CROSS-REACTIVITY OF CELL-MEDIATED IMMUNITY BETWEEN INTERSTITIAL (TYPE I) AND BASEMENT MEMBRANE (TYPE IV) COLLAGENS*

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Collagen is the major protein of skeletal and connective tissues. Genetically distinct types of collagen, although similar in macromolecular structure, differ in primary structure and contain diverse antigenic determinants (1). In addition, post-translational modifications result in further differences in collagens of similar primary structure. These modifications include hydroxylation of proline and lysine, glycosylation of amino acids involved in the glycopeptide bonds, and the subsequent assembly of carbohydrate units (2). Glucosylgalactosyl and galactosyl residues attached to hydroxylysine are the major forms of carbohydrates in collagen (3–7). These carbohydrate units may be considered a structural characteristic exclusive to the collagen family of proteins because hydroxylysine is found almost exclusively in collagen (8). However, both a subcomponent of the first component of the complement system, Clq (9), and acetycholine esterase (10, 11) contain collagen-like triple helical structures as well as significant quantities of these carbohydrate moieties. Basement membrane (type IV) collagen, in addition to the hydroxylysine-linked carbohydrate, contains asparagine-linked heteropolysaccharide in the nonhelical procollagen extensions; however, this carbohydrate-peptide linkage is also found in a large variety of proteins of diverse origin (6).

We previously demonstrated the ability of type IV collagen, isolated from syngeneic, murine Engelbroth-Holm/Swarm sarcoma, to induce a collagen type IV-specific cell-mediated immune response in a murine model (12). Mice sensitized to type IV collagen in adjuvant developed significant delayed-type hypersensitivity responses (DTH)[1] to type IV collagen; no response was observed in these mice after challenge with homologous type I collagen. This response was characterized by an intense mononuclear cell infiltrate in the footpad lesions of immune mice after challenge with

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[1] *Abbreviations used in this paper:* anti-Thy-1.2, monoclonal anti-Thy-1.2 antibody; C, guinea pig complement; CMI, cell-mediated immunity; DTH, delayed-type hypersensitivity; EHS, Engelbroth-Holm/Swarm sarcoma; ELISA, enzyme-linked immunosorbent assay; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; T cell, thymus-derived lymphocyte.

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type IV collagen and by the ability of type IV collagen immune T lymphocytes to adoptively transfer this DTH response to normal, syngeneic mice.

In the present study, we demonstrate DTH to homologous type I collagen that cross-reacts with type IV collagen. Mice receiving a single sensitizing injection of native or denatured type I collagen in adjuvant mount a significant DTH response to challenge with native or denatured type I collagen as well as native type IV collagen. The cross-reactive cell-mediated immunity (CMI) is eliminated by denaturation of the type IV collagen molecule or by the chemical or enzymatic removal of carbohydrate components. Treatment of type IV collagen with α-glucosidase appears to localize the cross-reactive antigenic determinant to a glucose-containing determinant. In contrast, antibodies generated to type I collagen in this model were collagen type specific and did not react with other collagenous or noncollagenous connective tissue components. Thus, these studies demonstrate that collagen-immune T lymphocytes are capable of recognizing antigenic determinants shared by genetically distinct types of collagen.

Materials and Methods

Animals. C57BL/6 female mice (Laboratory Animal Medicine, Medical University of South Carolina) were used at 8–10 wk of age.

Preparations and Characterization of Collagenous Proteins. Type I collagen was isolated from murine tail tendon as described previously (12). Denatured type I collagen was prepared by heating a solution of collagen (0.1 mg/ml) in 0.1 M acetic acid for 30 min at 50°C.

The Engelbroth-Holm/Swarm (EHS) sarcoma was the source of type IV collagen as previously detailed (12, 13). Briefly, homogenized tumor tissue was extracted with neutral salt buffers to remove soluble extracellular proteins and noncollagenous glycoproteins. The residue was solubilized in 0.5 M acetic acid and the insoluble collagenous material treated with pepsin (50 mg/g) for 24 h at 4°C. Solubilized protein was precipitated with 10% NaCl, dissolved in 2 M urea (0.05 M Tris-HCl, pH 8.6), and separated by DEAE-cellulose chromatography. The collagen that appeared with the buffer front was dialyzed against 0.05% acetic acid and lyophilized.

Before denaturation of type IV collagen, a solution of the collagen in 0.1 M sodium phosphate, pH 8.0, containing 0.4 M NaCl, was reduced by the addition of 0.02 M dithioerythritol and alkylated with 0.08 M sodium iodoacetate to block free sulfhydryl groups as described by Timpl et al. (14). These reagents were removed by dialysis against 0.1 M acetic acid. Denatured type IV collagen was prepared by heating a solution of the reduced and alkylated collagen (0.1 mg/ml) in 0.1 M acetic acid for 30 min at 50°C.

