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Anti-collagen antibodies in systemic sclerosis and in primary Raynaud's phenomenon

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SUMMARY

The frequency and specificity of antibodies to native and denatured collagens were evaluated in systemic sclerosis (SSc) and in primary Raynaud's phenomenon (PRP) by direct and competitive ELISA. Antibodies reactive with denatured collagen type I (CI) were found in 43% of the SSc sera, and anti-CIV and anti-CV in 31%. In PRP, anti-CI, anti-CIV and anti-CV antibodies were detected in 8% of patient sera. Anti-CI, anti-CIV and anti-CV antibodies reacted with determinants expressed on the native as well as on the denatured molecule. Anti-CI and anti-CIV were cross-reactive; a reactivity with CII and a lower one with CV were detected. Anti-CV antibodies also reacted with CI and CII and, in a smaller proportion of cases, with CIV. Anti-collagen antibodies, affinity-purified from blotted collagen IV and V and cyanogen bromide (CBr)-digested CI, displayed the cross-reactivities shown by inhibition studies on sera. Moreover, antibodies eluted from a CBr fragment of CI reacted with the other CBr fragments as well. These data show that one-third of SSc sera contain antibodies that react with epitopes expressed on native as well as on heat-denatured CI, CII, CIV and CV, and therefore have the potential to bind collagens *in vivo*.

Keywords systemic sclerosis Raynaud's phenomenon autoantibodies collagen

INTRODUCTION

The main proteins of the connective tissues are collagens, whose basic structure consists of three polypeptide α chains in the form of a triple helix with repeating triplets, often glycine-proline-hydroxyproline. At least 18 distinct types of collagens have so far been described, with specific distributions within individual tissues. Collagens type I (CI), II (CII), and V (CV) are members of the group 1, or fibrillar forming collagens; CV is a minor connective tissue constituent with a nearly ubiquitous distribution. Collagen IV (CIV) is a basement membrane component that forms a mesh-like network in the extracellular matrix.

Collagens are phylogenetically highly conserved proteins and, like many other molecules with this feature, can form the target of autoimmune responses [1].

Systemic sclerosis (SSc) is an autoimmune disease characterized by the excessive deposition of collagen and other matrix components in the skin and internal organs, including the kidneys, lungs, heart and gastrointestinal tract. In SSc patients serum antibodies and lymphocytes reactive with collagen have been found [2]. Autoimmunity to collagen has also been reported in primary Raynaud's phenomenon (PRP), a vasospastic disorder of the extremities consisting of an intermittent constriction of the small arterioles followed by venostasis and hyperaemia [3]. The exact role of the autoimmune response to collagen in these disorders is, however, still unknown.

While the presence of antibodies to denatured collagens seems to have been well established by different authors, conflicting results are obtained when antibodies to native collagens are measured [3,4]. The aim of this study was to evaluate the frequency and specificity of antibodies to native and denatured CI, CIV and CV in patients affected by SSc and PRP.

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PATIENTS AND METHODS

Patient population

Sera were obtained from 35 patients (six males and 29 females) with SSc and from 12 patients with PRP (one male and 11 females), ranging in age from 22 to 78 years (mean age 51 years). The diagnosis of SSc was established on the basis of the ARA criteria [5]; 24 patients suffered from a diffuse form of SSc and 11 from a limited form. The presence of Raynaud's phenomenon was established using a cold provocation test, the results of which were documented by photoplethysmography. Raynaud's phenomenon was classified as primary if

it had been present for at least 2 years and was not associated with clinical or serological findings of connective tissue diseases. All patients with PRP included in this study had normal nailfold capillaries.

Detection of antibodies to centromere and DNA topoisomerase I
Antibodies to DNA topoisomerase I (Scl70) were measured by counterimmunoelectrophoresis and anti-centromere antibodies by immunofluorescence on Hep2 cells. Anti-centromere antibodies were detected in 4/35 (11%) SSc patients and in 2/12 PRP patients; anti-Scl70 antibodies were detected only in SSc patients (21/35, 63%).

