Connective Tissue Autoantibodies in Dupuytren's Disease: Associations with HLA DR3

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Not true: Source article had no statistical analysis re: anticollagen antibodies and concluded "Our results do not bolster the hypothesis of collagen as the auto-antigen"

Introduction

The hypothesis that autoimmune phenomena might be associated with the pathogenesis of Dupuytren's contracture (DC) was advanced by Gay and Gay in 1972 [1]. This hypothesis was bolstered by Menzel et al. [2], who demonstrated the presence of circulating antibodies to collagen (ACA) in DC patients. These results were confirmed by Pereira et al. [3], showing a whole spectrum of autoantibodies to different collagen types in these patients. The mere demonstration of autoantibodies to connective tissue components, however, does not imply that these antibodies are instrumental in the pathogenesis of a disease. It only proves that autoimmune processes accompany the development of the disease, if only as innocent bystanders. The suggestion that autoantibodies to collagen might contribute to the perpetuation of DC is problematic, since the inflammatory component of this disease is not very pronounced. This is in contrast to the situation in rheumatoid arthritis (RA), which is characterized by circulating immune complexes, complement activation and ACA.

The basis of collagen autoimmunity seems to be an immunogenetic disposition. Thus, as shown by Klimiuk et al. [4], RA patients presenting with autoantibodies to native type II collagen (cartilage collagen) represent a distinct genetic subset characterized by an association with HLA DR3. In contrast, antibodies to denatured type II collagen are associated with HLA DR4, the class II histocompatibility antigen to which susceptibility for RA has been strongly linked [5]. Interestingly, an analogous association was demonstrated by Pereira et al. [3] in patients with DC. The condition itself may be familial. Two early studies [6,7] showing no association with the HLA system were followed by two investigations including the HLA DR locus [8,9]. According to these more recent publications there is a suggestion of an association with HLA DR4 and HLA B12, with a raised overall prevalence of the HLA-A1-B8-DR3 haplotype, although these associations did not reach statistical significance. A synopsis of HLA associations with collagen or elastin autoimmunity is presented in Table 1.

The immediate cause for the formation of autoantibodies to the various collagen types is unknown. Pereira et al. [3] suggested that the raised incidence

Table 1. Autoimmunity to collagen and the HLA system

Disease	Anti-collagen antibodies (type)	HLA association	Reference
Thromboangiitis obliterans	I	A1/B8	Smolen et al. [13]
RA	II den	DR 4	Rowley et al. [5]
RA	II	DR3/7	Sanders et al. [22]
RA	None	DR 4	Sanders et al. [22]
DC	II den	DR 4	Pereira et al. [3]
DC	I	DR 3	Menzel et al. (this chapter)
	Elastin	DR 3	

den, denaturated collagen.

of antibodies to native type III collagen may result from the considerable increase in the production of this collagen type at the site of the fibrotic lesion [10]. In contrast to collagen from the aponeurosis of normal adult subjects, the nodules, contracted bands and even the apparently uninvolved palmar fascia of DC patients contain substantial amounts of type III (fetal) collagen. These results were obtained by qualitative examination of SDS-polyacrylamide gels performed with pepsin digests of tissue samples. No information was given regarding how much total collagen was solubilized. The incidence of antibodies to type II collagen in DC patients [3] is intriguing, since this collagen type is rarely found even in the diseased palmar fascia [11]. The only possible interpretation of such a finding would be cross reactivity of ACA with different collagen types.

Here, we have attempted to correlate HLA typing results with ACA to collagens type I–IV and antielastin antibodies (ELAB) in DC patients and controls. Antibodies to kappa-elastin of bovine origin were first described in sera of severely atheromatous patients by Stein et al. [12]. ELAB are also found together with ACA in patients with thrombangiitis obliterans [13], another disease showing significant associations with the HLA system. In addition, we describe a sensitive enzyme immunoassay (EIA) for collagen type III and its utilization for determining the ratio of collagen type I: collagen type III in normal palmar aponeurosis and Dupuytren's lesions.

