M (membrane) collagen, are associated with the normal human fibroblast membrane, and that antibodies to one of these collagens, type M collagen, can induce C-mediated cytotoxicity.

Materials and Methods

Preparation of Collagen Antigens. Type I collagen was prepared by acid extraction of human skin and purified according to the method of Bornstein and Piez (6). To obtain type III and type M collagen antigens, human placentas were pepsin digested and the supernate was sequentially salt precipitated at neutral pH with 0.15 M, 1.5 M, and 2.5 M NaCl. The type III collagen was further purified by carboxymethylcellulose (CMC) chromatography (7-9). Type I and type III collagens were judged to be pure by sodium dodecyl sulfate (SDS)¹ polyacrylamide gel electrophoresis (10) and amino acid analysis (7). Pepsin-treated type I collagen was prepared as previously described (5, 8, 9).

Preparation of Antibodies to the Purified Collagens. Antibodies were raised by subcutaneous immunization of rabbits with 5-10 mg of heat-denatured collagen in 1.0 ml of 0.15 M NaCl, 0.05

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¹ *Abbreviations used in this paper:* CF, complement fixation; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; SDS, sodium dodecyl sulfate.

Tris, pH 7.5, to which was added an equal volume of complete Freund's adjuvant. Booster injections containing a similar amount of collagen were given at 3-wk intervals. Development of antibodies was monitored by Ouchterlony gel diffusion analysis (11) using the specific collagen immunogens. Hyperimmune antisera harvested 1 wk after the third and fourth booster injections were used for the studies in this report.

In order to prepare type-specific antibodies, the antisera were cross-absorbed on affinity columns prepared by coupling specific collagens to Sepharose 4B as described previously (12). Approximately 20 mg of collagen was bound to 5 ml of settled Sepharose. For absorption, 1 ml of antiserum was incubated with 5 ml of the collagen Sepharose slurry at room temperature for 2 h and then overnight at 4°C. The unbound antiserum was then separated by filtration, precipitated at 40% saturation with $(\text{NH}_4)_2\text{SO}_4$, redissolved in 1 ml of Hanks' balanced salt solution (HBSS) (Grand Island Biological Co., Grand Island, N. Y.), and dialyzed against this solution.

Radioimmunoassay. The titers of the antisera were assessed by the ability to bind ^{125}I -labeled collagen. Collagens were iodinated by the lactoperoxidase method (13). The reaction mixture consisted of 40 to 125 μg of collagen, 1 mCi [^{125}I]Na (Industrial Nuclear Co., Inc., St. Louis, Mo.), 5 μg of lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.), and 1 μl of 0.88 mM H_2O_2 in a total vol of 50 μl . Iodination proceeded for 1 min at room temperature and was stopped by the addition of 500 μl of 0.1% sodium azide in 0.15 M NaCl. The iodinated collagens were then separated from free ^{125}I on Sephadex G-100 and stored at -70°C in 1% albumin. The anticollagen antisera were titered by a double antibody precipitation reaction: 100 μl of serial doubling dilutions of antiserum made in 0.15 M NaCl, 0.05 Tris, pH 7.4, were incubated for 24 h at 4°C with 100 μl of ^{125}I -labeled collagen (approximately 15,000 cpm). Immediately before the addition of goat antirabbit IgG in excess, 5 μg of purified nonimmune rabbit gamma globulin was added as a carrier to ensure complete precipitation. The resultant precipitates were harvested after an additional 24 h at 4°C, washed twice, and counted in an automatic gamma scintillation spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.). Titers were expressed as the highest dilution of antiserum which bound 50% of the maximum counts precipitated. The collagen antigens after iodination, and the iodinated collagens after antibody precipitation had migration patterns similar to the uniodinated antigens on autoradiographs of SDS polyacrylamide gel electrophoresis, indicating that the initial collagen antigen, and not a minor contaminant was being assessed in the radioimmunoassay. C fixation was assessed by standard techniques (14).

Assay of Cytotoxicity. Cultured human skin fibroblasts (Robel, CRL 1187; Elman, CRL 1101; Fesin, CRL 1106; and HeLa, CRL 2) were purchased from the American Type Culture Collection, Rockville, Md. and were used between the fifth and tenth passage. All strains gave identical results in these experiments. Cells were grown to confluence in Dulbecco-Vogt modified Eagle's medium (GIBCO) containing 10% fetal calf serum. Human tendon and chick tendon fibroblasts were grown and used as primary cultures.

