Increased release of von Willebrand factor antigen from endothelial cells by anti-DNA autoantibodies

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Abstract

Objective—To determine whether antibodies to double stranded DNA (anti-dsDNA) have a pathogenic role in systemic lupus erythematosus (SLE).

Methods—IgG was purified from 17 patients with SLE (median anti-dsDNA titre 1212 IU/ml) and nine healthy controls (median titre 40 IU/ml). Anti-dsDNA depleted polyclonal IgG (median anti-dsDNA titre 17 IU/ml) was also prepared from sera of the 17 patients by affinity chromatography on a DNA cellulose column. Binding to antiendothelial cell antibodies (AECA) and expression of von Willebrand factor (VWF) antigen by cultured human umbilical vein endothelial cells (HUVECs) were studied by flow cytometry.

Results—The percentage of HUVECs binding to AECA or expressing VWF was greater for cells incubated with IgG from patients with SLE than for cells incubated with control IgG, though values did not reach statistical significance; nevertheless, HUVECs incubated with IgG from patients expressed a greater mean fluorescence intensity with AECA ($p = 0.0001$) and greater VWF expression ($p = 0.019$). Both the fluorescence intensity and percentage of HUVECs binding to AECA or expressing VWF were significantly greater in HUVEC incubated with IgG containing anti-dsDNA than in those incubated with anti-dsDNA depleted IgG. The concentration of VWF in the supernatant was significantly increased in HUVECs incubated with IgG containing anti-dsDNA compared with control IgG or anti-dsDNA depleted IgG. Pretreatment of HUVECs with native DNA before incubation with IgG from lupus patients did not increase binding to AECA, or expression or release of VWF.

Conclusions—Our study provides in vitro evidence that antibodies to DNA have a pathogenic role in the induction of inflammatory injury of the vascular endothelium in SLE.

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The pathogenic mechanism of vasculitis and nephritis in systemic lupus erythematosus (SLE) remains a subject of debate. Human and animal studies in B/W mice show the presence of anti-double stranded DNA antibodies (anti-dsDNA), DNA and complement in kidney

eluates, suggesting a possible pathogenic role for DNA-anti-DNA immune complexes.^{1,2} In addition, a correlation has been reported between pretreatment anti-dsDNA titre in serum and severity of the disease, particularly in the case of glomerulonephritis.^{3,4} Other evidence for a direct pathogenic function of anti-dsDNA is not strong.

Plasma concentrations of von Willebrand factor (VWF) antigen are increased in patients with SLE and other vasculitic lesions, and are considered to be an indicator of endothelial cell damage.^{5,6} There is, however, little information to suggest that antibodies to dsDNA release these endothelial products.

In this study, we examined the expression and release of VWF from, and the binding of antiendothelial cell antibodies (AECA) to, cultured human umbilical vein endothelial cells (HUVECs) in response to IgG containing anti-dsDNA and IgG depleted of anti-dsDNA from patients with SLE, to determine any direct pathogenic effect of anti-dsDNA on endothelial injury.

Materials and methods

IMMUNOGLOBULIN PREPARATION

Sera were obtained from 17 randomly selected patients with SLE (12 female; mean age 25 (SEM 4) years) before they received immunosuppressive treatment, and from nine healthy controls (eight female; mean age 28 (4) years). The patients with SLE fulfilled the criteria of the American Rheumatism Association⁷ and none of them had a clinical history of thrombosis.

Purified IgG preparations were prepared by affinity chromatography with protein-G sepharose (Pharmacia LKB, Uppsala, Sweden) and their purity ascertained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Endotoxin was not detected in the IgG preparations by Limulus amoebocyte lysate test as previously described.⁸ The possible cytotoxicity caused by these autoantibodies was studied by chromium release from ⁵¹Cr-labelled HUVECs incubated with different IgG preparations. The IgG preparations were adjusted to 10 mg/ml and the anti-dsDNA titre was determined by an enzyme linked immunosorbent assay (ELISA) (NovoPath anti-dsDNA kit, Biorad, Hercules, CA, USA). All IgG preparations were tested for anticardiolipin antibody, tumour necrosis factor α (TNF α), and interleukin 1 β (IL-1 β). IgG depleted of anti-dsDNA were prepared by passing 20 mg of IgG preparation through a native DNA-cellulose column (Pharmacia)

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and the flow through was collected, dialysed, and concentrated to 10 mg/ml. Complete removal of anti-dsDNA was confirmed by the NovoPath anti-dsDNA ELISA. The IgG and anti-dsDNA depleted IgG preparations were stored at -70°C until required for use.

