Anticoagulant antibodies in the synovial membranes of patients suffering from haemophilia, rheumatoid arthritis and other rheumatic disorders

SG RAINSFORD, A ARONSTAM, EJ HOLBOROW,* DS McLELLAN

*From the Haemophilia Laboratory, Lord Mayor Treloar Hospital, Alton, Hampshire, and the *Bone and Joint Research Unit, The London Hospital Medical College, Whitechapel, London*

SUMMARY This study has identified IgG and IgM anticoagulant antibodies in the synovial membranes of patients suffering from haemophilia and rheumatoid arthritis (RA) but not in synovial tissues from normal subjects or in patients with other arthritides. In the majority of cases the antibody appeared to have the specificity of the lupus-like anticoagulant (LLA) seen in patients with systemic lupus erythematosus (SLE). The importance of these findings with regard to the treatment of certain cases of haemophilia and RA and the possible relation between the presence of these antibodies and viral infections is discussed.

Approximately 6% of patients suffering from haemophilia develop a systemic antibody for factor VIII C¹ which seriously complicates the treatment of this disorder and patients with rheumatoid arthritis (RA) have been reported to have developed acquired haemophilia for the same reason.^{2,3} The cause of this complication is at present unknown. However, the histopathology of the synovial membrane in some RA cases closely resembled that of advanced haemophilic arthropathy, namely hyperplasia, round cell infiltration but more especially the presence of plasma cells together with deposits of haemosiderin.⁴ Those observations suggested the possibility that an anticoagulant antibody can arise in the joint perhaps becoming systemic in some patients.

However, although our preliminary investigations⁴ demonstrated anticoagulant activity in synovial extracts from RA and haemophilic patients, the specificity of these anticoagulants did not appear to be active against factor VIII C.

We report here a more comprehensive investigation of this phenomenon and the results of our attempts to characterise (a) the antibody class(es) and (b) the coagulation reactions involved.

Material and methods

One hundred and six specimens of synovial membrane from patients suffering from haemophilia,

Christmas disease, rheumatoid arthritis (RA), osteoarthritis (OA) and other rheumatic disorders have been examined. Six specimens of normal synovial membrane were also examined.

SYNOVIAL MEMBRANE EXTRACTS

The method of extraction has been described previously.⁴ In brief the synovial membranes were homogenised and extracted in physiological saline. All extracts were then heated at 56°C and absorbed with aluminium hydroxide. Each extract was concentrated fivefold then assayed for total protein.

AGAROSE GEL DIFFUSION TEST (AGDT)

The AGDT was employed as a preliminary test for the detection and measurement of coagulation inhibitors in these extracts. The methodology for this test is fully described elsewhere.^{5,6}

An antibody reference standard plasma containing 50 New Oxford Units (NOU)/ml of antifactor VIII C was employed to compare results.⁷ Before use the plasma was heated at 56°C for 30 min and absorbed with Al (OH)₃ to remove all clotting factors. Results were expressed as equivalents of factor VIII C antibody units (AGDT units of inhibitor).

An example of titration of inhibitors by this method is given in Fig. 1 which shows zones of clotting inhibition around wells containing different concentrations of inhibitor. The zone of inhibition is proportional to the antibody concentration.

Fig. 1 The detection and measurement of coagulation inhibitors in agarose gel.

Specimen	Well No	Serial dilutions	AGDT units/ml
Ref standard	1-5	1/1-1/16	50
A	6-9	1/1-1/8	66
B	10-12	1/1-1/4	16
C	13-14	1/1-1/2	7.5
Normal plasma	15 and 16	Undiluted	Nil
Saline diluent	17 and 18	—	Nil
Empty	19 and 20	—	Nil

CLOTTING SPECIFICITY TESTS

The specificity of the anticoagulant present in extracts positive by the AGDT was determined by incubating the extract with an equal volume of fresh frozen plasma (FFP). This mixture was then subjected to screening tests, followed by specific assays for the residual clotting factors.

Screening tests

The prothrombin time (PT) was measured by a standard procedure⁸ employing human brain thromboplastin. The effect of diluting this brain extract in tenfold dilutions, ranging from 1/10-1/1000 was also investigated.⁹ The kaolin cephalin time (KCCT) was measured by a standard method⁸ and the clotting

times were determined with a fibrometer (Becton and Dickinson). These two basic tests were employed to determine whether the clotting inhibition lay in the intrinsic or extrinsic pathway of the coagulation system, or the common link via a "lupus-like" anticoagulant activity (LLA).

