Effect of fibronectin on the *Crithidia luciliae* test for anti-double-stranded DNA antibodies

K E Herbert, E Jeffery, D L Scott

Abstract
The various tests for anti-double-stranded DNA antibodies do not always agree. Plasma fibronectin specifically binds DNA, is a component of immune complexes, and shows variations in concentration with disease activity in systemic lupus erythematosus. It may therefore interfere with the detection of DNA autoantibodies. This possibility was examined in a series of studies using the *Crithidia luciliae* test. Studies were based on serum samples received during one year (250 samples). Serum samples from 50 patients which were positive or weakly positive in the *C luciliae* test were used. In blocking experiments fibronectin was added either to the wells or to the serum. In a second series of experiments fibronectin was depleted by affinity chromatography from six serum samples with weak anti-DNA staining. Precipitation of wells with fibronectin or addition of fibronectin to serum invariably blocked the interaction of anti-DNA antibodies with the *C luciliae* kinetoplast. When fibronectin was removed from serum the intensity of staining was increased. These results indicate that fibronectin influences the detection of anti-double-stranded DNA antibodies using *C luciliae* and may explain the disparity between the results of different tests for DNA antibodies. Furthermore, the unmasking of positive reactivity when fibronectin is removed from serum has implications for the diagnosis and treatment of systemic lupus erythematosus.

Antibodies to DNA characterise systemic lupus erythematosus (SLE) and may have a pathogenic role in the disease. The detection of antibodies to double stranded (ds) DNA is a valuable diagnostic test for SLE. Such antibodies are found in roughly two thirds of SLE sera, the levels being more pronounced in severe disease. Anti-dsDNA antibodies are rare in other diseases, giving weight to their use in the diagnosis of SLE.

Immunomicroscopic procedures have been developed for the detection of anti-dsDNA antibodies using a variety of tissues and cells as substrates. The system developed by Aarden *et al* uses the haemoflagellate *Crithidia luciliae*. The mitochondrion or kinetoplast of this organism contains a high concentration of dsDNA; in the test antibodies to dsDNA bound to this organelle are detected by indirect immunofluorescence microscopy. A radioimmunoassay for dsDNA antibody detection and measurement, the Farr assay, has been widely used in clinical immunology laboratories. Although the *C luciliae* and Farr tests show good correlation, the various tests for anti-dsDNA antibodies do not always agree.

Fibronectin, a cell surface and plasma glycoprotein, has a specific binding site for DNA. It is a component of immune complexes in connective tissue disease and shows variations in concentration with disease activity in SLE. Fibronectin may therefore interfere with the detection of autoantibodies directed against DNA in SLE. We tested this hypothesis in a series of studies using the *Crithidia luciliae* test.

Methods and materials
Studies were based on serum samples screened during one year (250 samples). Serum samples from 50 patients which were positive or weakly positive in the *C luciliae* test were used.

PURIFICATION OF PLASMA FIBRONECTIN
Fibronectin was purified from citrated normal human plasma by affinity chromatography on Gelofusin-Sepharose 4B and stored at -20°C.

DEPLETION OF PLASMA FIBRONECTIN
Fibronectin was depleted from some serum samples by gelatin-Sepharose 4B affinity chromatography as described for the purification of fibronectin but on a smaller scale (column bed volume 5 ml). For control purposes Sepharose 4B alone was used. Diluted serum (50% v/v in phosphate buffered saline (PBS); 0.1 mol/l KH2PO4, 0.154 mol/l NaCl, pH 7.2) was applied to the appropriate column and allowed to sink into the gel bed.

Fibronectin depleted serum was eluted from the column with PBS; in total the serum was diluted 1/10 (v/v) in PBS. In all, fibronectin was depleted from six serum samples with weak anti-DNA staining in the *C luciliae* test. Removal of fibronectin was assessed by an enzyme linked immunosorbent assay (ELISA) for fibronectin.

FIBRONECTIN MANIPULATIONS
In a series of blocking experiments fibronectin was added either to the wells before the addition of known positive serum samples or to positive serum directly. In a second series of experiments fibronectin was depleted from six serum samples with weak anti-DNA staining.
ASSAY FOR ANTI-dsDNA ANTIBODIES
The test was performed with a Fluoro-kit II (Clinical Sciences Inc, USA) according to the manufacturer's instructions. Briefly, in blocking experiments some wells were overlaid for 30 minutes at room temperature with either purified normal human plasma fibronectin or bovine serum albumin (0.3 mg/ml in PBS) then washed with PBS for 10 minutes. Serum samples were diluted 1 in 10 (v/v) in PBS or in PBS containing fibronectin at the concentration found in human plasma (0.3 mg/ml), and 25 μl applied to the wells for 30 minutes at room temperature in a covered moist chamber. A DNA positive serum and a serum sample containing no autoantibodies were used in each experiment as controls. After careful washing in PBS for 10 minutes, 25 μl of reconstituted fluorescein labelled antihuman gammaglobulin was added to each well and the slides placed in the moist chamber for a further 30 minutes. After washing in PBS the wells were mounted in Citifluor mounting medium (Citifluor Ltd, London, UK). Slides were examined immediately with a Leitz Dialux fluorescence microscope (Leitz, UK). This was performed 'blind' by an experienced observer. Staining intensity was recorded as negative (−), weakly positive (wk), positive (+), or strongly positive (+++) (figure A–D).

