Clin Exp Immunol 1996; **106**:491–497

Antibodies to membranes of endothelial cells and fibroblasts in scleroderma

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(Accepted for publication 26 July 1996)

SUMMARY

Anti-endothelial and other cell membrane-reactive antibodies in scleroderma were characterized by immunoblotting sera with membrane and cytosol preparations of human umbilical vein endothelial cells (HUVEC), dermal fibroblasts and a T cell lymphoma HUT78. Antibodies reactive with HUVEC membranes were found in 17 of 20 patients with scleroderma (33 bands) in contrast to only two of 20 controls (two bands; $P<0{\cdot}01$) and three of 11 patients with myocardial infarction (four bands). Eleven of the 20 patients possessed antibodies that were specific for HUVEC membrane and did not cross-react with other cell lines. Analysis of patient subgroups showed that HUVEC membrane antibodies were present in nine of 11 patients with systemic sclerosis and in all nine with the CREST syndrome, and were HUVEC-specific in five and six of these cases, respectively. Although considerable heterogeneity was seen, antibodies to an 18–19-kD membrane epitope were found in 11 of the 20 patients but in none of the controls ($P<0{\cdot}01$). This antibody which reacted particularly with HUVEC ($n = 9$) and HUT78 membranes ($n = 9$) was associated with CREST syndrome rather than systemic sclerosis (9/9 *versus* 1/11; $P<0{\cdot}01$), and after elution was shown to possess anticentromere activity. In addition, antibodies reactive with both fibroblast ($n = 11$; 18 bands) and HUT78 membranes ($n = 18$; 42 bands) were detected and were specific for either fibroblast or HUT78 membranes in nine and 14 patients, respectively. There was no significant difference in the incidence of these fibroblasts and HUT78 membrane antibodies in the two patient subgroups. These findings support the concept that membrane-reactive antibodies, including anticentromeric antibodies, may play a central role in the pathogenesis of scleroderma, through their ability to react with endothelial cells.

Keywords immunoblotting endothelial antibodies scleroderma

INTRODUCTION

The clinical association or ‘overlap’ of scleroderma with other autoimmune connective tissue diseases such as systemic lupus erythematosus and polymyositis [1], together with its high incidence of distinctive autoantibodies [2,3], circulating immune complexes [4,5] and evidence of T cell activation [6] with elevated levels of cytokines [7,8], all strongly suggest that autoimmune mechanisms are involved in the pathogenesis of the disease. The development of scleroderma-like lesions as a feature of chronic graft-*versus*-host disease, both experimentally [9] and in man [10], further supports this concept. A distinctive vascular pathology [11], as well as increased collagen formation by fibroblasts [12], is a characteristic feature of the disease and has led to the recognition by ELISA of anti-endothelial cell antibodies [13], some of which are capable of causing antibody-dependent cellular cytotoxicity (ADCC) [14,15], in the disorder. This study therefore has further characterized antibodies reacting with endothelial and other cell membranes in the disease by immunoblotting sera with membrane and cytosol preparations of human umbilical vein endothelial cells (HUVEC), human dermal fibroblasts and a T cell lymphoma line HUT78.

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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 20 patients with scleroderma (16 women, four men; mean age $59{\cdot}9 \pm 9{\cdot}8$ years) who satisfied the preliminary criteria of the American Rheumatism Association [16], 11 patients with a recent myocardial infarction (four women, seven men; mean age $66{\cdot}5 \pm 6{\cdot}9$ years) and 20 normal controls (16 women, four men; mean age $56{\cdot}6 \pm 9{\cdot}2$ years). The patients with scleroderma were investigated by a routine protocol involving x-rays of chest and hands, barium swallow examination, electrocardiograph, lung function tests including carbon monoxide

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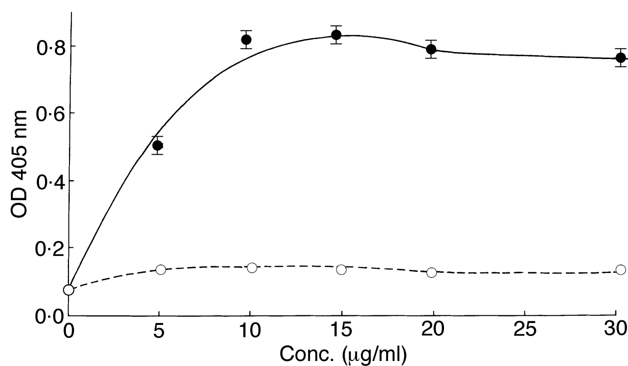

Fig. 1. Reactivity of human endothelial membrane-specific MoAb EN4 with increasing concentrations of membrane (○) and cytosol (●) preparations of human umbilical vein endothelial cells.

transfer factor, CPK estimations, creatinine clearance, urine protein estimations and examination of urinary sediment, together with clinical assessment of the extent of cutaneous involvement by the disease. Eleven patients of the scleroderma group had features of systemic sclerosis (eight women, three men; mean age $60{\cdot}1 \pm 8{\cdot}9$ years), while nine patients, with purely sclerodactyly without proximal or diffuse sclerosis, satisfied the criteria of the CREST syndrome [3] (eight women, one man; mean age $59{\cdot}5 \pm 11{\cdot}5$ years).

