Antibodies against reticulin and collagen

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Nowadays the idea that systemic rheumatic conditions such as rheumatoid arthritis, systemic lupus erythematosus, and scleroderma are disorders primarily affecting collagen—that is, that these conditions are ‘collagen diseases’—has been somewhat discredited. Fifteen to 20 years ago it attracted considerable support from the then striking revelation that autoimmunity directed at certain tissue antigens not only occurs but may be closely associated with local disease in organs where the antigens in question are found. Endocrine autoimmune diseases such as Hashimoto’s thyroiditis are classic examples.

It has turned out, however, at least in SLE and rheumatoid arthritis, that while it cannot be seriously doubted that autoantibodies do indeed make a major contribution to the pathogenesis of the typical arthritis, nephritis, or vasculitis reactions respectively they do so by their participation in the formation of immune complexes in which the antigens involved, although endogenous, are demonstrably not exclusively characteristic of the tissue sites affected. At least a significant part of the damage results from the inflammatory consequences of local deposition of immune complexes.

The demonstrable immunogenicity of collagen

FIG. 1 Cryostat section of rat liver. Staining of adventitial fibres in a portal tract and fibres in the parenchyma produced by serum from patient with coeliac disease. Indirect immunofluorescence. $\times 320$

FIG. 2 Cryostat section of rat kidney. Staining of peritubular and periglomerular fibres produced by same serum as in Fig. 1. $\times 320$

when injected into experimental animals refutes the early view that collagen is a non-antigenic substance, and the striking antigenic differences which have recently been shown to exist between the native and denatured forms of collagen molecules have refocused attention on the possibility that collagen may be an autoantigen in man. At the same time immunological observations in human gastroenterology and dermatology have indicated that another connective tissue component, reticulin, may also acquire autoantigenic properties, since anti-reticulin antibodies are found in coeliac disease, dermatitis herpetiformis (DH), and Crohn's disease.

This communication compares and briefly contrasts the autoantibodies found in man against these two connective tissue components—that is, reticulin and collagen—and considers some of the ideas that have been put forward to account for their presence and, indeed, to ascribe to them a functional significance.

Antireticulin antibodies

One of the most striking immunological abnormalities in gluten-sensitive enteropathy is the

presence of autoantibodies that give an immunofluorescence staining pattern similar in distribution to that of reticulin (Figs. 1, 2). Their highest incidence, about 85%, is in coeliac disease in children who are not on a gluten-free diet (Seah *et al.*, 1973). The sera of 60% of adults with untreated coeliac disease patients and 30% of patients with untreated DH also react by immunofluorescence with connective tissue fibres in cryostat sections of various organs from rats, humans, and other species. The best correlation of the immunofluorescence pattern is with the distribution of staining by silver impregnation. Since collagen stains yellow, brown, or pink by this method and reticulin stains black this suggests that the antibody in these patients' sera is antireticulin. The failure of pretreatment of sections with collagenase to affect their ability to display the characteristic staining pattern with these sera while removing their ability to stain by van Gieson's method supports this idea. Immunoperoxidase visualization of the antibody in the electron microscope shows that matrix not collagen is stained (Fig. 3).

Reticulin is the name given to a particular

FIG. 3 Electron micrograph showing cross sections of collagen fibres in a rat liver preparation treated with human serum containing antireticulin antibody. Note no staining of fibres but perifibrillar zones show positive staining. Immunoperoxidase method. $\times 62\,000$. (Kindly provided by Dr. P. P. Seah)