In these tests normal controls consisted of substrate normal plasma (SNP) and extracts of two normal synovial membranes, 91H and 92H. Known coagulation inhibitors were included in some tests as positive controls, either plasma from a case of SLE (LLA-positive) or the antifactor VIII C antibody (50 NOU)⁷ were used. The SLE plasma were pretreated as previously described for the antifactor VIII C standard.

Specific assays

In comparison with the AGDT, clotting tests are extravagant in their requirement of test material. The limited amount of test material available on some samples necessitated that specific assays be reduced to a minimum. The assays performed were in part governed by the pattern of screening coagulation test results. For the assay of factor IX an agarose gel technique was employed.⁴ This method only required 10 μ l of material and it was therefore possible to screen all extracts.

Standard methods⁸ were employed for other clotting assays which included the two stage factor VIII, assays of factor X using Russell viper venom (RVV) and factor II employing Taipan snake venom.

ANTIFIBRINOGEN ACTIVITY

This was tested for by the passive agglutination method of Poston.¹⁰ This test only required 50 μ l of test material and it was therefore possible to test all AGDT positive extracts.

ANTIBODY NEUTRALISATION TESTS

Specific antisera against human immunoglobulins (IgG and IgM) were heated at 56°C for 30 min and absorbed with Al (OH)₃ to remove all coagulant factors and were then incubated with an equal volume of inhibitor-positive extract. The mixture of antisera and extract was then incubated with an equal volume of fresh normal plasma and the KCCT estimated.

Normal controls consisted of plasma and antisera suitably diluted with saline. The antifactor VIII C standard was included as a positive IgG control. It was found that most batches of antisera obtained from several different commercial sources had anti-coagulant properties. These sera are expensive and the cost of the search for acceptable sera high. Because of these difficulties only a very limited number of serological tests were possible and other methods were investigated.

FRACTIONATION STUDIES

Extracts were fractionated on Sepharose S 200 Superfine column. Eluted fractions were pooled, re-concentrated and tested for immunoglobulin classes IgG, IgM, and IgA by immunoelectrophoresis and single radial immunodiffusion. Affinity chromatography based on a method which utilises protein A adsorbed on to Sepharose 4B¹¹ was also employed. The various fractions by these procedures were finally screened with the AGDT for the presence of coagulation inhibitor.

RHEUMATOID FACTOR (RF) ASSAY

The sera of all patients was tested for the presence of IgM, RF employing the Latex test.¹²

Results

AGDT SCREENING TEST

A high proportion of synovial extracts from cases of haemophilia, rheumatoid arthritis and Still's disease contained a coagulation inhibitor while those from normal synovial membranes and from cases of osteoarthritis and other rheumatic disorders were negative (Table 1). The sera from all patients from whom a positive extract was obtained were screened with the AGDT for inhibitors and all except one were negative. This positive specimen was obtained at a post mortem from a haemophilic patient known to have a systemic inhibitor for factor VIII C.

Table 1 Incidence of coagulation inhibitors (CI) in synovial extracts

Disease	No of cases	CI positive
Christmas disease	1	0
Haemophilia	14	9
RA	63	26
Still's disease	2	1
OA	23	0
PVNS	1	0
Gout	1	0
Psoriasis	1	0
Normals	6	0
Total	112	36

RA = rheumatoid arthritis.

The total amount of protein in any extract had an important influence on the results. No inhibitor was found in any extract containing 2.5 mg of protein/ml or less after concentration and the results from all such specimens have been excluded from the figures shown in Table 1. The mean figure of units of inhibitor/mg of protein was 1.5 with a range of 0.2-7.0. The sensitivity of the AGDT is such that is unlikely to detect less than one inhibitor unit/ml. Thus nine of 12 specimens which contained less than 5 mg protein/ml were negative but may have contained an inhibitor which was not detectable.

TESTS FOR FIBRINOGEN ANTIBODY

All 112 extracts of synovial membranes were tested for the presence of this antibody and all were negative.

CLOTTING SPECIFICITY TESTS

Figs 2 and 3 show the clotting reactions obtained with the KCCT and PT screening test and demonstrated the anticoagulant effect on normal plasma

Fig. 2 Kaolin cephalin time (KCCT) and prothrombin time (PT) screening tests showing lupus-like anticoagulant (LLA) characteristic reactions with haemophilic extracts. 91H is an extract of normal synovial membrane. Substrate normal plasma control (SNP).

