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Characterization and specificity of anti-endothelial cell membrane antibodies and their relationship to thrombosis in primary antiphospholipid syndrome (APS)

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SUMMARY

Immunoblotting was used to detect antibodies reacting with membrane and cytosol preparations of human umbilical vein endothelial cells (HUVEC), fibroblasts and a T lymphoma line HUT78 in 18 patients with anticardiolipin antibodies (ACA) (14 of whom had had a thrombotic event), 11 patients with a recent myocardial infarction and 17 controls. Multiple membrane-specific antibodies to HUVEC were found in 10 of the patients with ACA (28 bands) and in nine of the patients with thromboses (27 bands) in contrast to only three of the patients with myocardial infarction (four bands) and one control (one band). The most frequently recognized HUVEC membrane epitopes were at 33 kD (four sera), 61–63 kD (five sera) and 76–79 kD (four sera). Although cross-reactivity with fibroblast and/or HUT78 membranes was seen at 33 kD, binding at 61–63 kD and 76–79 kD was specific for endothelial membranes. Although no correlations with the presence and titre of ACA were seen, HUVEC membrane-specific antibodies showed a correlation with venous thrombotic events.

Keywords endothelial membranes immunoblotting anti-endothelial antibodies antiphospholipid syndrome

INTRODUCTION

Although anticardiolipin antibodies (ACA) are associated with the occurrence of thrombosis [1–5] in patients with primary APS, the mechanisms involved are likely to be complex and multifactoral, involving inhibition of thrombomodulin-induced activation of protein C [6–8], impairment of both antithrombin III [9] and fibrinolysis [10], as well as inhibition of prostacyclin release from endothelium [11–13]. Studies of some of these mechanisms have certainly produced conflicting results [13] and it is also clear that a single thrombogenic factor such as ACA does not satisfactorily explain either the triggering of thrombotic events or the fact that not all patients develop thrombosis [14,15]. Previous studies, involving small groups of patients with primary APS and systemic lupus erythematosus (SLE), indicated that patients with ACA possess anti-endothelial cell antibodies [16] and that these antibodies, detected by ELISA on human umbilical vein endothelium (HUVEC), showed a stronger correlation with inhibition of prostacyclin release in patients with thrombosis than did ACA [17]. In this study, we confirm that patients with primary APS do have anti-endothelial cell antibodies, and further characterize and define the specificity of these antibodies by immunoblotting sera with membrane and cytosol preparations of HUVEC, fibroblasts and a T lymphoma line HUT78 (ECACC, Porton Down, UK).

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PATIENTS AND METHODS

Patients and controls

Serum samples, aliquots of which were stored at −80°C before use, were obtained by venepuncture from 18 patients with positive tests for ACA (12 women, six men; mean age 56 ± 15 years), 11 patients with a recent myocardial infarction (four women, seven men; mean age 66 ± 7 years) and 17 normal controls (13 women, four men; mean age 55 ± 10 years). Of the 18 patients with ACA, 14 had had either a venous and/or arterial thrombosis and satisfied the criteria of primary APS [18] (eight women, six men; mean age 56 ± 12 years), while the remaining four patients had had minor manifestations such as migraine, leg ulcers or transient cerebral ischaemic episodes which had led to the finding of a positive test for ACA.

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Culture of HUVEC fibroblasts and human T lymphoma line HUT78
HUVEC were isolated as previously described [17] and cultured on a 0·2% gelatin matrix in M199 supplemented by 20% human AB serum (with antibiotics). Cells were harvested, when confluent, after the first passage, having been maintained for the last 4 days of culture in M199 supplemented by 5% IgG-depleted human AB serum in order to reduce the amount of non-specific binding of human IgG to the cell membranes.

Human dermal fibroblasts (obtained from a normal control) were cultured in Dulbecco's minimum essential medium (MEM) supplemented by 10% fetal calf serum (FCS) (with antibiotics).

The human T lymphoma line HUT78 (originating from a patient with Sezary syndrome) was grown in RPMI 1640 supplemented with 10% FCS (with antibiotics).