Materials and Methods

Enzyme Immunoassay for Antielastin Antibodies

Solubilized forms of elastin retain their immunological reactivity and can therefore be used for the detection of ELAB in solid phase assays. Among the several published methods to solubilize elastin we chose the procedure described by Robert and Poullain [14]. Starting material was a commercial preparation of insoluble elastin from bovine ligamentum nuchae (Sigma E

1625). What follows is a description of the preparation of soluble kappa 2 elastin: elastin powder (10 g) is suspended in 250 ml of 1 *N* KOH/ethanol (80:20, v/v) and stirred at 37°C for 60 min. After centrifugation at 16000 g for 15 min the pellet is resuspended in KOH/ethanol and again heated for 60 min. The neutralized supernatant is lyophilized and redissolved in 0.02 *M* acetic acid. The soluble elastin is chromatographed on a Sephacryl S 200 column (Pharmacia) of the dimensions 1.8 × 26.0 cm (Fig. 1). Calibration was performed with human IgG and bovine serum albumin as molecular weight markers. For the assay of ELAB the molecular weight fraction between 40000 and 65000 (corresponding to the maximal molecular weight eluted) was used as soluble elastin antigen in the EIA procedure. To this end, the wells of NUNC microtiter plates were coated with 0.2 ml of the kappa 2 elastin solution of defined molecular weight range (0.1 mg/ml in a pH 7.5 phosphate or Tris buffer, 0.02 *M*, containing 0.45 *M* NaCl and 0.02% sodium azide). Incubation was for 3 days at 4°C. The coated plates were washed and incubated for 2 h at room temperature with phosphate buffered saline (PBS), containing 0.4% bovine serum albumin (BSA), to reduce nonspecific binding effects. After washing, 0.2 ml of a 1:20 dilution with PBS-0.1% Tween of each sample to be tested was added to each well (triplicate assays). Incubation was for 30 min at 37°C and 60 min at 4°C. After a threefold washing procedure, the second antibody, a 1:1000 dilution of anti-human IgG coupled to horseradish-peroxidase, in PBS-BSA (1%) without azide was added. Incubation and washing cycles were as above. Finally, the substrate ABTS was added and extinction measured in an ELISA reader at 405 nm after 10–30 min, depending on the intensity of color developed.

Each result is reported as the mean of triplicate assays, converted to the number of standard deviations of the normal control population above the normal mean value, as described by Wener et al. [15].

Enzyme Immunoassay for Anticollagen Antibodies

Antibodies to collagen types I–IV were determined by an analogous procedure as for ELAB. Coating was done at a much lower antigen concentration (5 μg/ml). Collagen type I was prepared from human infant dura mater, type II from human cartilage, type III from human skin and type IV from a commercial preparation of human placenta (Sigma C 7521). All collagens were extracted by peptic digestion and purified by salt fractionation [16]. They were used only in native form as EIA antigens. Evaluation of results was as described above for ELAB. Only duplicate assays were performed.

Quantitation of Collagen Type III in Tissue Samples

Samples were obtained immediately postoperatively and deep frozen or immediately processed. After complete removal of blood by thorough washing