Cell counts were performed with a hemocytometer. For cytotoxicity experiments, 2.5×10^6 cells were trypsinized, washed, and suspended in 1 ml of HBSS. Depending on the experiment, 25–100 μl of cells (62,500–250,000 cells) were incubated with an equal aliquot of antiserum at 37°C for 15 min. An equal aliquot of a 1:5 dilution of guinea pig C (GIBCO) was then added. After incubation for an additional 45 min at 37°C, 0.2% trypan blue was added and viability was assessed.

In order to assess the specificity of the C-mediated cytotoxicity, cells were pretreated with collagenase, trypsin, or chymotrypsin before the addition of antibody and C. Bacterial collagenase (type III) was purchased from Advanced Biofactors. Since commercial collagenase preparations have shown to contain nonspecific proteases (15), the purity of this preparation was ascertained. The collagenase used in this study was judged to be pure by the following criteria: it gave a doublet band on 5% SDS gel electrophoresis of 105,000, released no [^3H]tryptophan counts from [^3H]tryptophan-labeled fibroblast proteins, and there was no alteration of the migration of albumin on 5% SDS gel electrophoresis after collagenase digestion. Trypsin and chymotrypsin were purchased from Sigma Chemical Co.

Indirect Immunofluorescence. For indirect immunofluorescence, 100 mg of a gamma globulin fraction of goat antirabbit IgG was reacted overnight at 4°C with 5 mg of fluorescein isothiocyanate (FITC) (Sigma Chemical Co.) in 0.1 M Na_2CO_3 , pH 9.0. Separation of FITC-labeled material was accomplished on Sephadex G-25. Staining was accomplished using 500 μl of trypsinized cells suspended in HBSS containing 0.61% NaN_2 which were incubated with 50- μl dilutions of the rabbit antibodies at 0°C for 30 min, washed three times in HBSS, 0.1% NaN_2 , and further

incubated with a 1:100 dilution of the fluorescent goat antirabbit IgG for 30 min at 4°C. The cells were washed three times in HBSS, 0.1% NaN₂, dried on cover slips, and mounted on slides in 90% buffered glycerol. Cells were observed and photographed in a Leitz orthoflux fluorescence microscope with epilumination (E. Leitz Inc., Rockleigh, N. J.).

Results

In an effort to immunologically dissect the types of collagen bound to the normal human fibroblast cell membrane, type I and type III collagens were purified to prepare type-specific antibodies. In the course of the placental preparation of type III collagen, it was found that another distinct collagen, type M collagen, which was more antigenic than type III collagen, copurified with type III collagen. Type III and type M collagen could be partially separated by differential salt fractionation, since the majority of the type III was precipitated with 1.5 M salt, while most of the type M was precipitated by 2.5 M salt. Amino acid analysis indicated that type M is a collagen, since approximately 1/3 of the amino acids are glycine, it has a high content of proline and hydroxyproline, and hydroxylysine is present. Since this collagen is chemically and immunologically distinct from type I and type III collagens, we refer to it as type M (membrane) collagen. A full report of the chemical and immunologic characteristics of type M collagen is in preparation.

Antisera and purified specific antibodies used for these studies were titrated using a double antibody radioimmunoassay. As shown in Table I, before absorption with type-specific collagen bound to Sepharose, antitype M collagen antiserum cross-reacts with ¹²⁵I-labeled type I collagen at a 1:200 dilution. Similarly, antitype M collagen antiserum binds ¹²⁵I-labeled type I collagen at a 1:700 dilution. Ouchterlony analysis showed that the unabsorbed antisera cross-reacted with both of these collagens. By absorption with type-specific collagens, it was possible to obtain specific antitype I and antitype M collagen as judged by monospecific precipitin lines in Ouchterlony plates and binding of only a single type of collagen in the radioimmunoassay (Table I). Native and denatured type I and type III collagens and pepsin-treated type I collagen failed to inhibit the binding of ¹²⁵I-labeled type M collagen by antitype M collagen in the radioimmunoassay.