histological form of extracellular connective tissue fibres that are fine, branched, and isotropic between crossed polars. They are unstained by van Gieson dyes and are blackened by silver impregnation techniques. Reticulin thus differs antigenically as well as histologically from collagen, as suggested by Taylor *et al.* (1961), and this conclusion receives support from the observation that a non-collagenous reticulin component could be extracted from the saline insoluble residue of pig or human renal cortex or liver. The extract contains no hydroxyproline but is 4% carbohydrate and is antigenic in rabbits (Pras and Glynn, 1973). The resulting antiserum gives a fluorescent staining pattern on cryostat sections indistinguishable from that obtained with human antireticulin antibody. Furthermore, the non-collagenous reticulin component of Pras and Glynn proved to be capable of absorbing the characteristic staining pattern imparted by human antireticulin antibody from 9 out of 11 sera tested (Pras *et al.*, 1974). It was also noted that human antireticulin antibody does not stain the argyrophilic fibres of the newly formed collagen fibres seen in healing wounds, and this is probably

due to the absence of a specific glycoprotein of reticulin from young collagen fibres in this situation. So far, however, the chemical nature of the antigenic determinants specific for reticulin has not been elucidated.

The reasons for the appearance of antireticulin antibodies in gluten enteropathy and their possible immunopathological significance are obscure. Their appearance may result from increased absorption of bacterial and food antigens in malabsorption states, and a report of raised antireticulin titres in heroin addicts may reflect a connexion with micro-organisms (Rizzetto and Doniach, 1973). This would put them in the class of autoantibodies such as smooth muscle and antinuclear antibodies which are more easily evoked by external agents such as viruses and drugs.

It is tempting to attribute the Ig and complement deposits found in the reticulin-rich dermal papillae in DH to the reaction of these antibodies, but neither these nor the immune complexes demonstrated in sera of both coeliac disease and DH patients (Mowbray *et al.*, 1973) have yet been linked with the presence of antireticulin antibodies,

Table I *Different specificities of anticollagen antibodies produced by immunizing animals (type I antibodies composite from rat, guinea-pig, rabbit, and chicken)*

Species specificity	Molecular region of determinants	Antibody reacts with:
Species specific	Helical (conformation dependent)	Native
	Non-helical (sequence dependent)	Native, denatured
Species cross-reactive	Central (sequence dependent)	Denatured (native)

Table II *Immunogenicity of native and denatured collagen in animal species*

Animal species	Immunogen		Authority
	Native collagen	Denatured collagen	
Rabbit	+	+	Lindsley <i>et al.</i> (1971) Beil <i>et al.</i> (1973)
Chicken	+	+	Beil <i>et al.</i> (1973)
Guinea-pig	+	—	Beard <i>et al.</i> (1976a) Gentner and Adelman (1976a, b)
Mouse (SWR; C3H.SW)	+	+	Nowack <i>et al.</i> (1975)
Mouse (C57BL/10; B10.BR)	+	—	Fuchs <i>et al.</i> (1974)

Anticollagen antibodies

Unlike antireticulin antibodies, we know a good deal about how the specificity of anticollagen antibodies is related to the primary structure and chain composition of collagen, as described elsewhere in this symposium. In the native molecule the triple-helical conformation is maintained throughout its length except for short, non-helical regions at both ends. The native conformation is lost on gentle heating and the resulting chains of denatured collagen assume random conformations as mono-, di-, or trimeric forms. Immunization of different animal species with heterologous collagen produces specific antibodies whose specificity varies according to the native or denatured state of the antigen (Tables I, II) and according to the species of animal immunized (Beard *et al.*, 1976a).

Anticollagen antibodies in disease

Humans have antibodies to denatured collagen (called antigelatin factor (AGF)) in their sera (Maurer, 1954), but recent attempts to measure human autoantibodies to collagen in health and disease have produced conflicting results concerning the presence of anticollagen antibodies in normal

people. Wolff *et al.* (1967) found that human AGF was composed of two macroglobulin components, one of which (the 'agglutinator') was heat labile. They suggested that AGF differs from true collagen autoantibodies in three ways: (1) it is found in all sera, (2) it is heat labile, and (3) it is macroglobulin in nature. Maurer (1954) found AGF in 65% of human sera and he was the first to report that these antigelatin antibodies were indeed heat labile (Maurer, 1958). However, Beard *et al.* (1976a) found heat stable agglutinators in all of a large number of normal human sera tested (Table III) which react with denatured human collagen in passive haemagglutination.