Detection of anti-collagen activity

ELISA. Levels of IgG antibodies to CI, CIV and CV were measured in all patients by ELISA, as previously described with minor modifications [6,7]. The cross-reactivity of collagen antibodies with CII was also tested. CI, CIV and CV from human placenta (all supplied by Sigma Chemical Co., St Louis, MO) and CII from bovine cartilage (supplied by SERVA Feinbiochemica GmbH, Heidelberg, Germany) were dissolved in 0·05 M acetic acid; heat-denaturated collagens were obtained by boiling the dissolved proteins for 5 min. The collagens were then diluted in 0·05 M carbonate buffer pH 9·6 to 20 μg/ml and used to coat Nunc (Roskilde, Denmark) microtitre plates (50 μl/well).

After blocking with 3% bovine serum albumin (BSA) for 1 h at room temperature, purified antibodies or human sera diluted 1 : 100 in PBS–0·05% Tween 20 containing BSA 1% (diluting buffer) were added. The plates were incubated for 4 h at room temperature, and then washed once with PBS–Tween 1% and twice with PBS; 50 μl of an alkaline phosphatase-conjugated $(Fab)_2$ fragment of goat anti-human IgG (Sigma) in diluting buffer were then added and the plates were incubated overnight at 4°C. After washings, bound enzymic activity was measured with *p*-nitrophenyl-phosphate. Each serum was tested on a control plate not coated with the antigen. This non-specific binding never exceeded 10% of the specific binding (e.g. to the antigen-coated plates). For the competitive assays, the amount of serum that gave 50% of the maximum binding was pre-incubated with different amounts of collagens or buffer for 1 h at 37°C and was then transferred to collagen-coated plates. The assay was then carried on as the direct binding assay.

Twenty-five sera from normal subjects (matched for age and sex with the patients) were used as the control group. Values higher than the mean + 3 s.d. of the control group were considered positive.

Immunoblot. Cyanogen bromide peptides (CBr-peptides) of CI were prepared by treating a 2 mg/ml solution in 70% formic acid with an excess of CBr as described by Miller [8].

The lyophilized CBr-peptides were dissolved directly into the sample buffer and loaded into adjacent wells of a 15% resolving gel. After electrophoresis, the CBr-peptides were electrophoretically transferred from the SDS-polyacrylamide gels into nitrocellulose [9]. After the transfer, the nitrocellulose membrane was cut into strips. Briefly, the filters were saturated by incubation for 1 h in 0·05 M Tris 0·15 M NaCl 5% dry non-fat milk. The same buffer was used for antibody dilutions and washings. Purified antibodies or sera diluted 1 : 500 were incubated on filters for 4 h at room temperature. After repeated washings, alkaline phosphatase-conjugated goat anti-human IgG (Sigma) was added and the filters were incubated overnight at 4°C. The immunoreactive bands were visualized using 5-bromo-4-chloro-indoxyl-phosphate and nitroblue tetrazolium as substrate [10].

Isolation of anti-collagen antibodies. To isolate the anti-CI antibodies, the nitrocellulose strips containing the lower molecular weight polypeptides targeted by serum antibodies were excised from the blotted CBr-peptides. To isolate the anti-CIV and anti-CV antibodies, collagens diluted at 200 μg/ml were dot blotted on nitrocellulose strips. The strips were saturated in 0·05 M Tris 0·15 M NaCl 5% BSA and incubated with sera diluted 1 : 5 in 0·05 M Tris 0·15 M NaCl for 1 h at room temperature. The strips were washed five times in the same buffer, then the bound antibodies were eluted by 0·1 M glycine pH 2·8 and immediately neutralized by Tris.

RESULTS

We measured IgG antibody binding to denatured CI, CIV and CV by ELISA in 35 SSc sera and in 12 PRP sera. Positive results (higher than the mean + 3 s.d. of the values for normal subjects) were obtained in 15/35 (43%) of the SSc patients on CI, and in 11/35 (31%) on CIV and CV (Fig. 1). In the diffuse form of the disease, anti-CI antibodies were detected in 13/24 patients (54%, confidence limit 33–74%); in the limited form of the disease they were detected in 2/11 (18%, confidence limit 2–52%). In PRP, anti-CI, anti-CIV and anti-CV antibodies were detected in 1/12 patients (8%) (Fig. 1).