IMMUNOASSAYS FOR CYTOKINES IN IgG PREPARATIONS

'Sandwich' ELISAs were used to measure TNF α (Genzyme, Boston, MA, USA) and IL-1 β (R & D System) in the IgG preparations. In all the assays, two separate monoclonal or polyclonal antibodies were used, each recognising a separate epitope of the respective molecule. Results were determined from a standard curve derived from the optical densities of the different dilutions of the standard samples. The lower limits of sensitivity for the assays are 0.03 pg/ml (IL-1 β) and 5 pg/ml (TNF α). All samples were assayed at the same time to avoid between batch variation. Intra-assay coefficient of variation was less than 5% for all assays.

ISOLATION AND CULTURE OF ENDOTHELIAL CELLS

HUVECs were isolated according to the method of Jaffe *et al.*⁹ and cultured on a 1% gelatin coated tissue culture flask (Falcon, Becton-Dickinson, Mountain View, CA, USA) with M199 culture medium containing 20% heat inactivated fetal bovine serum, 100 U/ml penicillin and streptomycin (Gibco, Chargin Falls, NY, USA), 0.25 mg/ml heparin, and 20 $\mu\text{g/ml}$ endothelial cell growth supplement (Sigma, St Louis, MO, USA). Endothelial cell phenotype was confirmed by a typical cobblestone morphology, and the presence of VWF by immunofluorescence staining using monoclonal antihuman VWF (Dako, Copenhagen, Denmark).

CYTOTOXICITY ASSAY

Second to third passage HUVECs were plated out into 24 well culture plates. Chromium-51 labelling was performed by incubating 1 μCi of chromium-51 (1 mCi/ml; Amersham, UK) in 300 μl culture medium for 24 hours at 37°C . After three washes with phosphate buffered saline (PBS) to remove excess radiolabel, the cells were incubated in triplicate with 0.5 mg/ml IgG in medium, M199, or 100 pg/ml TNF α for 16 hours at 37°C . The plates were centrifuged at 800 rpm for five minutes, the culture supernatant removed, and the released radioactivity measured by gamma-counter. The results were expressed as percentage specific release = $(\text{cpm experimental} - \text{cpm spontaneous}) / (\text{cpm total} - \text{cpm spontaneous}) \times 100\%$, where total release indicated the radioactivity collected after 100 μl of 2 mol/l NaOH was added, and spontaneous release was the radioactivity in the supernatant from cell cultures in medium alone.

Table 1 Preliminary study for determination of optimal concentration of IgG from lupus patients on expression and release of VWF from cultured HUVECs

IgG concentration (mg/ml)	VWF expression (mean fluorescence channel number)	VWF in supernate (mIU/ml)
0	450	3.352
0.125	463	4.176
0.25	478	5.165
0.5	510	6.153
1	523	6.562
2	533	7.013
4	540	7.152

Values represent mean of IgG preparations from three lupus patients.