These findings conflict with those of several other authors, who report antigelatin antibodies in no more than 10% of normal sera using either passive haemagglutination (Wells *et al.*, 1973; Hahn *et al.*, 1975; Michaeli and Fudenberg, 1974b) or the antiglobulin consumption test (Steffen, 1969, 1970), although it is not always stated in these reports whether or not the sera were heated. Unlike others (Wells *et al.*, 1973; Michaeli and Fudenberg, 1974a, 1974b), we (Beard *et al.*, 1976a) used unformalized tanned red blood cells in passive haemagglutination, and direct comparison of fresh and formalized

Table III *Reported incidence of anticollagen antibodies in normal human subjects*

Positive (%)	Native	Denatured	Authority
2.5	+	—	Wells <i>et al.</i> (1973)
1.0	+	—	Michaeli and Fudenberg (1974a)
9.0	—	+	Michaeli and Fudenberg (1974b)
56.0	+	—	Conochie and Faulk (unpublished)
100.0	—	+	Conochie and Faulk (unpublished)

tanned red blood cells in haemagglutination tests with animal sera indicate that fresh collagen-coated cells are sensitive to AGF whereas formalized collagen-coated red blood cells are not (Beard, 1974). Probably, therefore, heat stable antigelatin agglutinators are present in most normal human sera but are not detectable by antiglobulin consumption or by passive haemagglutination using formalized cells. Some of these sera also give precipitin reactions to denatured collagen by Ouchterlony testing.

Although there is general agreement that normal sera react poorly with native as compared with denatured collagen, the serum protein(s) responsible for these reactions have not been identified in most cases. The only difference we have found between test and control human sera (Fig. 4) is that the haemagglutinating titres to denatured collagen are somewhat raised in several 'collagen diseases' (Beard *et al.*, 1976b). It is relevant to comments in the opening paragraphs of this paper that both anticollagen antibodies (Cracchiolo *et al.*, 1975) and collagen-anti-collagen immune complexes (Steffen *et al.*, 1974) have been identified in synovial tissues and fluids. The results of some other studies of collagen autoantibodies in connective tissue disease are summarized in Table IV.

The antiglobulin consumption test has been used by Steffen's group (Steffen, 1970, 1969; Steffen *et al.*, 1971) to study anticollagen antibodies in a large number of conditions. They have found antibodies in about three-quarters of patients with rheumatoid arthritis, Felty's syndrome, systemic lupus erythematosus, Sjögren's syndrome, and scleroderma, while control sera are largely negative. Of particular interest is their finding of antibodies in 88% of cases with severe, quickly progressing rheumatoid arthritis and in only 29.2% of cases with slowly progressive disease. Positive reactions could be inhibited equally well with calf and human collagens, and could not be correlated with results of latex and Waaler-Rose testing in the same patients.

Steffen *et al.* (1973), however, subsequently showed that antinuclear factors were more commonly identified in sera of rheumatoid patients who also had anticollagen antibodies. Similar results have been obtained by Michaeli and Fudenberg (1971), who

report haemagglutinating antibodies (using tanned, formalized cells) to denatured collagen in 60% of the sera of patients with rheumatoid arthritis. Also 20% of their patients had antibodies to native collagen, while anticollagen antibodies were identified in only 8% of the control population. The antibodies in several of their sera were found to be IgG by chromatography, and this is also consistent with reported results using the antiglobulin consumption test, which primarily measures IgG antibody.