with PBS, the soaked samples were dried with paper towels and wet weight was determined. After mincing with scissors the tissue fragments were frozen in liquid nitrogen and homogenized in a Braun Dismembrator II (Teflon chambers, 3.0 ml, 9 mm balls from a ball bearing). All samples were completely transformed into powder. Digestion with pepsin of high purity (Sigma P 6887) was performed at an enzyme to substrate ratio of 1:10 in 0.5 *M* acetic acid for three days at 4°C. After ultracentrifugation the supernatants were assayed for type III collagen and total collagen. The collagen type III fraction was quantitated by a sandwich EIA method: briefly, anti-type III antibody from goat (Southern Biotechnology) was coated to NUNC microtiter wells at a dilution of 1:100. The antigen extract was then incubated with the solid phase and – after thorough washing – the bound type III collagen detected by second antibody (monoclonal anti-type III collagen from mouse, Heyl) at a dilution of 1:200, followed by an incubation step with sheep anti-mouse Ig in peroxidase-linked form (Amersham) at a dilution 1:2000–1:4000. Color was developed after adding ABTS. Different concentrations of pure type III collagen were analyzed in the same way to obtain a standard curve in the range 0.1–100 µg/ml type III collagen. Total collagen in the pepsin extract was assayed via Stegemann's method [17]. A direct quantitation of type I collagen by an analogous EIA method proved not feasible since the anti-type I antibodies available were of low quality. As control, immunofluorescence staining of formalin-fixed or frozen tissue sections was performed using anti-type III antibodies and anti-Ig antibodies in FITC-linked form (second antibodies). As an alternative, peroxidase-labeled second antibodies were used.

HLA Typing

The determination of HLA antigens of class I (HLA A, B, C locus) was performed using the microlymphocytotoxicity test (MLCT), NIH standard technique [18], while testing of the class II HLA antigens (only HLA DR subregion) was performed by the double fluorescence MLCT [19]. Every specificity was tested with three antisera of a different serum charge.

Statistics

As usual in HLA investigations, the chi^2 test obtained from 2 × 2 contingency tables was carried out. If one or more fields of the contingency tables were occupied by numbers <10, the Yate correction was performed. From the 2 × 2 contingency table relative risk was calculated as follows [20]:

$$RR = \frac{\text{Concordance} \times \text{absence of antigen and disease}}{\text{Discordance} \times \text{presence of antigen in absence of disease}}$$

Patients

There were 45 patients who suffered from DC and underwent surgical therapy; 59 healthy controls were selected for this study. HLA typing was performed in 42 DC patients and 47 controls; ELAB were determined in 38 patients and 50 controls; ACA in 22 DC patients and 50 controls.

Results

Autoantibodies to Collagen in DC Patients

The highest incidence of ACA in DC patients was observed for antibodies to type III collagen, closely followed by those to type I collagen. Autoantibodies to type IV collagen (basement membrane collagen) and type II collagen (cartilage collagen) were present in a minority of DC patients (Table 2). It is interesting to note that ACA-positive patients became negative upon reinspection 6 months after the first venipuncture (with the exception of one of seven patients assayed at two different postsurgical time intervals). This is

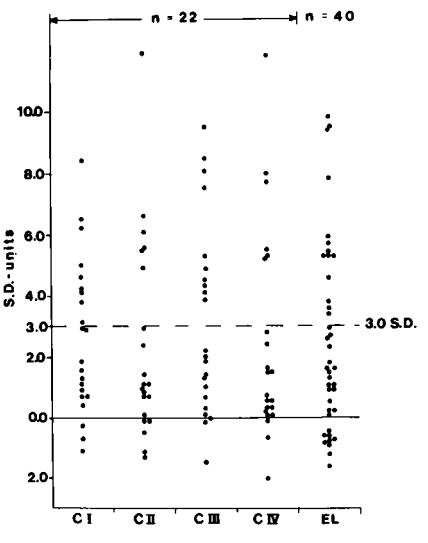

Fig. 1. Enzyme immunoassay results obtained by analysing Dupuytren's contracture (*DC*) sera for anti-collagen antibodies to collagen types I–IV and to elastin. *SD*, standard deviation

Table 2. Percentage of anti-collagen and anti-elastin antibodies in Dupuytren's contracture patients

Tissue antigen	Dupuytrens contracture (n = 22/40)	Controls[a] (n = 50)	Significance[b]
Collagen I	40.9	4.0	$p < 0.01$
Collagen II	27.3	8.0	$p < 0.01$
Collagen III	45.5	4.0	$p < 0.01$
Collagen IV	31.8	0	$p < 0.01$
Elastin kappa	40.0	15.1	$p < 0.05$

[a] For ELAB n = 53 controls.
[b] Dupuytren's contracture patients vs controls.