Fig. 3 KCCT and PT screening tests showing LLA characteristic reactions with RA extracts. 92H is an extract of normal synovial membrane. Substrate normal plasma control (SNP).

of the AGDT inhibitor in some extracts and the disproportionate prolongation of clotting times with dilute thromboplastin. This latter phenomenon has been described by Boxer⁹ in his studies of the lupus-like anticoagulant (LLA). In two specimens namely 47H and 118H this type of reaction could only be demonstrated after these extracts had been diluted with an equal volume of saline.

Twenty-two of the 36 AGDT inhibitor-positive extracts gave this LLA type of reaction (Table 2). Furthermore, specific assays indicated that none of these 22 extracts had any antifactor II or X activity. The inhibitor in five of the extracts (four RA and one haemophilia) produced only a prolonged KCCT. Fig. 4b shows the findings for three of these five extracts. No antifactor VIII or IX activity could be demonstrated with any of the four RA extracts, but extract H144 from a case of haemophilia, was found to have specific antifactor VIII C activity. This was the post-mortem specimen to which reference has already been made.

It therefore appears that in the four RA extracts that an antifactor XI or XII activity may be present. This possibility remains to be confirmed.

A summary of the results of these specificity tests is given in Table 2. It will be seen that in nine of 36 AGDT inhibitor-positive extracts, the inhibitor remained unidentified. In five there was insufficient test material available to permit adequate examina-

Table 2 Specificity of coagulation inhibitors in synovial extracts

Disease	Number of +ve cases	LLA	Others	Not identified
Haemophilia	9	6	*1 (anti-VIII)	2
RA	26	15	4 (anti-XI or XII)	7
Still's disease	1	1	—	—
Total	36	22	5	9

*Systemic antibody present.

LLA = lupus-like anticoagulant.

tion and the remaining four contained less than 4 inhibitor units/ml.

With the technique employed it was found that clotting tests with specimens containing less than 4 inhibitor units/ml were inconclusive. However, Fig. 4a shows a repeat of the KCCT and PT on extract H14 (shown in Fig. 2) which contained only 4 inhibitor units/ml and the similarity of the clotting reactions in the two separate tests is remarkable.

RHEUMATOID FACTOR TESTS

Table 3 shows the rheumatoid factor (RF) status of all "inhibitor-positive" patients and it will be seen that there was no correlation between the presence of RF in the blood and inhibitors in the synovial tissues.

Fig. 4a Repeat of test on extract H14 shown in Fig. 2.
Fig. 4b KCCT and PT screening tests showing a prolonged KCCT but normal PT compared with antifactor VIII C standard. H138 and H145 are RA extracts. H144 is a haemophilic extract.

Table 3 Rheumatoid factor status of cases with synovial membrane anticoagulant antibodies

Disease	No of cases	Serum latex positive
Haemophilia	9	2
RA	26	19
Still's disease	1	1

Fig. 5 Neutralisation of inhibitors with anti-IgG serum. H91 is a normal synovial extract. H16 and H47 are haemophilic extracts with LLA inhibitors. Antifactor VIII C is the reference standard (from Panayi GS, Johnson PN, eds. *Immunopathogenesis of rheumatoid arthritis*⁴).

ANTIBODY NEUTRALISATION TESTS

The results of three tests are shown in Figs 5, 6a and 6b. It will be seen that anti-IgG produced a reduction in the KCCT with all the extracts tested and with the antifactor VIII C standard. In the case of extract H118 (Fig. 6b) however, the anti-IgM had an even greater neutralising effect. Complete neutralisation was obtained with specimens containing ≤ 8 inhibitor units/ml but with more potent extracts only partial neutralisation was attained.

FRACTIONATION STUDIES

Sephacrose fractions of extract H47 and antifactor VIII C plasma were tested for the presence of inhibitor with the AGDT. Only fractions corresponding to IgG were found positive.

The heat-stable nature of the anticoagulant activity related to the molecular sizing obtained from the column fractionation studies and the neutralisation results with class specific anti-immunoglobulin sera, indicated that the anticoagulant is antibody IgG in most instances. Furthermore, the anticoagulant activity was not affected by prolonged exposure to 1/5000 concentration of sodium azide or neutralised by Polybrene.