Cell membrane preparation
Cell membranes from washed confluent cultures of HUVEC, fibroblasts and the T lymphoma line HUT78, were prepared by a method based on that of van der Zee *et al.* [20], involving freeze-thawing, sonication and differential centrifugation. Cells were harvested by mechanical scraping, lysed by freeze thawing three times in PBS containing as enzyme inhibitors EDTA 0·02 M, benzamidine HCl 0·01 M and Trasylol 70 μg/ml. The lysed cell membranes were harvested by centrifugation at 10 000 ***g*** for 30 min and the supernatant was retained as the cytosolic fraction which was concentrated by ultrafiltration using a Minicon filter (Amicon, Stonehouse, UK). The pelleted membranes were resuspended in inhibition medium and sonicated four times for 10 s before being centrifuged at 15 000 ***g*** for 30 min, resuspended in inhibition medium and finally recentrifuged at 4500 ***g*** for 15 min to remove cytosolic contamination from the final pelleted preparation. Both this final enriched membrane preparation and the concentrated cytosolic fraction were adjusted with inhibition medium to a final concentration of 800 μg/ml of protein and stored in aliquots at −80°C before use.

Integrity of membrane and cytosol fractions of HUVEC
The enrichment of the membrane preparations and freedom from cross-contamination of the cytosol fractions prepared from HUVEC were established by an ELISA which used a MoAb EN4 (Bradsure Biologicals, Loughborough, UK) which is known to react with membrane epitopes which are retained on cultured HUVEC [21]. ELISA plates were coated overnight at 4°C with 0–30 μg/ml of either membrane or cytosol preparation solubilized in 25 mM carbonate buffer pH 9·6 and then incubated, after washing and blocking with PBS, 0·1% Tween 20 and PBS 0·1%, Tween 20, 2% bovine serum albumin (BSA), with MoAb EN4 diluted 1:500. Bound EN4 was detected using affinity-purified human serum absorbed goat anti-mouse IgG–alkaline phosphatase conjugate (Sigma, Poole, UK). Absorbance at 405 nm was measured after the addition of *p*-nitrophenyl phosphate (Sigma). Typical results, illustrated in Fig. 1, indicated persistence of membrane epitopes detected by MoAb EN4 in the membrane preparations of the HUVEC without any evidence of cross-contamination in the cytosol preparations.

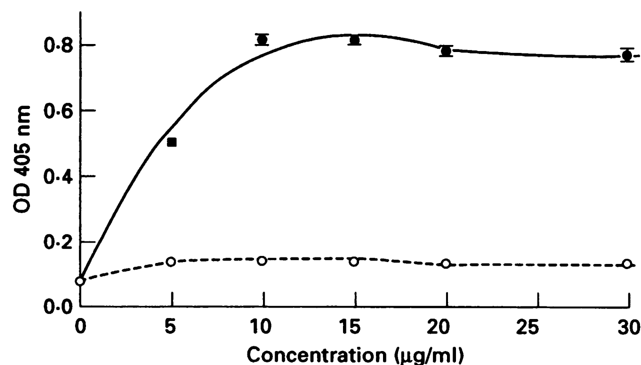

Fig. 1. Reactivity of human endothelial membrane-specific MoAb EN4 with increasing concentrations of membrane (——) and cytosol (-----) preparations of human umbilical vein endothelial cells (HUVEC).

Immunoblotting for the detection of membrane-specific antibodies
SDS–PAGE of 40 μg aliquots of membrane and cytosol preparations, together with molecular weight markers (Sigma) over the range 12·5–205 kD, was performed using (10%T, 2·67%C) bis-cross linked gels for 140 mA hours. Semi-dry electroblotting for 500 mA hours was used to transfer proteins to nitrocellulose membranes which were stained with 5% amido black in 40% methanol, 10% acetic acid, then de-stained and blocked with PBS, 1% casein. Strips of the nitrocellulose membranes were then incubated for 2 h with patient and control sera, diluted 1:300 in PBS, 2% FCS, 1% casein. After four washes in PBS, 0·1% Tween 20, strips were incubated for 1·5 h with peroxidase-conjugated c-chain-specific goat anti-human IgG (Sigma) diluted 1:300 in PBS 2% FCS, 1% casein. After four further washes with PBS, 0·1% Tween 20, bound peroxidase conjugate was detected following a final 1-min incubation with luminol (10% in DMSO with enhancer) diluted 1:200 and H_2O_2 (30%) diluted 1:400 in 0·1 M Tris–HCl pH 8·0 [22]. Chemiluminescence was detected using Fuji RX x-ray film (GRI Ltd, Dunmow, UK).