Table 3. Anticollagen antibody titer changes in 6 months[a]

Patient	I	II	III	IV
1	−/+	−/−	−/−	−/−
2	−/−	−/−	+/−	−/−
3	−/−	−/−	+/−	+/−
4	−/−	−/−	−/−	−/−
5	−/−	−/−	−/−	−/−
6	−/−	−/−	+/−	−/−
7	−/−	+/−	+/−	+/−

[a] Most antibodies disappear after 6 months.

shown in Tables 3 and 4. A graphical representation is given in Fig. 1, comparing high level ACA with low level ELAB. Individual results are shown in Table 5.

Autoantibodies to Elastin in DC Patients

Frequency and titer of ELAB are shown in Table 2 and Fig. 2. Individual results can be derived from Table 4. Interestingly, there was also a relatively high percentage of ELAB-positive control subjects (15% as opposed to 40% in the DC group).

Correlation with HLA Typing Results

HLA typing for class I antigens showed no remarkable difference between DC patients and controls or in ELAB-positive as compared to ELAB-negative individuals. Significant differences were found, however, for HLA class II antigen HLA-DR3, the only DR antigen with a statistically significant difference: 22 of 42 DC patients (52.4%) and 15 of 47 controls (31.9%) were

Table 4. Transitory nature of anti-collagen antibodies in a Dupuytren's contracture patient[a]

Collagen type	Anti-collagen antibody results (in SD units)	
	June 5, 1986	November 24, 1986
I	Negative	Negative
II	9.8	1.5
III	8.0	−1.1
IV	8.5	−2.0

[a] Patient number 7 (see Table 3); operated on Jan. 23, 1986.

Table 5. Autoantibodies to collagen and elastin in Dupuytren's contracture patients

Patient	Collagen type				Elastin (kappa)
	I	II	III	IV	
1	−	−	−	−	−
2	−	−	−	−	−
3	−	+	+	+	−
4	+	−	−	−	+
5	+	−	−	−	+
6	+	−	−	−	+
7	−	+	+	−	−
8	−	−	+	−	+
9	+	+	+	+	−
10	−	−	+	−	−
11	−	−	+	+	−
12	+	−	−	−	−
13	+	−	−	−	+
14	−	−	−	−	+
15	−	−	−	−	−
16	−	+	+	+	−
17	+	−	+	−	−
18	+	+	+	+	−
19	+	−	+	−	−
20	−	−	−	−	−
21	−	+	+	+	−
22	−	−	−	−	−

positive for HLA-DR3 ($chi^2 = 3.82$, of borderline significance; chi^2 value for $p < 0.05 = 3.84$; RR = 2.34). In addition, there was a significant correlation between presence of HLA-DR3 and increased concentration of ACA to type I collagen in DC patients (Table 6). Although no correlation of statistical significance was seen between ELAB positivity and HLA-DR3 in controls, the association of these autoantibodies with HLA-DR3 in the controls plus DC patients was statistically highly significant (Table 6).

Table 6. Association between Dupuytren's contracture (DC) and autoantibodies to connective tissue antigens and HLA DR3

Antibody	Dupuytren's contracture patients or controls	Significance
Collagen I	DC	$p < 0.05$
Collagen II	DC	n.s.
Collagen III	DC	n.s.
Collagen IV	DC	n.s.
Elastin	DC	n.s.
Elastin	DC and controls	$p < 0.001$
ELAB and ACA	DC	n.s.
ELAB	DC: controls	$p < 0.05$
ACA	DC: controls	$p < 0.01$
Dupuytren	DC: controls	$p = 0.05$

ELAB, anti-elastin antibodies; ACA, anti-collagen antibodies.