FLOW CYTOMETRIC ANALYSIS OF VWF

EXPRESSED ON AND AECA BOUND TO HUVEC

Second to third passage HUVECs were plated out into 24 well gelatin coated tissue culture plates (Costar, Cambridge, MA, USA) at a concentration of 2.5×10^5 cells per well for 24 hours before the experiment. The confluent cells were then incubated at 37°C in triplicate with 0.5 mg/ml IgG, M199, or TNF α (Boehringer, Mannheim, Germany) at a final concentration of 1000 pg/ml in complete culture medium for 16 hours; the optimal concentration of IgG was determined by preliminary studies of the expression and release of VWF by cultured HUVECs (table 1), and the HUVECs were incubated for 16 hours to achieve a near-maximal release of VWF (table 2). After incubation, supernatant samples were collected and stored at -70°C until required for VWF assay. The cells were washed with PBS, harvested at room temperature with 0.5 mmol/l EDTA in PBS, and transferred into 12×75 mm tubes. The cell suspensions were divided into two portions: one was stained for binding to human IgG using affinity purified fluorescein isothiocyanate (FITC) conjugated goat anti-human IgG F(ab')₂ preparation (Tago, Camarillo, CA, USA); the other was stained for VWF expression using monoclonal antihuman VWF (Dako) as primary antibody and affinity purified FITC conjugated F(ab')₂ fragment of goat antimouse immunoglobulins as secondary antibody (Dako). Background control staining of the HUVECs was achieved by reaction with either preimmune FITC conjugated goat IgG F(ab')₂ (Tago) or isotype matched mouse IgG (Dako). The stained cells were analysed using a FACScan analyser (Becton-Dickinson) and a technique adopted from Westphal *et al.*¹⁰ A minimum of 5000 unfixed cells for each sample was analysed. Fluorescence intensity in each

Table 2 Kinetic study of soluble VWF release of VWF (mIU/ml) from HUVECs incubated with 0.5 mg/ml IgG

Duration of incubation (hours)	Control IgG	Anti-dsDNA depleted IgG	Anti-dsDNA IgG
0	3.54	3.81	3.69
2	3.84	4.26	4.79
4	3.92	4.53	4.96
6	3.96	4.62	5.26
8	4.05	4.86	5.48
16	4.20	5.05	6.55
24	4.22	5.21	7.14
48	3.98	5.08	6.98

Values represent mean of IgG preparations from three lupus patients and three controls.

experiment was evaluated by comparing the mean fluorescence channels without subtraction of control staining. The percentage of HUVECs with fluorescence intensity greater than the background from isotypic control antibodies was also determined. All staining for a single molecule was done in a single batch to avoid between batch variation.

ELISA FOR MEASURING VWF IN SUPERNATE

Supernatant concentrations of VWF in HUVECs cultured with IgG, medium, or TNF were assayed using a modified technique of Lindsey *et al.*¹¹ ELISA plates were coated with monoclonal antibodies against VWF (Dako) in 0.05 mol/l carbonate buffer, pH 9.6, overnight at room temperature. After washing with PBS containing 0.05% Tween 20, the plates were blocked for non-specific binding with 3% bovine serum albumin in PBS for one hour. After washing, the culture supernate and VWF standards (Immuno, Sevenoaks, Kent, UK) were added to the wells and further incubated for two hours. The plates were washed three times with PBS/Tween and then incubated with a peroxidase labelled polyclonal antibody against human VWF (Dako) for one hour. After a further three washes, the plates were incubated with 0.05% orthophenylenediamine hydrochloride and 0.01% hydrogen peroxide and the resulting colour reaction measured at 492 nm using an ELISA reader.

PREINCUBATION OF HUVEC WITH DNA

In order to determine whether immune complexes formed by anti-dsDNA with DNA on the surface of HUVECs were necessary for endothelial activation, all experiments were repeated in cultured HUVECs pretreated with calf thymus native DNA (Sigma) at a concentration of 50 µg/ml for one hour before further incubation with IgG preparation.

STATISTICS

Because the distribution of the data appeared not to be normal, group results were expressed as median and range unless stated otherwise, and non-parametric tests for significance were performed. The data between the IgG preparations from patients with SLE and healthy controls were tested using the Wilcoxon rank sum test, and data between IgG containing anti-dsDNA and anti-dsDNA depleted IgG were analysed with the signed rank test. Relationships between the expression and release of VWF, AECA, or anti-dsDNA titre were examined by Spearman Rank Correlation. Statistical significance was assigned at $p < 0.05$.