Eight LLA-positive extracts including H47 and the antifactor VIII C standard were fractionated on a protein A column. This material preferentially adsorbs the IgG 1, 2 and 4 subclasses.¹¹ Eluates and unretained material were reconcentrated to the original volume and tested for presence of inhibitor. With the exception of the antifactor VIII C standard, inhibitor was only found in the unretained material. Neutralisation tests with class specific anti-immunoglobulin sera showed that three of these extracts contained some IgM antibody but all contained an IgG component. These findings therefore suggest that the antibody activity was confined to IgG subclass 3.

Discussion

This investigation shows that anticoagulant antibodies can be extracted from a high proportion of both rheumatoid and haemophilic synovial membranes.

In the majority of these specimens the anticoagulant was identified as an antibody similar to the LLA⁹ and in four cases antifactor XI or XII was inferred. Antibodies to the latter two factors have been reported to be occasionally present in the blood of SLE patients.^{13 14}

The LLA inhibits clotting by blocking the activation of prothrombin to thrombin by the complex of factors Xa, V and phospholipid. It derives its title from the frequency with which it has been reported in the blood of patients with SLE.^{2 9} The LLA has been shown to be IgG or IgM antibody but can contain both these components of immunoglobulin.² This would seem to have been confirmed by this study. However, it would appear from the protein A studies that the IgG component was restricted in some cases to the subclass IgG₃ which would be indicative of an antibody response of limited heterogeneity. Such an atypical response is of interest in view of the antibody made against factor VIII C in haemophiliacs, for this antibody has been shown on

Fig. 6a Neutralisation of LLA inhibitors with anti-IgG serum in a haemophilic extract H14 and an RA extract H48. H91 is an extract of normal synovial membrane. Fig. 6b Neutralisation of an LLA inhibitor by both anti-IgG and IgM serum in an RA extract H118 and only by anti-IgG serum of an antifactor VIII C inhibitor in a haemophilic extract H144.

many occasions to be restricted to a single subclass, IgG₄.^{2 15}

Although the LLA is probably the most prevalent of all systemic inhibitors^{2 9} the identification of this antibody in the majority of synovial extracts from patients with haemophilia and RA was quite unexpected and raises the possibility that there is some common factor operating in these two diseases. Clinically, the presence of LLA in the blood seldom gives rise to spontaneous bleeding. It is only when it is accompanied by an additional haemostatic defect that haemorrhage occurs.^{2 9 16} Nevertheless, the deposits of haemosiderin seen in the synovial membrane from some cases of RA would seem to indicate that isolated haemorrhages into the synovial tissues do occur, and bleeds of this nature could perhaps explain the acute exacerbations seen in this disease and the phenomena of palindromic rheumatism.

In haemophilia these localised haemorrhages would initiate a more severe type of bleeding episode and such cases would be expected to bleed more frequently.¹⁷ If this is so, both local and general treatment with steroids or some other form of immunosuppressive treatment should prove beneficial for such cases.

Boxer *et al*⁹ found a coincidental prothrombin deficiency associated with the presence of LLA and raised transaminase and alkaline phosphatase activities. No explanation has been offered for these

phenomena though normally a deficiency of vitamin K-dependent clotting factors with a raised activity of transaminase would be regarded as suggestive of hepatitis. It is therefore of considerable significance that Levo *et al*¹⁸ found 7 of 21 RA cases were seropositive for hepatitis B virus (HBV) antibody which suggests that HBV infection could be a common factor in these two diseases. The annual incidence of jaundice in haemophilic patients ranges between 2 and 3%.¹ In most cases the hepatitis in haemophilia has been due to HBV. These patients are transfused frequently with factor VIII concentrates and therefore receive material collected from many thousands of blood donors annually. Most factor VIII concentrates are prepared from the cryoprecipitate fraction of plasma and it has been found that HBV in the plasma becomes concentrated in this fraction.¹⁸

It can be assumed, therefore, that most haemophilic patients have been exposed to infection with HBV. This is confirmed by the fact that other than a few boys who are HBV antigen carriers, the remainder of the 50 boys suffering from haemophilia in residence at the Lord Mayor Treloar College are seropositive for HBV antibody and have raised transaminase activities. Finally, these investigations indicate the possible role of host responses to virus in the aetiology of chronic inflammatory arthritis.

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Requests for reprints to: Dr A Aronstam, The Treloar Haemophilia Centre, Lord Mayor Treloar College, Holybourne, Alton, Hampshire, England.