Collagen types I and IV were assayed for purity by amino acid analysis, sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and susceptibility to protease-free bacterial collagenase (15). No evidence of other collagenous proteins or noncollagenous impurities was observed in the collagen preparations, as previously described (12).

Chemical and Enzymatic Removal of Carbohydrate Moieties from Collagen. Periodate oxidation of types I and IV collagen was carried out in 0.05 M acetate buffer (pH 4.5), as described by Spiro (16). To each of the samples (2.5 mg/ml), an equal volume of 0.04 M $NaIO_4$ in 0.05 M acetate buffer (pH 4.5) was added, and the samples were kept in the dark for 90 min at 4°C. The samples were subsequently dialyzed against phosphate-buffered saline (PBS).

Types IV and I collagen (2 mg/ml) were dialyzed against PBS and then incubated with an equal volume of mixed glycosidase (0.05 mg/ml) from *T. Cornutus* (Miles Laboratories, Elkhart, IN) in the presence of 2 mM $MgCl_2$ (17). The mixed glycosidase was previously assayed for proteolytic and collagenolytic activity by the release of radioactive label ([^{3}H]tryptophan and [^{3}H]proline) from chick protein, as previously described (12, 15). No activity was demonstrated by the mixed glycosidase on these protein substrates.

Treatment of type IV collagen with α-glucosidase or β-galactosidase was modified from methods described by Spiro (18). α-Glucosidase (Brewer's yeast; Sigma Chemical Co., St. Louis,

MO) at a concentration of 10 U/ml was added to a solution of type IV collagen (1 mg/ml) in 0.2 M sodium acetate buffer (pH 5.0) and incubated at 37°C for 3 h. β-Galactosidase (25 U/ml; *Escherichia coli*, Sigma Chemical Co.) was incubated with type IV collagen in 0.5 M potassium phosphate (pH 7.0) in the presence of 0.01 M $MgSO_4$ for 3 h at 37°C. Sequential treatments of type IV collagen with α-glucosidase and β-galactosidase were performed as described above; after α-glucosidase treatment, type IV collagen samples were dialyzed against 0.5 M potassium phosphate (pH 7.0) before the addition of β-galactosidase. All collagen samples were dialyzed against PBS. Control samples and the glycosidase-treated type IV collagen preparations were prepared by the addition of appropriate concentrations of the mixed glycosidase, α-glucosidase, or β-galactosidase immediately before dialysis.

Collagen samples treated with periodate or glycosidases were assayed for total hexose by the anthrone reaction (19). All samples displayed a significant reduction in the amount of hexose present when compared with type IV collagen controls.

Delayed-type Hypersensitivity. 5 μg of antigen in 0.1 ml 0.1 M acetic acid was emulsified with an equal volume of complete Freund's adjuvant (CFA; Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) and injected subcutaneously in the abdomen. Control mice were untreated. DTH was measured by footpad swelling in response to antigenic challenge, as described previously (12). On days 3, 6, 10, and 13, mice were injected intradermally in the plantar surface of the hindfoot with 5 μg antigen in a volume of 0.03 ml. Footpad thickness was determined 4 and 24 h post-challenge with a micrometer and compared with measurements observed before antigen injection. The data are expressed as the mean percentage footpad swelling ± SE: percent footpad swelling = ([mm after challenge − mm before challenge]/[mm before challenge]) × 100.

Passive Transfer. Spleens removed from collagen-sensitized mice 7 d after immunization were passed through 60-mesh stainless steel screens into RPMI 1640 (Gibco Laboratories, Grand Island Biological Co.) with 2.5% fetal calf serum to obtain single cell suspensions. Erythrocytes were lysed by hypotonic shock, and cells were washed three times with RPMI. The sensitized spleen cells were left untreated or were incubated with monoclonal murine anti-Thy-1.2 antibody (anti-Thy-1.2; New England Nuclear, Boston, MA) plus guinea pig complement (C; M. A. Bioproducts, Walkersville, MD), as previously described (12). 25 million untreated or T cell-depleted spleen cells were injected intraperitoneally in 0.5 ml PBS into normal mice. 2 d after cell transfer, recipient mice were challenged in the footpad with 5 μg antigen, and footpad swelling was assayed 24 h later.

Preparation of Antisera. Rabbit gamma globulin fractions against types I and IV collagen were prepared as described previously (20). Lyophilized antibodies were used as a stock solution of 1 mg/ml.

Mice were immunized at weekly intervals by subcutaneous injections of 50 μg native types I or IV collagen dissolved in 0.1 ml 0.1 M acetic acid and emulsified with an equal volume of incomplete Freund's adjuvant (IFA, Gibco Laboratories, Grand Island Biological Co.). Control mice were injected with 0.1 ml 0.1 M acetic acid emulsified with an equal volume of IFA. Immune and normal mice were bled from the ophthalmic venous plexus on day 42. Sera from each group (at least six mice per group) were pooled at stored at −70°C.