Indirect immunofluorescence, whether performed with antitype M antiserum, antitype I antiserum, specific antitype M, or with specific antitype I collagen antibodies, showed a speckled fluorescence pattern in all cells (Fig. 1). Immunofluorescence with antitype I collagen antiserum and specific antitype I collagen antibodies was abolished at a dilution of 1:50, while immunofluorescence with antitype M collagen antiserum was present to a dilution of 1:6,250. Antibodies to both type I and type M collagens bound C as assessed by standard C fixation techniques (Table I).

Since certain antibodies to fibroblast membrane proteins have been shown to elicit C-mediated cytotoxicity (16), we attempted to ascertain whether the anticollagen antibodies were cytotoxic. Antitype M collagen antiserum in the presence of C completely lysed cultured human skin fibroblasts to a 1:8 dilution, while antitype I collagen antiserum had maximally a 20% cytotoxic effect, and control rabbit serum had essentially no cytotoxic effect (Fig. 2). Specific antibodies to type M collagen retained the potent cytotoxic effect. The decrease in

TABLE I
Titers of Antibodies to Type I and Type M Collagens

Antiserum	Radioimmunoassay			
	¹²⁵ I-labeled type I collagen	¹²⁵ I-labeled type M collagen	Indirect immunofluorescence	Complement fixation
1) Antitype M collagen, nonspecific	1/200	1/12,800	1/6,250	1/2,400
2) Antitype I collagen, nonspecific	1/2,400	1/700	1/50	1/600
3) Antitype M collagen, specific	No binding	1/4,800	1/1,250	Not done
4) Antitype I collagen, specific	1/600	No binding	1/50	Not done

Specific activity of the ¹²⁵I-labeled type I collagen was 4.4×10^5 cpm/ μ g and of the ¹²⁵I-labeled type M collagen was 2.1×10^6 cpm/ μ g. 15,000 cpm were used in each tube for the radioimmunoassay. Indirect immunofluorescence was performed as indicated in the Materials and Methods. Data are expressed as the dilution at which immunofluorescence last appeared. CF test was performed as previously described (14) with 0.1 μ g of type I or type M collagen as antigens.

cytotoxicity with the specific antibodies could be due to either the decrease in titer to type M collagen with purification (Table I) and/or the removal of endogenous C in the purification of antibodies. In order to assess the effect of the removal of endogenous C, control rabbit serum and heat-inactivated rabbit serum were incubated with the specific type M antibodies. Control rabbit serum in a 1:1 mixture with specific antitype M antibodies effected complete cytotoxicity, while control rabbit serum treated at 56°C for 30 min to denature C and incubated with the specific type M antibodies in a 1:1 mixture had no cytotoxic effect above that of specific type M antibodies in a 1:1 dilution. These results suggest that both the removal of endogenous C and the lower titers account for the difference in cytotoxicity between the antitype M antiserum and the purified antitype M collagen antibodies.

The inability of antitype I collagen antibodies to effect C-mediated cytotoxicity was apparently not due to the failure to bind C, since antitype I antiserum had a 1/600 C fixation (CF) titer compared with a 1/2,400 CF titer for antitype M collagen antiserum (Table I). The fourfold difference in CF titer is probably not enough to explain the marked differences in C-mediated cytotoxicity (Fig. 2). The inability of antitype I collagen antibodies to induce cytotoxicity was not due to differences in titer since a sixfold concentration of the antibodies by 40% ammonium sulfate precipitation had no C-mediated cytotoxicity above that of the unconcentrated antiserum.

To further confirm the specificity of the cytotoxicity, blocking experiments were performed in which type I, type III, or type M collagen antigens were preincubated with antiserum to type M collagen before addition of the trypsinized cultured fibroblasts. Type M collagen effectively blocked cytotoxicity, while the addition of native or denatured type I collagen or denatured type III collagen had no effect on cytotoxicity (Table II).