The membrane specificity of antibodies reacting with the three cell lines was established by discounting any antibodies that showed binding common to identical molecular weight epitopes in both cytosol and membrane fractions.

ELISA for ACA
ACA were detected as previously described [23], by an assay calibrated against IgG and IgM ACA standards (kindly donated by Dr N. Harris, Rayne Institute, London, UK) with a reference range of 0–8 units and 0–5 units for IgG and IgM antibodies, respectively, based on determinations (mean ± 3 s.d.) in 40 normal controls. For analysis, results were subdivided into high titre ($>$25 units) and low titre ($<$25 units) groups.

Statistical analysis
Comparison of the incidence of antibodies reactive with membrane preparations of HUVEC in patients and control groups was made by the χ^2 test after applying Yate's correction.

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RESULTS

Immunoblotting with membrane and cytosol preparations of HUVEC, fibroblasts and human T lymphoma line HUT78

Binding of IgG to HUVEC membrane preparations, usually with multiple bands, was detected with 10 of the 18 sera (55%) containing ACA. In the 14 sera from patients with primary APS (with venous and/or arterial thrombosis) binding to membrane preparations was found with nine sera (64%), producing a total of 27 bands in contrast to one sera, producing one band, in the 17 normal controls (9/14 *versus* 1/17; $P < 0{\cdot}01$). The most frequently recognized membrane antigens were at 33 kD (four sera), 61–63 kD (five sera) and 76–79 kD (four sera) (Fig. 2). Binding at both 61–63 kD and 76–79 kD was specific for endothelium, whereas cross-reactivity with either fibroblasts or T lymphoma HUT78 membranes was seen with all four sera binding at 33 kD (Fig. 3). Similar cross-reactivity with either fibroblasts and/or HUT78 membranes was seen with only two of the additional HUVEC membrane antibodies detected at 37 kD and 18 kD, respectively.

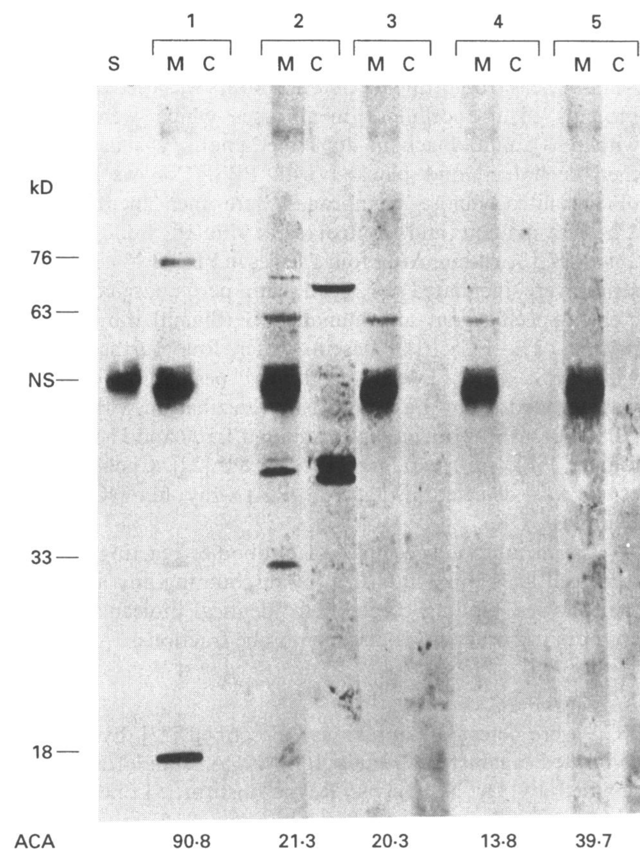

Fig. 2. Immunoblot of five sera containing 90·8, 21·3, 20·3, 13·8 and 39·7 units of IgG anticardiolipin antibodies (ACA), respectively, with membrane (M) and cytosol (C) preparations of human umbilical vein endothelial cells (HUVEC). IgG binding to 18 kD (1), 33 kD (1,2) 63 kD (2,3,4) and 76 kD (1) membrane epitopes is seen with sera from patients with venous and/or arterial thrombosis in contrast to the absence of antibodies to HUVEC membranes in patient 5 with purely migraine. The HUVEC membrane preparations also show non-specific binding of IgG fragments (NS) derived from the culture medium as indicated by the saline control (S).