Enzyme-linked Immunosorbent Assay (ELISA). Antibodies to types I and IV collagen were detected using the ELISA, as described previously (20–22). Serial dilutions of rabbit antibodies and immune mouse sera in PBS (pH 7.8) containing 0.05% Tween 20 and 1% bovine serum albumin (PBS-Tween-BSA) applied to polystyrene microtiter wells (Flow Laboratories, McLean, VA), previously coated with 0.25 μg type IV collagen or 1.25 μg type I collagen, were incubated for 45 min at room temperature. After extensive washing, 100 μl of peroxidase-conjugated goat anti-rabbit immunoglobulin (IgG, M, A) antibodies (1:500; N. L. Cappel Laboratories Inc., Cochranville, PA) or peroxidase-conjugated goat anti-mouse Ig (IgG, M, A) antibodies (1:250; N. L. Cappel Laboratories Inc.) in PBS-Tween-BSA were added. The plates were incubated for an additional 45 min and then washed. 100 μl 0.03% 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonate) (Sigma Chemical Co.) was added to each well in 0.1 M citrate buffer (pH 4.0) with 0.05% H_2O_2, and, after incubation for 1 h, absorbance was read at 414 nm on a Titertek Multiskan (Flow Laboratories Inc., Rockville, MD). Assays were performed in duplicate and results expressed as mean absorbance values.

Absorption Assays. Repeated absorptions of normal and immune mouse sera (1:20 dilution) were performed to remove collagen-specific antibody activity, as previously described (20).

Results

The ability of C57BL/6 mice to mount a DTH response to homologous type I collagen is shown in Fig. 1. Mice sensitized with native type I collagen and challenged with native or denatured type I collagen or native type IV collagen displayed significant footpad swelling peaking 7 d postsensitization (Fig. 1 A). Mice immunized with denatured type I collagen in CFA displayed significant footpad swelling 24 h after challenge with native or denatured type I collagen as well as native type IV collagen (Fig. 1 B). No significant reactivity was observed in either group when challenged with denatured type IV collagen. Normal control mice displayed no significant footpad swelling 24 h after challenge with the native or denatured types I

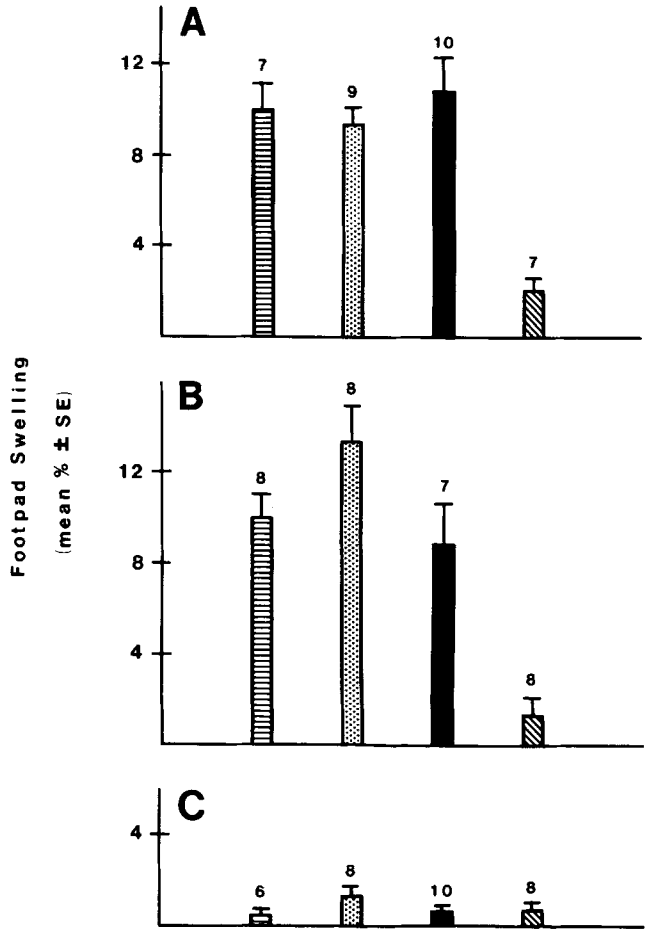

FIG. 1. DTH response of mice sensitized with 5 μg (A) native type I collagen, or (B) denatured type I collagen, and (C) normal mice challenged on days 3, 6, 10, and 13 postsensitization with native type I collagen (▤), denatured type I collagen (▨), native type IV collagen (■), or denatured type IV collagen (▧). The percent footpad swelling was assayed 24 h post-challenge, and the results are expressed as mean percentage footpad swelling ± SE. The number of mice assayed is indicated above each bar.

or IV collagen (Fig. 1 C). No significant swelling was observed 4 h after challenge with types I or IV collagen (data not shown), indicating the response was not the result of an immediate hypersensitivity response or an antibody-mediated Arthus reaction. These data indicate that a CMI response to type I collagen may recognize similar antigenic determinants on type IV collagen; the cross-reactive DTH response is specific for the native form of type IV collagen.