Since the antigen-blocking experiments seemed to indicate that cytotoxicity was dependent upon type M collagen on the cell surface, an attempt was made to

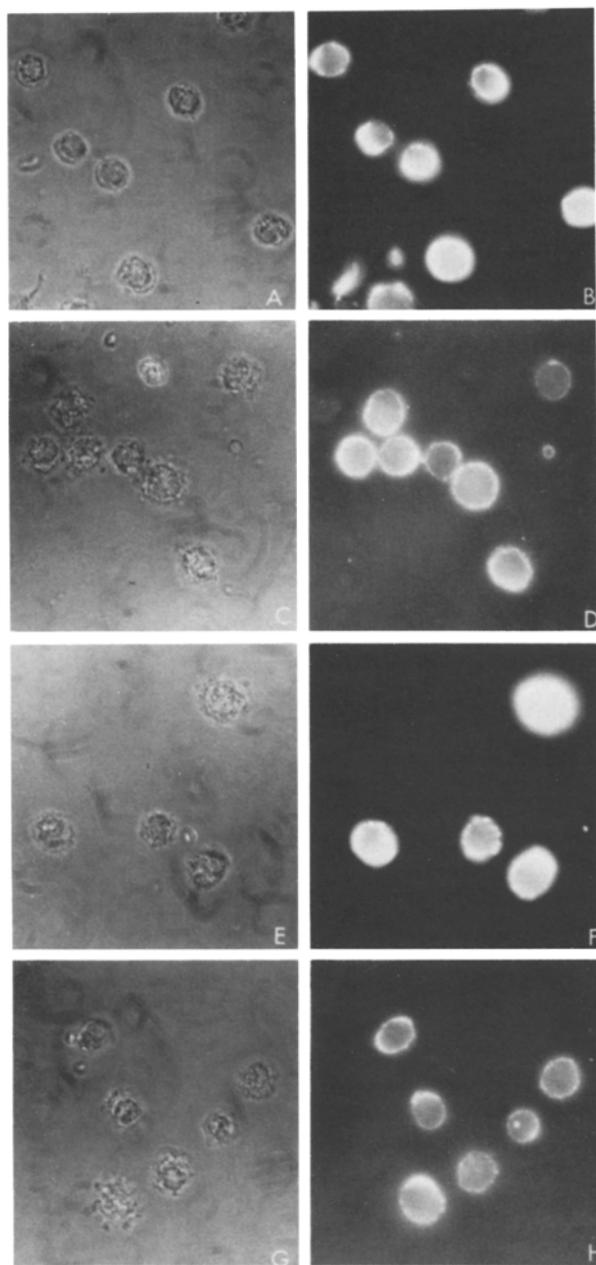


FIG. 1. Immunofluorescent staining of human fibroblasts with antitype I and antitype M collagen. Fibroblasts were stained by indirect immunofluorescence as described in the Materials and Methods and examined by phase-contrast and fluorescence microscopy. (A and B) antitype M, (C and D) antitype I, (E and F) antitype M absorbed with type I, and (G and H) antitype I absorbed with type M (original magnification 320 on all photographs).

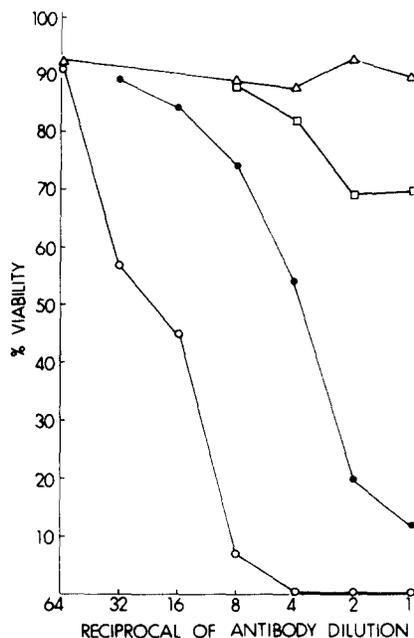


FIG. 2. The C-mediated cytotoxicity of control rabbit serum (Δ - Δ), antiserum to type I collagen (\square - \square), antiserum to type M collagen (\circ - \circ), and specific antibodies to type M collagen (\bullet - \bullet).