In the SSc group most of the sera containing anti-CI antibodies reacted with all of the collagens. In fact, out of the 15 sera reactive with CI, 11 also contained antibodies to CIV and to CV. These results can be explained either by the presence in the sera of different antibodies, each one reactive with a single collagen type, or by the reactivity of the same antibody population with different collagen types. To test these two possibilities, we performed the cross-inhibition studies summarized in Table 1. Antibody binding to denatured CI, CIV or CV on the solid phase was evaluated after pre-incubation of the sera with native or denatured CI, CII, CIV and CV. As shown

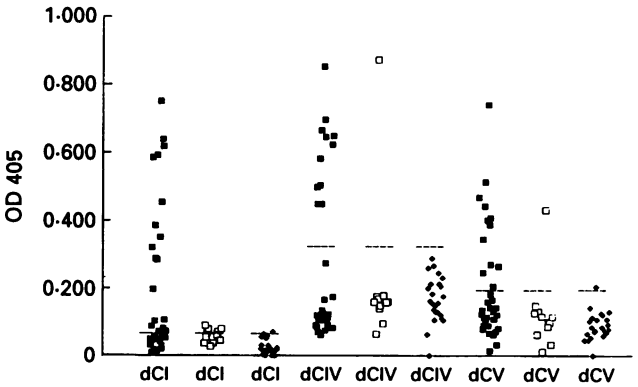

Fig. 1. Antibodies to denatured collagens in systemic sclerosis (SSc), primary Raynaud's phenomenon (PRP) and controls. Sera were incubated on collagen-coated plates and bound IgG antibodies were detected by alkaline phosphatase-labelled anti-human IgG. Results are expressed as absorbance at 405 nm, 30 min after substrate addition. The broken lines indicate the upper limit of normal (mean + 3 s.d. of the control group). ■, SSc; □, PRP; ◆, controls.

Table 1. Specificity of anti-collagen antibodies in systemic sclerosis (SSc)

Patient no.	nCI	dCI	nCIV	dCIV	nCV	dCV	nCII	dCII
Inhibition of binding to dCI								
1	19	3	123	19·3	95·6	81	ND	ND
2	21·4	1·8	200	26·8	97·8	77	>200	<3
3	12	3	>200	35·5	87·6	94	>200	3
4	22	0·8	>200	12	80·3	78	76	1
5	29	<0·8	175	27·6	88·4	58	100	3
6	3	0·8	60	6·7	98·6	81	>200	<3
Inhibition of binding to dCIV								
1	<0·8	<0·8	50	<3	>200	112	ND	ND
2	<3	<3	50	4·9	>200	100	78	0·3
3	<3	<3	50	6·6	>200	112	38	0·45
4	<3	<3	>200	12	138	ND	40	ND
5	<3	<3	200	12	50	ND	50	5
6	5·5	<3	12	13·2	187·2	14·9	50	ND
7	<3	<3	>200	12	200	ND	ND	0·3
8	12	0·8	>200	5·3	86·4	7·3	78	0·3
9	5·3	0·8	>200	10·3	127·5	16·6	56	2·2
10	2·3	0·8	90	8·6	110·8	15·0	73	7·8
11	ND	12	29	12	73	100	40	1·25
12	8·3	3	>200	12	ND	ND	ND	ND
Inhibition of binding to dCV								
1	ND	50	55·4	3·4	21	1·19	ND	ND
2	ND	25	ND	3	50	16·9	25	ND
3	ND	50	ND	>200	50	1·1	17	<1
4	26	33	ND	3	26·0	2·1	32	0·7
7	100	50	ND	>200	>200	33·7	ND	5
8	ND	<0·8	ND	>200	5·4	1·4	60	0·6

The binding of SSc sera to denatured (d) CI, CIV, CV was tested after preincubation with native or denatured collagens. Results are expressed as micrograms of antigen required to inhibit 50% of the binding.

in Table 1, antibody binding to CI was inhibited by liquid phase denatured CI, by native CI and by denatured CIV and CII. Native CIV, denatured CV, native CV and native CII were much less efficient inhibitors.