Although no collagenous or noncollagenous contaminants were observed in our collagen preparations by biochemical and immunological analyses (12, 20–22), the possibility that a noncollagenous protein contaminant was contributing to the observed cross-reactive DTH response between types I and IV collagen was investigated by challenging type I collagen-sensitized mice with collagenase-treated antigens. As shown in Table I, mice sensitized with type I collagen and challenged with either collagenase-treated type I or type IV collagen failed to show significant footpad swelling on day 7. Challenge of these same mice with untreated types I or IV collagen, immediately after assay of the footpad swelling on day 7, resulted in significant DTH responses 24 h later (day 8) in both groups of mice, indicating that mice immune to type I collagen respond to collagenous antigenic determinants.

The ability of type I and type IV collagen immune spleen cells to adoptively

TABLE I

*Collagenase Treatment of Antigen Preparations Used for DTH Challenge**

Sensitization	Challenge	Footpad swelling	
		With collagenase	Without collagenase
		mean % ± SE	
I	I	0.4 ± 0.1	11.0 ± 1.0
I	IV	0.4 ± 0.1	8.3 ± 0.8

* Three mice per group were sensitized with 5 μg type I collagen in CFA and challenged on day 6 with 5 μg collagenase-treated collagen preparations. Footpad swelling was determined on day 7 (24 h post-challenge), and the mice were immediately challenged with untreated collagen preparations. Footpad swelling was again determined after 24 h (day 8).

TABLE II

Adoptive Transfer of DTH to Type IV Collagen Using Untreated and T Cell-depleted Sensitized Spleen Cells

Sensitization	Cell treatment	Challenge	Number of mice	Footpad swelling
				mean % ± SE
IV	None	IV	4	9.2 ± 0.8
IV	Anti-Thy-1.2 + C	IV	4	1.1 ± 0.5
I	None	IV	5	7.4 ± 0.8
I	Anti-Thy-1.2 + C	IV	5	2.3 ± 0.4
I	None	I	4	7.2 ± 0.2
I	Anti-Thy-1.2 + C	I	4	1.8 ± 0.6

* Donor mice were immunized with 5 μg antigen in CFA. At day 7, 25×10^6 treated or untreated sensitized spleen cells were transferred per normal recipient.

‡ Recipient mice were challenged with 5 μg types IV or I collagen 48 h after cell transfer. Footpad swelling was assayed 24 h later.

transfer DTH responsiveness to normal syngenic mice is shown in Table II. Spleen cells were obtained from type I or type IV collagen sensitized mice on day 7, the peak of the DTH response (Fig. 1). Mice receiving an injection of untreated type IV collagen-sensitized spleen cells displayed significant footpad swelling 24 h after challenge with type IV collagen. This response was eliminated by T cell depletion of the immune spleen cells with a monoclonal murine anti-Thy-1.2 antibody plus C, as previously described (11). Mice receiving type I collagen-sensitized spleen cells exhibited significant DTH responsiveness after challenge with both types I and IV collagen. Type I collagen-sensitized spleen cells treated with the anti-Thy-1.2 antibody plus C were unable to adoptively transfer a DTH response. These data indicate that sensitized T lymphocytes are responsible for mediating the observed DTH responses to types I and IV collagen.

Immunological studies involving an oligosaccharide are difficult to perform using the classical procedures developed for pure carbohydrates, such as acid hydrolysis, because denaturation of the molecule results. However, periodate oxidation can be performed on the native protein under relatively mild conditions of temperature and pH, and has been shown to degrade the carbohydrate units of glycopeptides (16). Mice sensitized with type I collagen displayed no significant footpad swelling on day 7, when challenged with periodate-treated type IV collagen, as shown in Table III. Periodate treatment of type IV collagen did not significantly alter the previously described (12) DTH response in type IV collagen-sensitized mice. Normal mice exhibited no significant reactivity at any time when challenged with periodate-treated

TABLE III

Periodate Treatment of Antigen Preparations Used for DTH Challenge

Sensitization*	Challenge‡	Number of mice	Footpad swelling
			mean % ± SE
None	Periodate-treated IV	4	1.0 ± 0.5
I	Periodate-treated IV	8	2.5 ± 0.3
IV	Periodate-treated IV	4	9.4 ± 1.1

* Mice were sensitized on day 0 with 5 μg types I or IV collagen in CFA; normal controls were untreated.

‡ Mice were challenged on day 6 with 5 μg antigen, and the footpad swelling was assayed 24 h later (day 7), as indicated in Materials and Methods.

TABLE IV

Glycosidase Treatment of Collagen Preparations

Sensitization	Challenge	Number of mice	Footpad swelling*
			mean % ± SE
None	Glycosidase-treated IV	6	1.5 ± 0.4
I	Glycosidase-treated IV	11	1.1 ± 0.3
Glycosidase-treated I	IV	8	2.9 ± 0.7
I	Glycosidase-treated IV (control)	4	9.2 ± 0.5
IV	Glycosidase-treated IV	4	7.6 ± 0.5
Glycosidase-treated IV	IV	4	7.4 ± 1.1

* Mice were challenged on day 6 post-sensitization, and the footpad swelling was assayed 24 h later, as indicated in Materials and Methods.