Results
Preincubation of test wells with fibronectin or addition of fibronectin to serum blocked the
Crithidia luciliae test for anti-dsDNA antibodies

Effect of fibronectin depletion on the intensity of staining of C luciliae in the anti-double-stranded DNA test

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial staining</th>
<th>Staining after galactose-Sepharose 4B column</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>wk</td>
<td>++</td>
</tr>
<tr>
<td>B</td>
<td>wk</td>
<td>+ ++</td>
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<td>F</td>
<td>wk</td>
<td>wk</td>
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</tbody>
</table>

Staining intensities: -= negative; wk = weak positive; + = positive; and ++ = strong positive.

Interaction of anti-DNA antibodies with the C luciliae kinetoplast. When fibronectin was added to the C luciliae before addition of known positive sera the intensity of staining was attenuated, resulting in a less positive reaction or negative staining (figure E). Similarly, addition of fibronectin, at a concentration corresponding to that found in human plasma (0.3 mg/ml) to previously positive sera followed by assay on C luciliae as before caused a reduction in the staining intensity, which was then seen as negative or weakly positive (figure F). In contrast, the interaction between antibody and DNA was not blocked when bovine serum albumin was used.

These supplementation experiments suggested that endogenous fibronectin may be masking the interaction of anti-dsDNA antibody with DNA. Therefore we performed a series of experiments aimed at removing fibronectin from sera. As our hypothesis suggested that an increased staining would be found the depletion of fibronectin from known positive sera would not have been useful. If we were performing a series of titres to determine the end point then positive sera would have been used, but we were simply investigating the effect of fibronectin on a routine clinical immunology assay and screening at a serum dilution of 1 in 10. Clearly the use of negative sera in an attempt to unmask anti-DNA antibodies may not yield results as such serum samples may not actually contain these antibodies. Therefore known weakly positive serum samples were used for the fibronectin depletion experiments. The efficiency of depletion was assessed by ELISA for fibronectin and was between 75 and 92% of total fibronectin. The staining in the anti-DNA assay became strongly positive in two cases and positive in a third (table). Two remained weakly positive and a third was negative; the latter was also negative after passing through a Sepharose 4B column.

Discussion

Our results indicate that fibronectin concentrations influence detection of anti-dsDNA antibodies using C luciliae and may explain the disparity between different tests for DNA antibodies. Furthermore, the unmasking of positive reactivity on removing fibronectin from serum has implications for the diagnosis and treatment of SLE.

The results for blocking experiments were conclusive—fibronectin blocks the detection of dsDNA antibodies. In the second series of experiments an increase in fluorescence staining intensity was related to the removal of fibronectin from weakly positive sera. Although not all the serum samples became more positive after fibronectin depletion, the results are still consistent with our hypothesis that removal of fibronectin allows an increase in staining of weakly positive sera.

In SLE fibronectin concentrations in the circulation vary with disease activity in individual patients. This may explain some of the differences observed between assays for dsDNA autoantibodies in these patients. Fibronectin blocks the detection of antibodies to DNA in the C luciliae test but in theory should increase radioactive DNA binding, and hence precipitation, in the Farr assay. The latter effect remains to be investigated.

Weakly positive staining in the assay is reported as negative by routine clinical immunology laboratories; this is widely accepted and stated within the kit's instructions. Our results indicate that at least some of these serum samples would give a positive reaction in the absence of serum fibronectin. Fibronectin concentrations in serum are lower than in plasma owing to fibrin binding, but this is not reproducible and may be a further factor in the detection of anti-DNA antibodies.

We have shown that there is interference by fibronectin in the Crithidia luciliae test for anti-dsDNA antibodies. As circulating levels of both antibody and fibronectin vary with disease activity in SLE we are currently investigating the interactions of these three variables in a longitudinal study.

We acknowledge the financial support of the Arthritis and Rheumatism Council, the Joint Research Board of St Bartholomew's Hospital, and the North East Thames Regional Health Authority locally organised research scheme.

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doi: 10.1136/ard.49.9.705

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