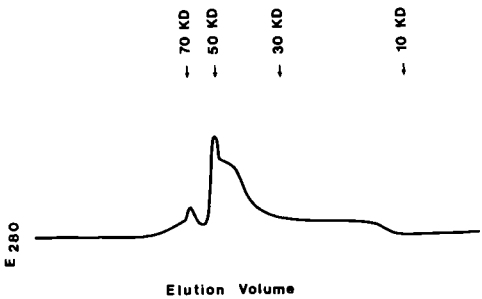

Fig. 2. Elution pattern of kappa-elastin chromatographed on Sephacryl S 200. *KD*, kilodaltons

Collagen Type III in DC Tissue

The type III sandwich EIA proved highly specific for this collagen type. Even a hundredfold excess of type I collagen did not significantly interfere with the quantitation of type III collagen (Figs. 3, 4). A much greater problem is the fact that collagen can be only partially solubilized by pepsin, even at the extreme enzyme to substrate ratio of 1:10 used in our study (Table 7). Therefore, only the percentage of type III collagen in the soluble fraction of total tissue collagen can be determined correctly, while the extrapolation to percentage of total collagen remains speculative. A significantly more efficient collagen solubilization may be achieved by peptic digestion at 14°C instead 4°C and/or by reducing pH to 2.5 or even 2.0 by addition of dilute hydrochloric acid.

Table 7. Type III collagen in Dupuytren's contracture tissue and control tissue as verified by sandwich ELISA technique

Sample	Localization	Collagen solubility by pepsin (%)	Type III in pepsin extract (%)[a]	Immuno-fluorescence
Carpal tunnel syndrome tendon	Long palmar muscle	5–38	0.3–4.2 (0.1–0.9)	Traces III
DC patient (apparently normal)	Third finger	11	3.3 (0.3)	III < I
Thickening of fibers, bands	Fourth finger	25–45	43–79 (10–35)	III > I
Contracture and nodules	Fifth finger	25	30 (7.5)	III > I
Rat tail tendons		95		
Human skin	Mammary	25	7	III < I

DC, Dupuytren's contracture.
[a] In estimated amount type as III percent of total collagen.

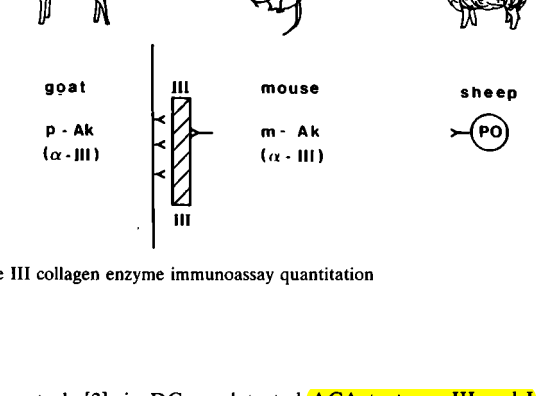

Fig. 3. Principle of type III collagen enzyme immunoassay quantitation

Discussion

In contrast to Pereira et al. [3], in DC we detected ACA to types III and I collagen at much higher frequency than ACA to type II collagen. This seems logical, since both these interstitial collagens are subject to an intense remodeling process in the course of DC, including neosynthesis and degradation by collagenase and other tissue proteases. Type II collagen, by contrast, is lacking in the normal palmar fascia and its appearance in involved aponeurosis is a rare finding (four out of 32 tissue samples contained type II collagen according to immunofluorescence studies by Meister et al. [11]). Why then should there be a significant titer of autoantibodies to this collagen type,