TABLE V

Reactivity of α-glucosidase and β-galactosidase Treated Type IV Collagen in DTH Response

Sensitization	Challenge	Number of mice	Footpad swelling*
			mean % ± SE
None	α-glucosidase-treated IV	5	2.4 ± 0.7
None	β-galactosidase-treated IV	5	1.5 ± 0.6
None	α-glucosidase/β-galactosidase-treated IV	6	1.2 ± 0.4
I	α-glucosidase/β-galactosidase-treated IV	11	3.1 ± 0.8
I	α-glucosidase-treated IV	11	1.9 ± 0.3
I	β-galactosidase-treated IV	10	8.5 ± 1.1
I	α-glucosidase/β-galactosidase-treated IV (control)	4	8.5 ± 0.6
IV	α-glucosidase/β-galactosidase-treated IV	4	8.0 ± 1.0

* Mice were challenged on day 6 post-sensitization, and the footpad swelling was assayed 24 h later, as indicated in Materials and Methods.

type IV collagen. These data indicate that the cross-reactive determinants on types I and IV collagen are periodate sensitive and probably related to carbohydrates. The carbohydrate moieties on type IV collagen, however, do not appear to be the sole antigenic determinants responsible for DTH in type IV collagen-sensitized mice.

Because of the heterogenous effects of periodate on glycoproteins (23), types I and IV collagen were treated with a mixture of glycosidases before use in DTH assays. Mice sensitized with type I collagen showed no significant footpad swelling when challenged with glycosidase-treated type IV collagen; in addition, no significant reactivity was observed in groups of mice immunized with glycosidase-treated type I collagen and challenged with untreated type IV collagen (Table IV). However, type I collagen-sensitized mice did display significant reactivity when challenged with a glycosidase-treated type IV control (enzyme added before dialysis). Mice sensitized with untreated type IV collagen or glycosidase-treated type IV collagen did exhibit significant footpad swelling responses when challenged with either treated or untreated type IV collagens. Normal controls showed no significant footpad swelling when challenged with glycosidase-treated type IV collagen preparations. These data give more definitive proof that carbohydrate moieties are involved in the cross-reactive DTH response between types I and IV collagen; however, carbohydrate molecules do not appear to be the major antigenic determinants responsible for mediating the DTH reactivity in type IV collagen-immune mice.

Because the major carbohydrate component found on types I and IV collagen is a dissaccharide unit containing glucose and galactose (3, 6), type IV collagen was treated with specific glycosidases (α-glucosidase, β-galactosidase) and assayed for its ability to elicit a DTH response in mice sensitized to types I or IV collagen (Table V). Mice sensitized with type I collagen in adjuvant and challenged with type IV collagen treated with α-glucosidase alone or a sequentially treated α-glucosidase/β-galactosidase preparation displayed no significant footpad swelling response; however, challenge with β-galactosidase-treated type IV collagen or an α-glucosidase/β-galactosidase type IV collagen control (enzymes added before dialysis) did not alter the previously observed DTH responses (Table V). Furthermore, the α-glucosidase/β-galactosidase-treated type IV collagen preparation was capable of eliciting significant DTH responsiveness in mice sensitized to type IV collagen. Normal mice challenged with