Similarly, antibody binding to denatured CIV was inhibited by denatured CIV, by native and denatured CI, and by denatured CII. Native CIV, native CV, native CII and denatured CV either inhibited only at high concentrations or did not inhibit binding at all. Cross-reactivity with CV was detectable in some of the sera (cases 6, 9, 10).

Anti-CV antibodies recognized determinants present on both the native and the denatured molecule; they also bound CI and CII, and (in cases 1, 2, and 4) CIV.

No inhibition of antibody binding was observed when the antigen solution was pre-incubated in the coated plates before addition of serum (data not shown). This result shows that the liquid phase collagen does not inhibit antibody binding through an interaction with the solid-phase collagen. We can therefore conclude that the inhibitions observed were dependent only on the binding of the antibodies to collagen in the liquid phase.

To analyse further the specificity of anti-collagen antibodies, we isolated anti-denatured CIV or CV antibodies by acid elution from dot blotted collagens and tested their reactivity by ELISA. Purified anti-CIV antibodies bound to CIV, CI and, to a lesser extent, to CV (Fig. 2a). Purified anti-CV antibodies bound CI and CV (Fig. 2b). On the whole, these results confirmed the cross-reactivities shown by our cross-inhibition studies.

To map the epitopes recognized by the anti-CI antibodies on the molecule, we tested sera reactivity with CBr-digested CI. Since the aI(I) and a2(I) chains were not separated prior to digestion, the fragments obtained originated from both chains. Their molecular weights could not be estimated on the basis of the usual reference curves, since collagen molecules migrate more slowly than globular proteins of similar size, and fragments of the same size do not always migrate at the same rate [11]. Thus, the molecular weight values reported here represent only the internal standards of the experiment.

Eight normal human sera, eight PRP and 14 SSc sera, 10 of which contained anti-CI antibodies by ELISA, were tested on blotted CBr-digested CI. Eleven SSc sera (10 positive on ELISA and one negative) reacted in the immunoblot and detected the same set of polypeptides, whose molecular weight ranged between 100 kD and 38 kD. A representative example of sera reactivity is given in Fig. 3. No binding was observed to the lower molecular weight polypeptides, although protein staining of blotted CBr-digested CI revealed significant bands of 29, 25 and 18 kD. Antibodies specific for the three lowest molecular weight bands (1, 58 kD; 2, 45 kD; 3, 38 kD) were acid-eluted and used to probe the blotted CBr-digested CI. The results, shown in Fig. 4, indicate that the same antibody