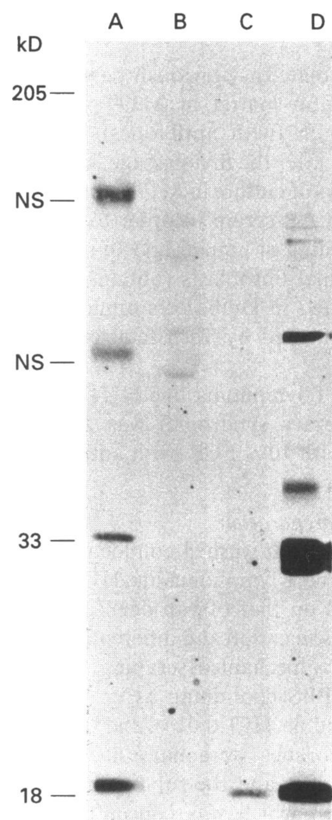

Fig. 3. Immunoblot of anticardiolipin-positive serum with membrane (A) and cytosol (B) preparations of human umbilical vein endothelial cells (HUVEC) and membrane preparations of human dermal fibroblasts (C) and lymphoma HUT78 (D) illustrating IgG binding to 33-kD and 18-kD epitopes on HUVEC membrane as well as additional reactivity or cross-reactivity with fibroblast and/or HUT78 membranes. The HUVEC membrane preparation also shows non-specific binding of IgG fragments derived from the culture medium (NS).

By contrast, binding of IgG to HUVEC membrane preparations was found with only one of the four sera (a single band) from patients with ACA without thrombotic events, and with three of 11 sera (four bands) from patients with myocardial infarction (Fig. 4). Only one of these 11 patients with myocardial infarction possessed a low titre ACA. In addition, epitopes confined to either fibroblast or HUT78 membrane preparations were detected by sera from seven (eight bands) and 10 (19 bands) of the 18 patients with ACA, respectively. Occasional single membrane epitopes from either fibroblasts or the lymphoma line were also detected by four of the 17 control sera and by six of the 11 sera from patients with myocardial infarction.

Association of ACA and anti-endothelial membrane antibodies with thrombosis

HUVEC membrane-specific antibodies were detected in seven of the eight patients (86%) with venous thrombosis, only three of whom had high titre ACA. HUVEC-specific membrane antigens at 76–79 kD and 61–63 kD were detected in four

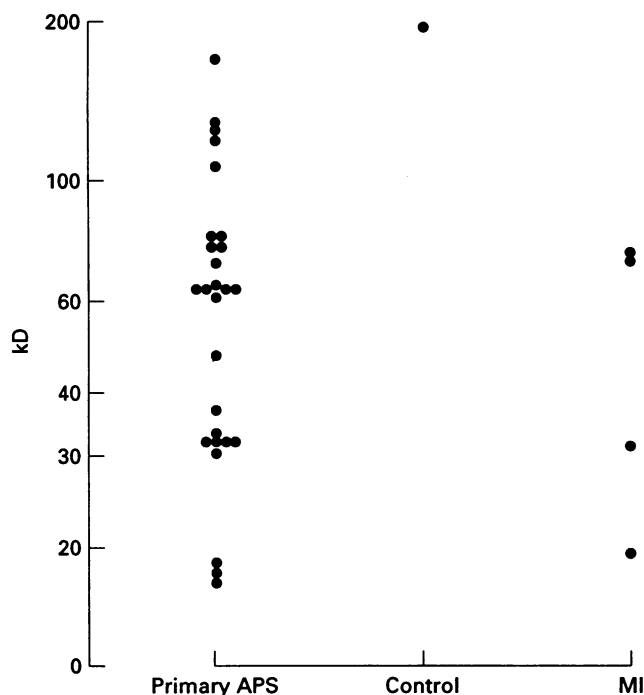

Fig. 4. Distribution of human umbilical vein endothelial cell (HUVEC) membrane epitopes detected by IgG antibodies in 14 patients with primary APS, 11 patients with a recent myocardial infarction (MI) and 17 normal controls. Nine primary APS sera reacted with HUVEC membranes in contrast to one control serum ($P < 0{\cdot}01$).