TABLE II
Antigen Inhibition of Cytotoxicity

Antiserum	Dilution	Antigen	Viable cells
			%
Control rabbit serum	Undiluted	-	93
Antitype M collagen	1/4	-	0
+	1/4	10 ng denatured type I collagen	0
+	1/4	10 ng native type I collagen	0
+	1/4	10 ng pepsin-treated type I collagen	0
+	1/4	10 ng denatured type III collagen	0
+	1/4	10 ng denatured type M collagen	60

Cytotoxicity assay was performed as described in the Materials and Methods. For the inhibition experiments, the added antigen was preincubated with the antibody for 30 min at 37°C before the incubation of antibody with cells, the mix was centrifuged and the supernate was added to trypsinized cells in the presence of C.

block the reaction by pretreating the cells either with bacterial collagenase, trypsin, or chymotrypsin. Preincubation of the cells with bacterial collagenase devoid of nonspecific protease activity (see Materials and Methods) completely abolished the cytotoxic effect (Table III). In addition, EDTA, an inhibitor of collagenase but not serine proteases, abolished the ability of collagenase to inhibit cytotoxicity. In contrast, preincubation with trypsin or chymotrypsin did not abolish the C-mediated cytotoxicity of antitype M collagen antiserum. These results indicate that it is specifically the collagen on the fibroblast cell membrane which elicits the cytotoxic response and that the membrane-bound type M collagen is in the triple helical conformation, since chymotrypsin or trypsin under the conditions employed would digest denatured collagen chains.

TABLE III
Enzymatic Inhibition of the C-Mediated Cytotoxicity of Antitype M Collagen Antiserum

Antiserum dilution	Enzyme preincubation	Preincubation time	Viability
		<i>min</i>	<i>%</i>
Antitype M 1/4	—	—	5
+	Bacterial collagenase	30	75
+	+	60	91
+	Bacterial collagenase and EDTA	30	0
—	EDTA	60	96
Antitype M 1/4	—	—	9.5
+	Trypsin	30	8
+	+	60	25
—	+	60	92.3
+	Chymotrypsin	30	13.4
+	+	60	33
—	+	60	88

Cells were preincubated with bacterial collagenase (5 units), 0.2% trypsin, or chymotrypsin for the indicated times. To inactivate the collagenase, 25 mM EDTA, 0.05 Tris, pH 7.4, was premixed with the collagenase. The cells were then centrifuged, washed three times with HBSS, and resuspended in HBSS before the addition of antiserum. Cytotoxicity was then assessed as indicated in the Materials and Methods.

In order to assess whether other proteins normally synthesized by human skin fibroblasts could effect a similar cytotoxic response, C-mediated cytotoxicity was tested using specific antibodies to human fibroblast collagenase, a protein secreted into the culture medium (17). No cytotoxic effect on fibroblasts was observed with anticollagenase antisera.

Finally, the species specificity of the C-mediated cytotoxicity was tested using chick tendon fibroblasts. Antisera to human type I and type M collagens had no effect on the viability of these cells beyond that of normal rabbit serum. Cultured human tendon cells were susceptible to C-mediated cytotoxicity with the same titers of antitype M collagen as used with skin fibroblasts.

Discussion

Our results indicate that both type M collagen and type I collagens are membrane bound in cultured human skin fibroblasts but that only antibodies to type M collagen in the presence of C mediate cytotoxicity. Type M collagen is immunologically and chemically distinct from type I and type III collagens since: (a) Specific antibodies to type M collagen bound only ^{125}I -labeled type M collagen. (b) Specific antibodies to type I collagen failed to bind ^{125}I -labeled type M collagen. (c) Type I collagen and type III collagen failed to inhibit the binding of specific antibodies to ^{125}I -labeled type M collagen. (d) Only type M collagen inhibited the C-mediated cytotoxicity of antibodies to type M collagen. (e) Type I collagen and type III collagen failed to inhibit the C-mediated cytotoxicity of antibodies to type M collagen. (f) Type M collagen could be separated from type I and type III collagen by differential salt precipitation and ion-exchange chromatography.

The amino acid content (not shown) and the mode of preparation preclude type M collagen from being either a basement membrane collagen, C1q, or

procollagen. Thus, we conclude that type M is either a new genetic type of collagen or an antigenically and chemically altered form of type I or type III collagen.

The obliteration of cytotoxicity by pretreatment of the fibroblast with purified bacterial collagenase and the failure of pretreatment with trypsin and chymotrypsin to modify cytotoxicity indicates that the membrane antigen is native, triple helical collagen. The correlation between the immunofluorescence titer and the radioimmunoassay titer suggests that the antibody prepared with denatured pepsin-treated type M collagen is able to bind both the denatured and native forms of the molecule.