FIG. 4 Haemagglutination of collagen-coated red blood cells by sera from normal people and patients (Conochie and Faulk, unpublished)

Table IV Reported incidence of anticollagen antibodies in patients

Condition	Positive (%)	Native	Denatured	Authority
Rheumatoid arthritis	60.0	—	+	Michaeli and Fudenberg (1974a)
Rheumatoid arthritis (severe)	88.0	+	—	Steffen (1969)
Rheumatoid arthritis (mild)	29.0	+	—	Steffen (1969)
SLE	68.0	+	—	Steffen (1969)
Scleroderma	71.0	+	—	Steffen (1969)
Emphysema	17.0	+	—	Michaeli and Fudenberg (1974b)
Emphysema	70.0	—	+	Michaeli and Fudenberg (1974b)
IgA deficiency	44.0	+	—	Wells <i>et al.</i> (1973)

An unusual group of patients lacking IgA have been studied by Wells *et al.* (1973). An overall incidence of 44.4% of these patients had antibodies to native human skin collagen as measured by tanned-cell agglutination, and similar antibodies were found in only 2.5% of the control population. Although other autoantibodies are often found in patients who lack IgA there was no correlation between the presence of anticollagen antibodies and autoantibodies to IgG, IgA, IgM, or antibodies to ruminant proteins. These investigators used tanned, formalized cells and antibodies to denatured collagen were not studied.

Michaeli and Fudenberg (1974a) investigated the class and antigenic specificity of antibodies to denatured human collagen in rheumatoid arthritis and found them to be primarily IgG. Two sera were studied using cyanogen-bromide (CB) fragments of collagen to inhibit the tanned-cell agglutination reaction, and a CB fragment from the N-terminus of the α_1 -chain and a CB fragment from the helical part of the α_2 -chain were found to inhibit.

Specificity for an N-terminal α_1 -peptide helps to explain why some human sera can recognize both native and denatured collagen, because non-helical sites are presumably available in both (Beard *et al.*, 1976b). On the other hand, collagen autoantibodies are known to cross-react between species (Steffen, 1969) and since species cross-reactivity is usually attributed to central sites available only on denatured collagen the inhibitory effect of the CB fragment from the helical part suggests an anti-denatured specificity for these sera.

Identical results for α_2 -chain specificity have been obtained by Michaeli and Fudenberg (1974b) in their study of anticollagen antibodies in the sera of 422 patients with emphysema. They found that neither whole α_1 -chain nor α_1 CB fragments inhibited tanned-cell agglutination, but fragments from either central or N-terminal sites in α_2 -chains inhibited haemagglutination.

In addition to serum antibodies, Steffen has claimed that antibodies to collagen may be detected in the synovial fluid from rheumatoid joints by agglutination of collagen-coated red blood cells (Steffen, 1970). In view of the findings of Conochie *et al.* (1975) the agglutinations obtained were probably not due to anticollagen antibodies but to the presence of glycosaminoglycans. These authors showed that the non-covalent binding between collagen and various polyanions, especially chondroitin-4-sulphate, closely simulates an immunological agglutination, and it seems likely that such substances are present in synovial fluid of the rheumatoid joint.

With regard to reactivity against native and denatured collagen, therefore, human autoantibodies against the latter seem to be well authenticated and to be present in raised titre in some systemic rheumatic diseases and some other chronic conditions which are associated with collagen breakdown. The true incidence of anti-native collagen autoantibodies in health and disease needs further investigation and biochemical characterization of AGF is needed.

Finally, a word about the possible functional role of anti-denatured collagen autoantibodies. Faulk *et al.* (1975) have shown that collagen is a membrane-bound component of fibroblasts. Exposure of these cells to anticollagen antisera causes patch-and-cap reactions of the membrane collagen analogous to that produced in B-lymphocytes by anti-Ig sera. In addition, Faulk and Temple (1975) have shown that fibroblasts grown in the presence of a rabbit antifibroblast antibody are stimulated to incorporate more radio-labelled proline into TCA-precipitable protein than are control cells grown in the presence of normal rabbit serum. The possibility that one effect of anticollagen antibody might be to stimulate fibroblasts to synthesize and secrete more cell product deserves study. Finally, the possible role of AGF-macrophage interactions in collagen catabolism merits further investigation (Hopper *et al.*, 1976).