and three of these eight patients, respectively. In patients with arterial thrombosis, HUVEC membrane-specific antibodies were detected in five of the nine patients (56%), but in only two of the six cases who had not had an associated venous thrombosis. High titre ACA were again found in only two of the six cases in this latter subgroup. None of these trends, however, was statistically significant, possibly due to the small size of the patient subgroups.

DISCUSSION

This investigation has shown, by the use of an anti-endothelial cell MoAb, that the HUVEC membrane preparations used to detect anti-endothelial cell antibodies have contained membrane-specific epitopes. The use of enriched cytosol as well as membrane preparations has emphasized the membrane specificity of the antibodies detected, while the use of multiple cell lines has indicated, in some instances, a degree of cross-reactivity between cell types.

On this basis, our study has shown that membrane-specific endothelial cell antibodies are an important feature of patients with primary APS, confirming and extending the findings of two earlier studies [16,17] which both used ELISA to detect the antibodies. The original paper of Cervera *et al.* [16] detected anti-endothelial cell antibodies in 13 of 21 patients with primary APS and seven of nine patients with SLE, all of whom had had thrombotic events; an incidence very similar to this investigation. No correlation between the presence of anti-endothelial cell antibodies and other autoantibodies, especially ACA, was noted, which again resembles our current findings. The coincidental finding of an increased incidence of antibodies reacting with both fibroblasts and T lymphoma HUT78 probably reflects partly the well recognized tendency for anti-endothelial cell antibodies to cross-react with fibroblasts [24], and partly the underlying defect in immune regulation which leads to the emergence of autoantibodies in these patients, including the potentially pathogenic ACA and anti-endothelial cell antibodies. Despite some heterogeneity and cellular cross-reactivity, it is quite clear that a high proportion of the endothelial cell antibodies recognized specific membrane epitopes, especially at 33 kD, 61–63 kD and 76–79 kD. Although the antibodies reacting with the 33-kD epitope cross-reacted with fibroblast and/or lymphoma HUT78 membranes, the antibodies to the 61–63-kD and 76–79-kD epitopes were entirely specific for endothelial cell membranes, and were found in seven of the 14 patients with primary APS. The particular finding of antibodies to HUVEC membranes in seven of the eight patients with venous thrombotic events is a stronger association than the presence of high titre ACA and is supportive of our earlier observation that anti-endothelial cell antibodies (detected by ELISA) showed a stronger correlation with inhibition of thrombin-induced prostacyclin release from endothelial cells than did the titre of ACA, especially in patients with thrombotic events [17]. The much poorer relationship between arterial thrombotic events and anti-endothelial cell antibodies detected by immunoblotting with venous endothelium, may be due to well recognized antigenic differences between venous and arterial endothelium [25]. Such a possibility could be further investigated by immunoblotting studies which compared endothelium obtained from various arterial sources with those of venous origin. Further investigation of the functional effects of the 33-kD and especially 61–63-kD and 76–79-kD endothelial cell membrane antibodies is also indicated. Although the observed associations of these antibodies with venous thrombotic events was not significant due to the size of the subgroups studied, they may be of pathogenic importance through their ability to cause damage and/or dysfunction of vascular endothelium. Effects such as complement-mediated or antibody-dependent cellular cytotoxicity and inhibition of prostacyclin release [17] may be more crucial in the initiation of a thrombotic episode than the possible effects that ACA may have on anti-thrombin III, fibrinolysis or thrombomodulin-induced activation of protein C [6–10]. Such a concept would not exclude the possibility of a synergistic interaction between ACA with their differing sites of actions and the anti-endothelial cell antibodies that this and previous studies [16,17] have shown to be such a prominent feature of primary APS.

ACKNOWLEDGMENTS

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