Results

The anti-dsDNA titres of the IgG preparations from lupus patients (median 1212 IU/ml, range 377–9662) were significantly greater than those of the healthy controls (median 40 IU/ml, range 23–52; $p < 0.0001$). After passage

through a native DNA-cellulose column, the anti-dsDNA titres of the flow through IgG (anti-dsDNA depleted IgG) decreased to negligible values (median 17 IU/ml, range 1–64; $p < 0.0001$) compared with the original IgG preparations. Anticardiolipin antibody, TNFα, and IL-1β were not detected in these IgG preparations. No cytotoxicity was detected in HUVECs exposed to IgG preparations. The average release of chromium-51 from HUVECs incubated with IgG preparations (4.8%) was similar to that observed in HUVECs incubated with culture medium (5.0%). However, release of chromium-51 from HUVECs incubated with TNFα (1000 pg/ml) was 15%, suggesting cytotoxicity induced by this dose of TNFα.

The AECA were determined indirectly as the binding of IgG to the HUVECs: there was an apparent increase in the percentage of HUVECs binding to IgG from patients with SLE compared with IgG from healthy controls, though the difference failed to reach statistical significance (fig 1A). The concentration of

Figure 1 A: Percentage of human umbilical vein endothelial cells (HUVECs) binding to antiendothelial cell antibodies after incubation with IgG containing anti-dsDNA (anti-dsDNA IgG), anti-dsDNA depleted IgG, or control IgG. Horizontal bar represents median value. Mean (SD) values of five experiments incubated with medium and tumour necrosis factor α (TNFα) 1000 pg/ml were 10.12 (3.1)% and 61.4 (10.8)%, respectively. Patients with SLE and healthy controls compared using the Wilcoxon rank sum test; anti-dsDNA IgG and anti-dsDNA depleted groups compared by signed rank test. B: Percentage of HUVECs expressing von Willebrand factor after incubation with different IgGs. Horizontal bar represents median value. Mean (SD) values of five experiments incubated with medium and TNFα were 28.2 (2.9)% and 55.4 (6.4)%, respectively.

AECA measured by the fluorescence intensity on HUVECs was significantly increased in IgG from patients with SLE compared with IgG from healthy controls (fig 2A).

The expression of VWF on cultured HUVECs in response to stimulation by different IgG preparations was determined as binding to antihuman VWF antibody measured by flow cytometry. Again, there was an apparent increase in the percentage of HUVECs expressing VWF when incubated with IgG from patients compared with control IgG, but the difference failed to reach statistical significance (fig 1B). As observed in AECA, the concentration of VWF measured by fluorescence intensity on HUVECs was significantly increased in HUVECs incubated with IgG from lupus patients compared with those incubated with control IgG (fig 2B).

To validate the selective effect of anti-dsDNA on expression of VWF by HUVECs, IgG containing anti-dsDNA and anti-dsDNA depleted-IgG were prepared from the 17 lupus

patients by affinity chromatography. AECA was significantly reduced in anti-dsDNA depleted-IgG compared with IgG containing anti-dsDNA from the same patient, as shown by the lower percentage and lower fluorescence intensity of HUVECs binding to AECA (figs 1, 2). Similarly, VWF expression was significantly reduced on HUVECs incubated with anti-dsDNA depleted IgG compared with HUVECs incubated with IgG containing anti-dsDNA from the same patient (figs 1, 2). Except for fluorescence intensity of binding to AECA ($p = 0.0165$), HUVECs incubated with anti-dsDNA depleted IgG showed no increase in percentage of binding to AECA or expression of VWF compared with those incubated with control IgG. The supernatant concentration of VWF was significantly increased in HUVECs incubated with IgG containing anti-dsDNA compared with control IgG or anti-dsDNA depleted IgG ($p = 0.009$ and 0.0003 , respectively) (fig 3). Pretreating HUVECs with native DNA did not result in an additive effect of binding to AECA or expression and release of VWF (data not shown).