In contrast, there is a disparity between the radioimmunoassay and the immunofluorescence titers of the antibodies to type I collagen. One explanation for this disparity and the inability of antitype I collagen to elicit C-mediated cytotoxicity is that there is a paucity of type I collagen molecules on the cell membrane. Another possible explanation is that these antibodies to denatured type I collagen have a low affinity for the native collagen molecule. Antibodies which are prepared with extracted, denatured type I collagen are primarily directed to the nonhelical amino and carboxyterminal portions of α -chains, most often the $\alpha 2$ -chain (18-20), and have a low affinity for the native molecule (20). Since we have not determined the affinity of the type I collagen antiserum for the native collagen molecule, or if the membrane-bound type I collagen is the native molecule, we can not distinguish between the aforementioned possibilities. The inability of antiserum to type I collagen to effect C-mediated cytotoxicity can not be due either to insufficient titers of antibody, since a sixfold increment of titer still did not induce cytotoxicity, or to the failure to bind C, since CF titers were comparable to the radioimmunoassay titers.

Previous studies of cell membrane collagen (1-3) have used acid or neutral salt extractable collagen in the preparation of anticollagen antibodies. Such preparative methods yield only type I collagen (7), and, thus, these studies have presumably utilized antiserum to type I collagen. Assuming that the antiserum utilized was to type I collagen, these studies indicate that type I collagen is membrane bound in chick (1), rat (2), and mouse L-cell fibroblasts (3). Our data indicate that type I collagen is also bound to the normal human fibroblast membrane, and, thus, is both a membrane-bound and an extracellular protein. The previous studies also demonstrated that a species-specific antiserum to type I collagen was strongly cytotoxic to chick fibroblasts (1), only weakly cytotoxic for rat fibroblasts (2), and had little cytotoxicity for rat osteoblasts (2). The cytotoxic reaction with antibodies to type M collagen also appears to be species specific, since these antibodies were not cytotoxic for cultured chick tendon fibroblasts but were cytotoxic for cultured human tendon cells. Thus, the predominant membrane-bound type of collagen may vary in different species and in different tissues.

Although type I collagen and type M collagen could be demonstrated on the cell membrane, it was not clear whether the collagens were in the process of being secreted or were a stable membrane protein. The fact that type I collagen is the major collagen synthesized and secreted by cultured human fibroblasts, but type M collagen seems to be more prevalent on the cell membrane, suggests

that the collagens detected are membrane bound. This conclusion is supported by the failure of antibodies to human fibroblast collagenase, another protein secreted by the fibroblast, to evoke C-mediated cytotoxicity.

The results suggest similarities between the collagens and the immunoglobulins. Although IgG is the major humoral antibody secreted by lymphocytes and plasma cells, IgM is the primary membrane-bound immunoglobulin (21). So also, type I collagen is the major secreted collagen and extracellular fibrillar collagen comprising 80-90% of the collagen synthesized by fibroblasts, while type M collagen may be the major collagen detected on the cultured skin fibroblast membrane.

These studies have relevance to human connective tissue diseases (22). Antibodies to collagen have been detected in the serum and joint fluid of patients with rheumatoid arthritis (23), in the serum from lupus erythematosus (23), and in the serum from patients with emphysema (24). Antibodies to fibroblast membranes which are cytotoxic at high concentrations have been shown to be immunostimulatory at low concentrations (16). It is possible that antibodies directed against type M collagen *in vivo* may be pathogenic in these diseases by either C-mediated cell destruction at high antibody titers or stimulating fibrosis at low antibody titers.

Summary

Collagen, the major extracellular matrix protein, is also a membrane protein. Two types of collagen are detected on the normal human fibroblast membrane in culture, type I collagen and a new immunologically and chemically distinct collagen, type M (membrane) collagen. Antibodies to type M collagen elicited complement-mediated cytotoxicity, which could be blocked by pretreatment of the cells with bacterial collagenase or the antibody with type M collagen. Pretreatment of the cells with other proteolytic enzymes or the antibody with type I collagen or type III collagen had no effect on this complement-mediated cytotoxicity. Although type I collagen is the major collagen synthesized by normal human fibroblasts type M collagen may be the major cell membrane collagen and may be a major cell membrane component.

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