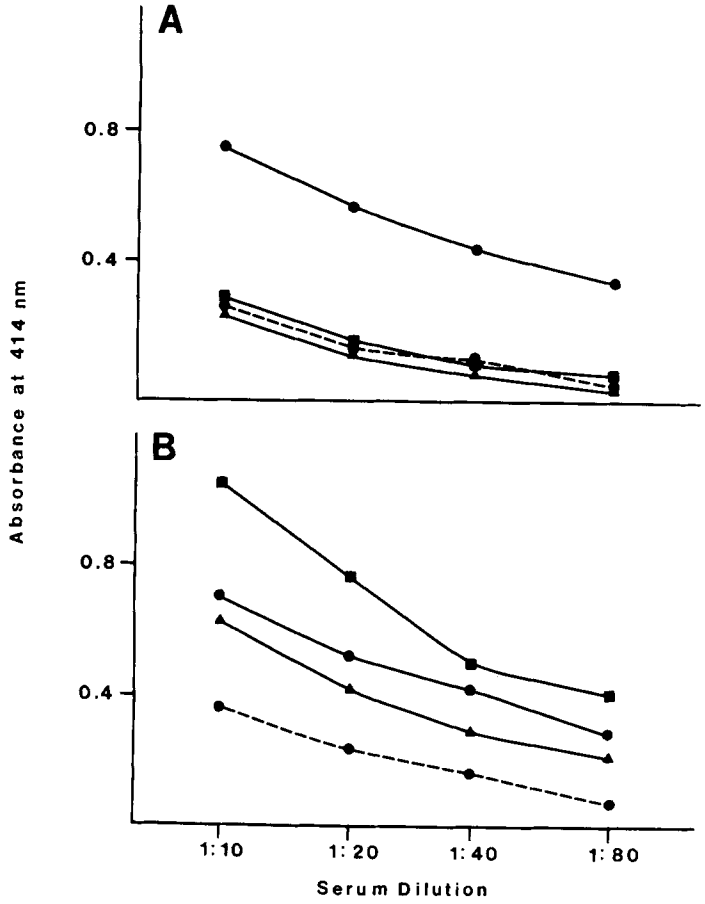

FIG. 2. Analyses of immune and normal mouse sera on (A) type IV and (B) type I collagens using the ELISA. Serial dilutions of mouse anti-type IV collagen sera (●——●), mouse anti-type I collagen sera (■), adjuvant control sera (▲), and normal mouse sera (●----●) were assayed on microtiter wells coated with (A) 0.25 μg type IV collagen and (B) 1.25 μg type I collagen. The results are expressed as the absorbance value at 414 nm.

type IV collagen treated with either α-glucosidase alone or a combination of α-glucosidase and β-galactosidase displayed no significant footpad swelling on day 7. These data indicate that carbohydrate-containing antigenic determinants, specifically those containing glucose, are responsible for cross-reactivity to type IV collagen in type I collagen-sensitized mice.

The ability of C57BL/6 mice to elicit an antibody response to homologous collagens was examined using ELISA. No antibody was detected in sera of mice displaying DTH as a result of a single sensitizing injection of types I or IV collagen (data not shown); however, repeated injections of 50 μg types IV or I collagen in IFA elicited significant antibody responses. The anti-type IV collagen-immune sera displayed significant antibody activity (titer >80) when assayed on wells coated with type IV collagen (Fig. 2A). No significant activity above the level of normal mouse sera was exhibited by the type I collagen-immune sera or adjuvant control sera when assayed on type IV collagen. Significant antibody activity to type I collagen was exhibited,

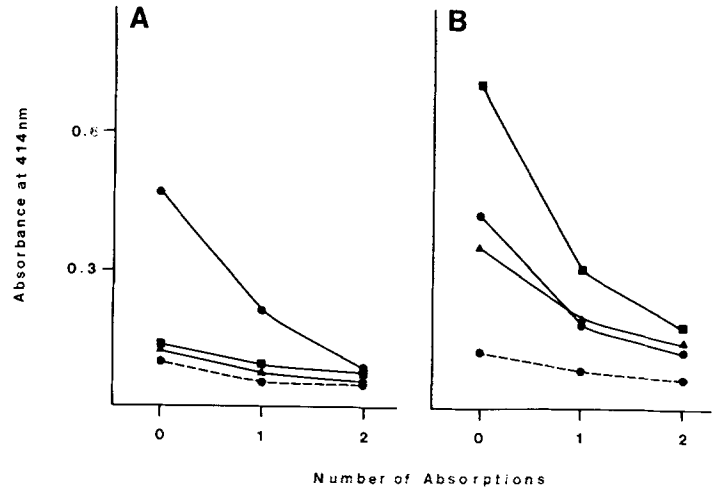

FIG. 3. Repeated absorptions of anti-type IV collagen sera (●——●), anti-type I collagen sera (■), adjuvant control sera (▲), and the normal mouse sera (●----●) on (A) type IV and (B) type I collagen-coated wells were performed to remove all collagen type-specific activity. All sera were assayed at a 1:20 dilution on wells coated with (A) 0.25 μg type IV collagen or (B) 1.25 μg type I collagen. The results are expressed as indicated in Fig. 2.

however, by both type I and type IV collagen-immune sera as well as adjuvant control sera as shown in Fig. 2 B. The high antibody activity of the IFA control sera indicate sensitization to type I collagen as a result of immunization in adjuvant. The intense inflammatory reaction observed in the skin after subcutaneous injection of adjuvant may lead to sensitization to type I collagen with antibody induced to this connective tissue protein.

Sera of mice immune to collagen were assayed for antigen specificity by absorption on types I or IV collagen-coated wells. Repeated absorptions of these sera on types IV and I collagen were performed to determine whether all collagen type-specific activity could be removed. As shown in Fig. 3 A, the reactivity of the type IV collagen-immune sera to type IV collagen was eliminated by sequential absorptions on type IV collagen; no significant reduction in reactivity to type I collagen was observed by the immune sera after absorption on type IV collagen (absorbance values, >0.400). ELISA activity of the anti-type I collagen, anti-type IV collagen, and adjuvant control sera with type I collagen were reduced to the level of the normal mouse sera by sequential absorptions on type I collagen (Fig. 3 B); the reactivity of the type IV collagen-immune sera with type IV collagen was not significantly affected by repeated absorptions on type I collagen (absorbance values, >0.300). These data indicate that antibodies to types IV and I collagen in the sera of collagen-immune mice are antigen specific and do not recognize cross-reactive determinants.