After incubation with IgG containing anti-dsDNA from patients with SLE, the percentage of HUVECs binding to AECA correlated significantly with the fluorescence intensity of AECA binding to HUVECs ($r_s = 0.53$, $p = 0.027$). Similarly, the percentage of HUVECs expressing VWF correlated significantly with the fluorescence intensity of VWF expression on HUVECs ($r_s = 0.79$, $p = 0.0015$). The fluorescence intensity of VWF expression on HUVECs following incubation with IgG containing anti-dsDNA correlated with the supernatant concentration of VWF ($r_s = 0.50$, $p = 0.044$). There was no correlation between the anti-dsDNA titres and binding to AECA, or expression or release of VWF by HUVECs. No significant correlation was demonstrated between AECA levels and

Figure 2 A: Fluorescence intensity of human umbilical vein endothelial cells (HUVECs) binding to antiendothelial cell antibodies after incubation with IgG containing anti-dsDNA (anti-dsDNA IgG), anti-dsDNA depleted IgG, or control IgG. Horizontal bar represents median value. Mean (SD) values of five experiments incubated with medium and tumour necrosis factor α (TNF α) were 357.6 (29.3) and 535.9 (43.8) respectively. Patients with SLE and healthy controls compared using the Wilcoxon rank sum test; IgG containing anti-dsDNA and anti-dsDNA depleted IgG groups compared by signed rank test. B: Fluorescence intensity of HUVECs expressing von Willebrand factor after incubation with different IgGs. Horizontal bar represents median value. Mean (SD) values of five experiments incubated with medium and TNF α were 424.2 (35.9) and 541.4 (43.5)%, respectively.

Figure 3 Supernatant concentrations of von Willebrand factor in human umbilical vein endothelial cells after incubation with IgG containing anti-dsDNA (anti-dsDNA IgG), anti-dsDNA depleted IgG, or control IgG. Horizontal bar represents median value. Mean (SD) values of five experiments incubated with medium and tumour necrosis factor α were 2.1 (1.5)% and 13.2 (4.1)%, respectively. Patients with SLE and healthy controls compared using the Wilcoxon rank sum test; IgG containing anti-dsDNA and anti-dsDNA depleted groups compared by signed rank test.

expression or release of VWF on HUVECs incubated with IgG from lupus patients.

Discussion

Circulating autoantibodies present in SLE patients, especially those against native double stranded DNA, have been shown to serve as an underlying substratum for the disease manifestation. A pathogenic role for anti-dsDNA of IgG isotype was indirectly established by the correlation of serum antibody titres with disease activity in some patients and the demonstration of DNA-anti-DNA immune complex deposition at sites of tissue damage such as kidney.¹⁻⁴ However, it is now becoming clear that anti-DNA antibodies are not always nephritogenic:¹² in NZB/NZW mice, administration of syngeneic anti-DNA monoclonal antibody often suppresses the development of murine lupus nephritis.¹³ In addition, most patients, even those with active SLE, may not have free circulating DNA or DNA-anti-DNA complexes in the blood or eluates from the affected kidney.^{14 15} Thus it remains questionable whether anti-dsDNA merely represent epiphenomena or have a direct pathogenic role.

Preliminary data in support of a direct pathogenic role for anti-dsDNA are few, but most intriguing. Sera from patients with SLE increased endothelial cell adherence, aggregation, and superoxide generation by neutrophils,¹⁶ and increased major histocompatibility complex class I antigen expression on cultured HUVECs.¹⁷ IgG from thrombosis susceptible patients with SLE stimulated the release of VWF from endothelial cells.¹¹ Anti-dsDNA altered the mitogenic responses of mononuclear cells¹⁸ and exerted a cytotoxic effect on cultured rat mesangial cells.¹⁹ Binding of IgG in sera from lupus patients to endothelium initiated complement activation that may lead to vascular injury.²⁰ In the present study, we have examined the effect of anti-dsDNA on the expression and release of VWF by HUVECs. Increased plasma concentrations of VWF are well recognised features of patients with vasculitis and autoimmune disorders, and have been taken to indicate the occurrence of in vivo endothelial cell damage.^{5 6} Lindsey *et al*¹¹ reported that IgG from thrombosis susceptible patients with SLE stimulated the release of VWF from endothelial cells, and provided evidence that IgG isolated from patients with SLE was capable of acting as an agonist for VWF release. In this study, we purified IgG free of complement or cytokine that could release VWF from HUVECs.^{21 22} Compared with IgG from healthy controls, the concentrations of AECA present in the IgG prepared from patients with SLE (measured by FACScan) were increased as determined by fluorescence intensity of HUVEC binding to IgG. Similarly, there was an increase in VWF expression on HUVECs incubated with IgG prepared from patients with SLE. The percentage of HUVECs binding to AECA or expressing VWF when incubated with IgG from SLE patients failed to reach statistical