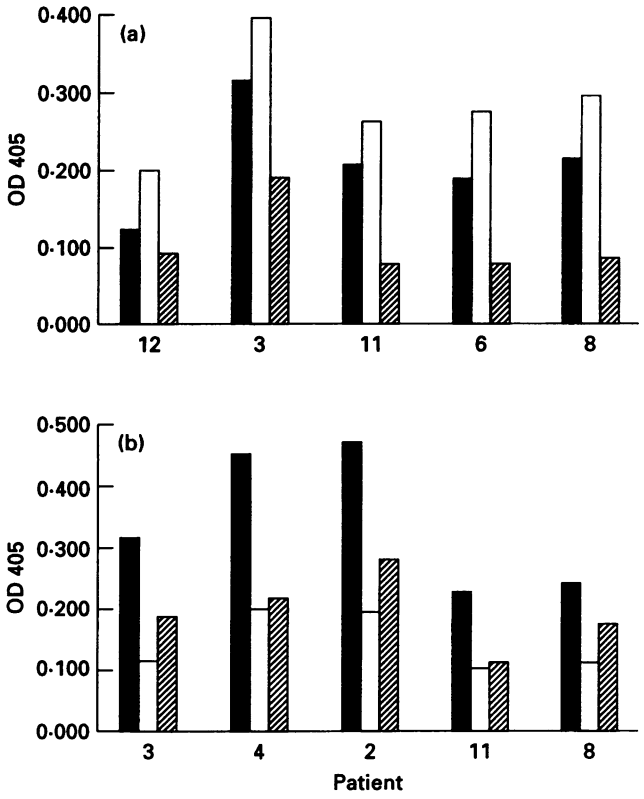

Fig. 2. Binding of affinity-purified anti-denatured collagen type IV (CIV) (a) and anti-denatured CV antibodies (b) to denatured CI, CIV, CV. Anti-CIV and anti-CV antibodies, affinity-purified from blotted denatured CIV and CV, were tested by ELISA on denatured CI (■), CIV (□) and CV (▨). Bound IgG antibodies were detected by alkaline phosphatase-labelled anti-human IgG. Results are expressed as absorbance at 405 nm, 30 min after substrate addition. Anti-denatured CIV antibodies bound to denatured CIV, CI and, to a lesser extent, to CV; anti-denatured CV antibodies bound to denatured CI and CV and, to lesser extent, to CIV.

population reacts with determinants shared by different fragments, and suggest that the epitopes are repeated along the collagen chains. Antibodies eluted from the three bands were tested by ELISA on denatured CI, CIV and CV (Fig. 5). Antibodies specific for any one of the three bands bound to a similar extent to CI and CIV and displayed a lower cross-reactivity with CV.

DISCUSSION

The data presented in this study show that anti-collagen antibodies are detectable in one-third of SSc sera and in 8% of PRP patients. Their frequency is higher in the diffuse than in the limited form of the disease. The presence in the serum of anti-collagen antibodies might herald the onset of SSc, and in particular of a severe form of the disease. A follow-up study of apparently primary Raynaud's phenomenon and SSc patients should be performed in order to evaluate the predictive value of the detection of anti-collagen antibodies.

Gabrielli *et al.* [3] reported the presence of anti-CIV antibodies in 10/48 (21%) PRP patients. However, in 8/10 patients positive for anti-CIV antibodies, serological and clinical features of a systemic disorder were present, and it is conceivable that some of these patients will develop SSc. The PRP patients whom we studied, on the contrary, showed no signs or symptoms of systemic disease, and only two patients had low-titre anti-centromere antibodies. The differing percentages of positivity for anti-CIV antibodies found in the two studies might therefore be due to different patient selection procedures. It should also be noted that in our study the upper limit of normal was the mean + 3 s.d. of the control group, while a limit of the mean + 2 s.d. was chosen by Gabrielli *et al.*

The frequency of anti-collagen antibodies detected in our SSc patients was also lower than the values reported by others. Gabrielli *et al.* [3] detected anti-CIV antibodies in 68% SSc patients; Mackel *et al.* [4] detected anti-CIV antibodies in 50% of their SSc patients and anti-CI in 60%. A different patient population (i.e. the proportion of limited *versus* diffuse cases) and/or a number of differences in the assays employed and the antigens used could account for these discrepancies. Murine collagens were in fact employed by these authors, while human

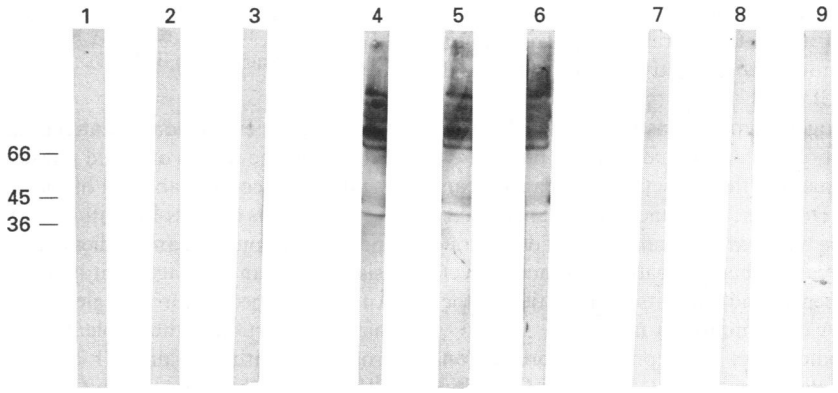

Fig. 3. Binding of sera to cyanogen bromide (CBr)-digested collagen type I (CI). CBr-digested CI was separated by SDS gel electrophoresis and transferred to nitrocellulose. Arrows indicate molecular weight markers. Three normal sera (1–3), three systemic sclerosis (SSc) sera (4–6) and three primary Raynaud's phenomenon (PRP) sera (7–9) were incubated on blotted CBr-digested CI. The same set of polypeptides, whose molecular weight ranged between 100 kD and 38 kD, was detected by the three SSc sera.