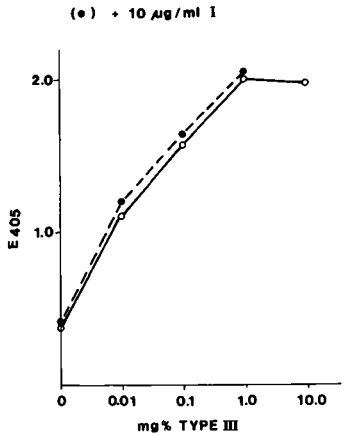

Fig. 4. Influence of type I collagen on type III sandwich enzyme immunoassay

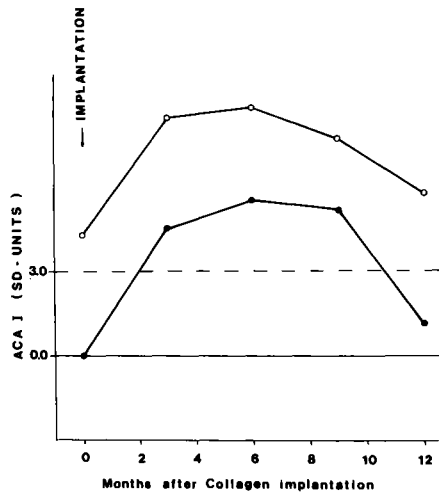

Fig. 5. Transitory nature of anti-collagen antibodies (*ACA*) in patients undergoing collagen implantation for diverse reasons. *Upper curve*, already existing autoantibody titer before collagen implantation; *lower curve*, autoimmunization to collagen as a consequence of collagen implantation

Table 8. Importance of anti-collagen antibodies Dupuytrens contracture (DC) and rheumatoid orthritis (RA)

Immune involvement	DC	RA
Local complement activation	−	+
Systemic compklement activation	−	+
Local immune Complexes	−	+
Systemic immune complexes	−	+
Systemic manifestation of disease	−	+ (EAM)
Other than connective tissue antibodies	−	+ (Rf)
HLA association	+	+
Anticollagen antibodies	I,III	II
Antibody class	?	IgG 2,3

as DC is a local and not a systemic disease like RA? The only explanation for ACA to type II collagen would be cross-reacting ACA, which should be more stringent for denaturated collagens than native collagens. Another possibility is a genetic predisposition; HLA DR4 seems to be associated with ACA to denatured collagen type II and HLA DR3 with ACA to native type II collagen in RA patients [5,21,22].

ELAB were seen in a considerable percentage of DC patients. Here, however, the control population of healthy persons also displayed an astonishingly high percentage of ELAB-positive individuals (15%), in confirmation of the results of Stein et al. [12]. ELAB and ACA positivity was not correlated in DC patients. However, autoantibodies were significantly correlated with HLA DR3 in DC patients (this is valid only for ACA to type I collagen). The association between the presence of HLA DR3 and DC was only of borderline significance, meaning that there is a suggestion of an association. Accordingly, relative risk is low.

In seven patients ACA were assayed at two time intervals postoperatively (time increment approximately 6 months). We noted a disappearance of ACA positivity in six out of seven cases, suggesting that ACA were triggered by surgical therapy, much as ACA can be induced in HLA DR3- or DR4-positive individuals by implants of bovine collagen implanted for cosmetic or reconstructive reasons (Fig. 5).

Several facts point to a marginal importance of ACA and ELAB in the development of DC. First, in contrast to RA, inflammatory mechanisms are involved only to a minor degree in DC. As shown in Table 8 immune reactions such as complement activation or immune complex formation are not seen in this fibromatous process. Second, ACA appear after surgical intervention and disappear again (Tables 3, 4), indicating their transitory nature. Lastly, ELAB are also found in healthy controls of the right genetic background, although a strict association with HLA DR3 was not observed.

The increased presence of type III collagen in involved or even uninvolved regions of the DC aponeurosis is evident from our results and those of others [23,24]. The major problem in an assay for type III collagen that depends on

the native collagen molecule is the restricted degree of collagen solubility, even in pepsin solutions of high enzyme activity, low pH and reaction temperatures above the normally used 4°C (e.g., 14°C). Most authors are rather reticent about the efficiency of collagen extraction procedures. The type III collagen EIA presented here proved, however, to be of high sensibitivity and good overall reproducibility and may be recommended for type III collagen quantitation, especially in the presence of the large excesses of type I collagen one usually has to cope with when analyzing connective tissue extracts of skin, tendon or dura mater.

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