significance, probably because of the small number of controls. It may be argued that the effect of IgG from patients with SLE on HUVECs may represent the difference in a property of IgG from the individual patient or control, rather than a specific effect of anti-dsDNA. We approached this issue by preparing IgG containing anti-dsDNA and anti-dsDNA depleted IgG from the same patient, and used the latter as control for IgG containing anti-dsDNA. Instead of testing anti-dsDNA eluted from the DNA-cellulose column, we used IgG containing anti-dsDNA for comparison with anti-dsDNA depleted IgG, as the recovery of anti-DNA activity and loaded IgG in the eluate was often less than 40%.²³ Similarly, we failed to recover more than 35% of anti-DNA activity and loaded IgG, raising the possibility that the eluted anti-dsDNA were of low affinity. The anti-dsDNA titres of the anti-dsDNA depleted IgG were comparable to those of healthy controls. Our data demonstrated significantly greater AECA concentrations in IgG containing anti-dsDNA compared with anti-dsDNA depleted IgG. There was also increased expression of VWF on HUVECs incubated with IgG containing anti-dsDNA compared with those incubated with anti-dsDNA depleted IgG prepared from the same patient. These data indicate strongly that the effect of anti-dsDNA on the HUVEC is highly specific. The flow cytometry results relating to VWF were confirmed by the increased supernatant concentration of VWF in HUVECs incubated with IgG containing anti-dsDNA. AECA is considered to be a potential marker for nephritis and vasculitis in SLE,²⁴ whereas VWF is a large adhesive glycoprotein that has an important role in the interaction of platelets and the blood vessel wall and is released after in vivo endothelial cell damage.^{21 25} Hence, our data provide additional evidence that anti-dsDNA directly stimulates endothelial cells and has a pathogenic role in the development of vasculitis in SLE. The direct stimulatory effect of anti-dsDNA on endothelium may have important pathological significance, as the release of VWF approximates 50% of the effect of TNF α at a concentration of 1000 pg/ml. We confirmed the absence of correlation between anti-dsDNA titre, AECA concentrations, and expression or release of VWF^{11 21} that may suggest a different pathogenic role of anti-dsDNA and AECA.

Previous studies suggested that the binding of anti-dsDNA to endothelial cells was mediated through DNA, which formed a bridge between the immunoglobulin and the DNA binding protein in the plasma membrane.^{26 27} However, more recently Tsai *et al*¹⁹ reported direct binding of IgG containing anti-dsDNA to cell membrane of rat mesangial cells. Shibata *et al*²⁸ have demonstrated that circulating anti-dsDNA can cross react with vascular heparan sulphate glycosaminoglycans, suggesting that at least a percentage of these autoreactive antibodies may be directed against epitopes in heparan sulphate that are conformationally related to those found in DNA. In

this study, we have demonstrated no additive effect of expression of adhesion molecules in HUVECs pretreated with native DNA. These findings tend to suggest that anti-dsDNA can bind directly to the plasma membrane of HUVECs, perhaps via heparan sulphate glycosaminoglycans, without bridging through the DNA binding protein. The exact mechanism for VWF release by anti-dsDNA is not certain, and we have shown that direct cytotoxicity is unlikely to play a significant part. Complement dependent effects of AECA in VWF release did not operate in our *in vitro* studies, as the IgG preparations were free of complement.

It should be emphasised that our studies were performed with endothelial cells from umbilical vein. The recent rediscovery of heterogeneity in human aortic endothelium and variant endothelial cell populations^{29,30} raises the possibility that our observed effect of anti-dsDNA may not be completely identical in these endothelial cells. Further studies of endothelial cells with different phenotypes are required. In conclusion, our study provides preliminary evidence that anti-dsDNA has a direct pathogenic role in the development of vasculitis in SLE.

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