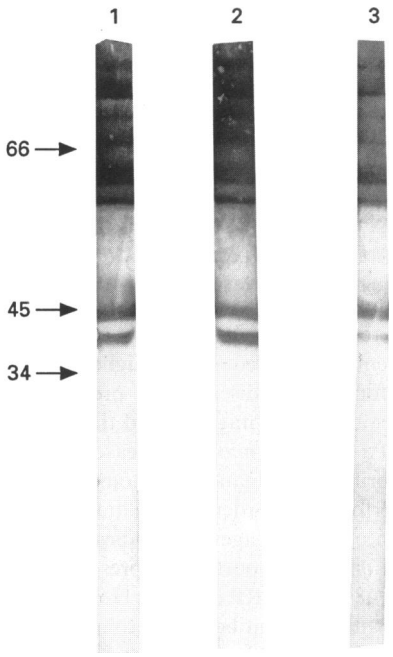

Fig. 4. Binding of affinity-purified anti-collagen type I (CI) antibodies to cyanogen bromide (CBr)-digested CI. CBr-digested CI was separated by SDS gel electrophoresis and transferred to nitrocellulose. Arrows indicate molecular weight markers. Antibodies specific for the three lowest molecular weight bands (1, 58 kD; 2, 45 kD; 3, 38 kD) were acid-eluted from patient 2 serum and used to probe CBr-digested CI. The results indicate that the same antibody population reacts with determinants shared by different fragments.

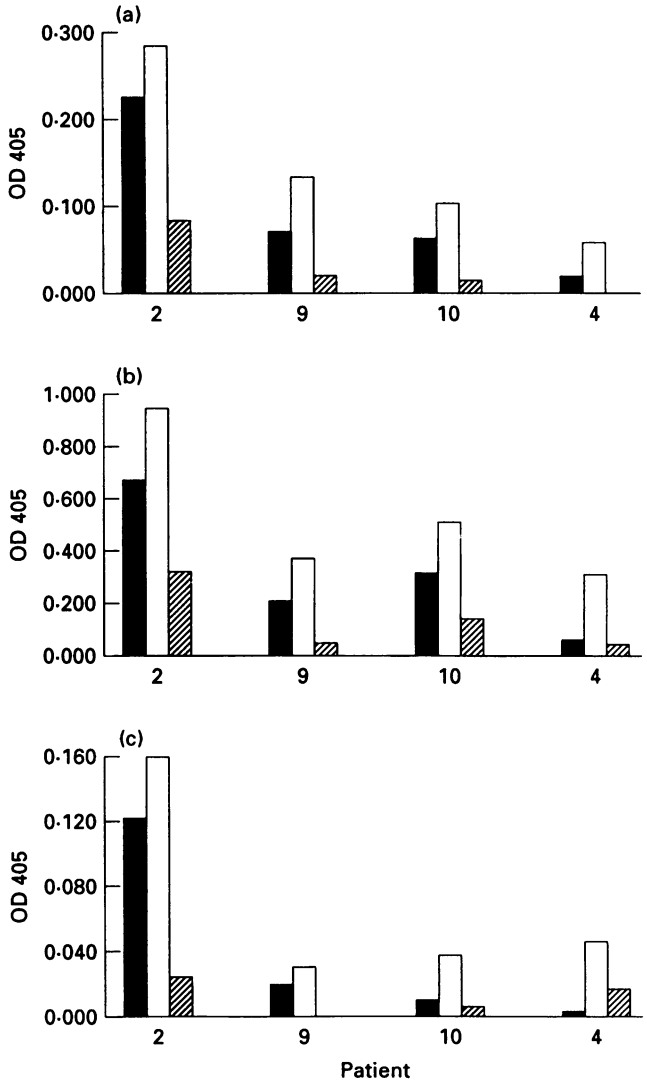

Fig. 5. Binding of antibodies eluted from three bands of blotted cyanogen bromide (CBr)-digested collagen type I (CI) to denatured CI, CIV, CV. Antibodies specific for the 58-kD (a), the 45-kD (b) and 38-kD (c) bands were acid eluted from blotted CBr-digested CI and tested by ELISA on denatured CI, CIV, CV. Results obtained with four patients' sera are shown. Antibodies specific for any one of the three bands showed a similar reactivity to denatured CI (■) and dCIV (□), but a lower reactivity to dCV (▨).

collagen was used in our assay. Although collagens show striking inter-species similarities, the existence of species-specific epitopes on CIV [12] has been previously described.