Discussion

After Watson et al. (24) demonstrated collagen to be immunogenic, studies were initiated to investigate the immunological characteristics of the different collagen types, the relation of collagen structure to antigenicity, and the role of collagens as potential autoantigens. Cell-mediated and/or humoral immunity to interstitial collagens (types I, II, and III) and basement membrane collagen (type IV) have been

demonstrated in human disease as well as in experimental animal models (1, 21, 25). Type I collagen has been implicated as an autoantigen in both rheumatic (20, 26–31) and nonrheumatic diseases (32–35). Humoral immunity and CMI to types I and II collagen have been suspected to contribute to the tissue destruction observed in rheumatoid arthritis (26–30, 36). Autoantibodies to type IV collagen have been reported in patients with basement membrane-associated diseases, including scleroderma (20), Goodpasture's syndrome (37), and epidermolysis bullosa simplex (38).

Antibodies to collagen generated in experimental animal models and in human disease appear to recognize collagen type-specific antigenic determinants, as demonstrated by competitive inhibition assays. Although collagenous proteins have been observed to elicit CMI, few studies have attempted to discern the antigen specificity of these responses, primarily because of the experimental limitations that exist in measuring antigen recognition by cells (1). Antigen-specific CMI to interstitial collagens (types I, II, and III) has been demonstrated in experimental systems (39); however, recent studies suggest that CMI to collagen may also result in the induction of cross-reactive cellular responses with other collagenous and collagen-like proteins. Champion and Poole (40) recently reported that rabbits immunized with homologous type III collagen mount a CMI response to the immunizing peptides that also cross-reacts with types I and II collagen. Furthermore, Menzel et al. (41) demonstrated cross-reactivity between human type I collagen and Clq (containing collagen-like domains) in guinea pigs at the level of delayed-type hypersensitivity. In addition, cellular and humoral cross-reactivity have been shown between rat tail tendon type I collagen and the triple helical structure of the electric eel acetylcholinesterase (42), a structure that appears to resemble type IV collagen with respect to amino acid composition and presence of disulfide bonds (10).

In the present study, we demonstrate DTH in type I collagen-sensitized mice that cross-reacts with type IV collagen. Mice sensitized to native or denatured type I collagen in CFA developed significant footpad swelling responses, peaking 7 d postsensitization, 24 h after challenge with native or denatured type I collagen, or native type IV collagen; no response was observed after challenge with denatured type IV collagen or collagenase-treated collagen preparations. Our studies are consistent with previous observations by Adelmann (43) that indicated that sensitization with native or denatured type I collagen results in positive DTH after challenge with native or denatured antigen. The absence of a cross-reactive DTH response between type I collagen and denatured type IV did not appear to be a result of protein modification after reduction and alkylation of type IV collagen, a procedure used to prevent the α chains from reannealing after heat denaturation, as challenge of type I collagen-sensitized mice with reduced and alkylated heat-denatured type I collagen did not significantly alter the DTH responses observed in Fig. 1 (data not shown). Thus, these observations suggest that the cross-reactivity between types I and IV collagen at the level of DTH involves multiple polypeptide chains of the type IV collagen molecule. Although significant antibody reactivity to type I collagen was observed in mice receiving repeated immunizations of either type I or type IV collagen in IFA, these anti-type I collagen antibodies appeared to be a result of immunization in adjuvant, as adjuvant control sera displayed significant activity against type I collagen. Absorption experiments performed to determine the collagen type-specificity of the antisera indicated that the antibodies to types IV and I collagen are antigen

specific and that the antigenic determinants of these two collagens are distinct in this system.

In an effort to further investigate the nature of the cross-reactive antigenic determinant(s) present on types I and IV collagen observed at the level of DTH, a common structural component shared by each collagen species was sought. Carbohydrate components are known to occur in all collagens as a result of a post-translational modifications. The major carbohydrate components of the interstitial collagens are glucose and galactose. Structural investigations (18, 44, 45) indicate that these carbohydrates are glycosidically linked to hydroxylysine in the form of either a glucosylgalactosyl disaccharide (2-*O*-α-*D*-glucopyranosyl-*O*-β-*D*-galactopyranosyl-hydroxylysine) or a galactosyl monosaccharide (*O*-β-*D*-galactopyranosyl-hydroxylysine). Studies conducted by Spiro et al. (18) suggest that basement membranes contain carbohydrate moieties similar to those found in the interstitial collagens. Evidence for a potential antigenic role of collagen carbohydrate moieties was provided by Mahieu et al. (46) in patients with Goodpasture's syndrome. These investigators demonstrated that the hydroxylysine-linked disaccharide of the glomerular basement membrane could specifically react with anti-glomerular basement membrane antibodies from these patients. These antibodies primarily reacted with the antigenic site formed by the β-D-galactosyl-hydroxylysine unit. Furthermore, this disaccharide-containing glycopeptide was able to elicit an in vitro cell-mediated immune response in these patients, as measured by the leukocyte migration inhibition assay. Recent carbohydrate analyses (13, 47, 48) of basement membrane collagen (type IV) derived from several tissues revealed a high concentration of glucosyl and galactosyl groups bound to hydroxylysine. The 7-S region of the type IV collagen, a major structural constitutent of type IV collagen, has been shown by Ristelli et al. (47) to contain ~22% carbohydrate, mainly as glucosylgalactosyl-hydroxylysine.