In our assay, we measured only IgG anti-collagen antibodies, while in the other cited papers the G, A and M immunoglobulins were also determined. The different procedures used in the ELISA assay should also be taken into account: harsher conditions for the washings (PBS–1% Tween) prevailed in our assay and may have limited the detection of the lower affinity antibodies. On the other hand, in a group of rheumatoid arthritis patients (data not shown) the frequency of anti-CII antibodies detected by us (10%) was very close to the values reported in the literature [1]. These antibodies display a very limited cross-reactivity with native and heat-denatured CI and CIV.

The purity of the antigen preparations used in our laboratory excludes a contribution of contaminants in the binding of the sera. Pepsin, which is used in the extraction of collagens, can bind immunoglobulins. However, an intact three-dimensional structure of the enzyme is required for binding. Heat denaturation or SDS and β-mercaptoethanol treatment should affect the tertiary structure of pepsin and make its contribution to the binding of sera in ELISA or immunoblot negligible.

Anti-collagen antibodies react with epitopes expressed on native as well as on heat-denatured CI, CIV, CV and CII. This conclusion is based on the results of liquid phase inhibition studies, in which the antibodies bind to truly native antigens in solution. Adsorption to the solid phase can in fact cause changes in the conformation of the molecules, and it is debatable to what extent coated antigens can be considered 'native'. Moreover, the results of liquid phase assays are not affected by the different coating abilities of the various antigens.

The results of cross-inhibition studies and the analysis of the specificity of purified antibodies are consistent with the hypothesis that the anti-collagen antibodies are widely cross-reactive. Antibodies specific for a single collagen type represent only a minor proportion of the whole. In fact, most anti-CI antibodies cross-react with CIV and CII; anti-CIV also react with CI and CII and, in a few cases, with CV; anti-CV react with CI, CII, CIV. This is not surprising, since the structural similarities among collagens are well known.

Analysing the reactivity of sera with fragments of CI, we found that the SSc sera react with several CBr-peptides.

Similarly, sera from women with silicone breast implants who developed anti-CI antibodies reacted on immunoblot with several CI fragments [13]. A similar reactivity with multiple CBr-peptides was also observed by the authors, who mapped the epitopes recognized by rheumatoid arthritis and systemic lupus sera on CII [14,15]. Multiple polypeptides were recognized by these sera and no disease-specific pattern could be identified. Cyonomolgous monkeys, which develop arthritis after CII immunization, show a similar pattern of response to multiple CBr-peptides [16]. In contrast, in mice and rats susceptible to collagen-induced arthritis an arthritogenic epitope, CBr 11, has been identified in one of the CBr-peptides of CII [17,18]. It is likely that a polyclonal response to CII characterizes human arthritis; the possible existence of an epitope common to several CBr-peptides, however, has not been excluded. Studying the anti-CI immune response in SSc patients, we showed that antibodies eluted from individual CBr-peptides react with all of the polypeptides detected by whole sera. These results suggest the existence of recurrent epitopes on CI molecules.

In conclusion, this study shows that IgG antibodies reactive with native and denatured collagens are detectable in one-third of SSc sera. Antibodies reactive with basement membrane collagens may contribute to tissue damage: whenever any injury exposes the basement membrane constituents, local immune complex formation and complement fixation may occur. Passive transfer of anti-collagen antibodies should be performed to elucidate the pathogenic role of the humoral immune response to collagens.

Alternatively, anti-collagen antibodies could represent the by-product of a cellular immune response taking place in SSc, a response which may play an important role, through interleukin release, in the abnormal collagen deposition that characterizes this disorder.

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