To evaluate the role of carbohydrate moieties in the cross-reactive DTH response observed between types I and IV collagen, the carbohydrate components were chemically or enzymatically removed from the collagens before immunological analyses. Mice sensitized to type I collagen in adjuvant were unable to mount a DTH response to type IV collagen treated with periodate, a mixture of glycosidases, or α-glucosidase. In addition, mice sensitized to glycosidase-treated type I collagen did not display a footpad swelling response after challenge with untreated type IV collagen. Although β-galactosidase treatment of type IV collagen did not significantly alter the DTH response in type I collagen-immune mice, galactose cannot be ruled out as being a component of the antigenic determinant because previous studies by Spiro et al. (18) indicated that galactose can only be removed enzymatically from the disaccharide unit when the glucose is eliminated initially, Thus, these studies indicate that a glucose-containing antigenic determinant found on both types I and IV collagen is responsible for the cross-reactive DTH response between these two species of collagen.

In light of the previously demonstrated collagen-specific CMI response to type IV collagen in mice sensitized to homologous type IV collagen (12), the "one-way" cross-reactive DTH response between types I and IV collagen is unexplained. Because type IV collagen is heterogenous and contains diverse structural regions, immunization with type IV collagen may result in induction of a CMI response preferentially directed against non-carbohydrate-containing antigenic determinants. Carbohydrates do not appear to play an essential role in the DTH response after sensitization to type

IV collagen because glycosidase-treated type IV collagen, when used to sensitize or challenge, retains its ability to induce a footpad swelling response. In addition, the cross-reactive DTH response does not appear to be the result of lectin-like interaction of T lymphocytes with the collagen carbohydrates. If such a reaction were relevant, a positive footpad swelling response would have been observed in normal mice or glycosidase-treated type I collagen-sensitized mice when challenged with type IV collagen (Fig. 1, Tables IV and V).

Although previous investigations have demonstrated CMI to type I collagen in a number of pathological processes, little is known of the role of cellular immunity to type IV collagen in disease. Tissues containing a high concentration of types I and IV collagen, such as the lung, are frequent sites of pathological fibrosis. We previously demonstrated (20) antibodies to types I and IV collagen in the sera of patients with scleroderma that correlated directly with the extent of interstitial lung disease. Furthermore, Kravis et al. (35) reported that fibrotic lung disease was associated with sensitization of CMI to type I collagen, as evidenced by the production of collagen-induced macrophage inhibition factor. Our observations suggest that the induction of CMI to type I collagen might result in the recognition of antigenic determinants shared by type IV collagen. Such a concomitant immune response to these collagenous proteins could potentially result in the perpetuation of disease processes, resulting in a chronic inflammatory response. Studies are currently underway to examine the possible immunopathological sequelae that might result after the induction of a cross-reactive CMI response to types I and IV collagen in this animal model. These studies might lead to a better understanding of the role of anti-collagen CMI in the pathogenesis of human connective tissue diseases.

Summary

In the present study, we demonstrate delayed-type hypersensitivity (DTH) to homologous type I collagen that cross-reacts with type IV collagen. Mice immunized with native or denatured type I collagens and challenged with these same antigens or native type IV collagen develop a peak DTH response on day 7. Challenge with denatured type IV collagen or collagenase-treated type IV collagen failed to elicit DTH in type I collagen-sensitized mice. Type I collagen-sensitized spleen cells adoptively transferred DTH to types IV and I collagen to normal recipients; T cell-depleted spleen cells failed to transfer immunity. Periodate-treated type IV collagen did not elicit DTH in mice sensitized to type I collagen; however, mice sensitized with type IV collagen displayed significant DTH when challenged with periodate-treated type IV collagen. Furthermore, treatment of type IV collagen with a mixed glycosidase or α-glucosidase before challenge eliminated the DTH response in type I collagen-sensitized mice; β-galactosidase treatment of type IV collagen had no effect on this response. Mice sensitized with type IV collagen, however, displayed significant DTH when challenged with these glycosidase-treated antigens. Antibodies produced to types I and IV collagen by repeated immunizations were specific for the sensitizing antigen and did not react with other connective tissue antigens. These studies indicate that a CMI response to type I collagen recognizes similar antigenic determinants on the type IV collagen molecule. These cross-reacting determinants are dependent on conformation and contain carbohydrates, particularly glucose residues.

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