Culture of HUVEC fibroblasts and human T lymphoma line HUT78
Human umbilical vein endothelial cells 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 (DMEM) supplemented by 10% fetal calf serum (FCS) (with antibiotics).

The human T lymphoma line HUT78 (ECACC, Porton Down, UK), 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 human umbilical vein endothelial cells, fibroblasts and the T lymphoma line HUT78, were prepared by a method based on that of van der Zee *et al.* [18] 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 500 kIU/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 using MoAb EN4 (Bradsure Biologicals, Loughborough, UK) which is known to react with membrane epitopes retained on cultured HUVEC [19]. ELISA plates were coated overnight at 4°C with 0–0·31 g/well 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. 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.

Immunoblotting for detection of membrane-specific antibodies
Initial 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% bis-cross-linked gels for 140 mA h. Further resolution and definition of membrane antigens smaller than 20 kD were subsequently performed using 12% bis-cross-linked gels. Semi-dry electroblotting for 500 mA h 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 successively with patient and control sera, diluted 1:300 in PBS, 2% FCS, 1% casein. After four further washes in PBS 0·1% Tween 20, strips were incubated for 1 h with peroxidase-conjugated γ-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 by 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 [20]. 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.

Detection of antinuclear antibodies
Antinuclear antibodies were detected by indirect immunofluorescence using a rat liver substrate and FITC-conjugated polyvalent swine anti-human immunoglobulin (Nordic Immunologicals, Tilburg, The Netherlands) [21]. All sera were screened at a dilution of 1:20 and the result expressed as the reciprocal of the end-point titre.

Centromere antibodies were detected by indirect immunofluorescence using a HEp2 epithelioid cell line preparation

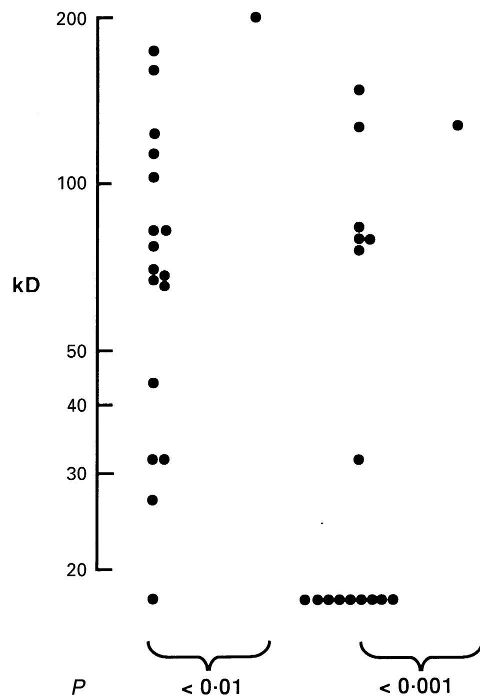

Fig. 2. Distribution of human umbilical vein endothelial cell (HUVEC) membrane antigens binding IgG antibodies in 11 patients with systemic sclerosis (SSc), nine patients with CREST syndrome, 11 patients with recent myocardial infarction (MI) and 20 age- and sex-matched normal controls.

(Sanofi Diagnostics, Guildford, UK) as substrate and FITC-conjugated polyvalent swine anti-human immunoglobulin [22].

Antibodies to native DNA were detected by a commercial ELISA kit (Sigma Diagnostics) [23] calibrated in terms of the WHO 1st International Standard [24].

Antibodies to extractable nuclear antigens were detected by double diffusion [25] using rabbit thymus and sheep spleen extracts. The specificity of precipitin lines seen on initial testing were confirmed by 'lines-of-identity' with type reference sera (PRV Procurement, Sheffield UK). Positive sera were additionally confirmed by immunoblotting.

Appropriate positive and negative control sera were included in each analytical batch.

Elution and antinuclear reactivity of antibodies binding to endothelial cell membrane

Endothelial membrane preparations (1·13 mg/gel) were separated on 12% bis-cross-linked polyacrylamide gels. After semi-dry blotting onto nitrocellulose, strips were cut containing proteins at 18 kD, 80 kD and 40 kD (control strip). These were incubated for 2 h with sera (1:100 dilution) from a CREST patient sample, previously shown to contain reactivity to antigens at 18–19 kD and 80 kD only. After confirming this binding pattern, bound antibody was eluted by incubating strips with 0·1 M glycine–HCl pH 2·8, neutralized and dialysed against PBS. After concentration to the original volume eluted antibody was tested for anticentromeric activity by indirect immunofluorescence on HEp2 cells as outlined above.

Preparation of nuclear fraction

In attempts to obtain a defined nuclear preparation for comparative immunoblotting, the alternative cell fractionation method of Chan *et al.* [26], was used. Both this method, and a modification of our standard procedure, involving early harvesting of nuclei released by homogenization, failed to produce a nuclear fraction which was free of membrane contamination as defined by EN4 MoAb binding in ELISA.

Statistical analysis

Comparison of the incidence of antibodies reactive with the various membrane preparations in patient and control groups was made by the χ^2 test after applying Yates' correction.

RESULTS

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

Binding of IgG to HUVEC membrane preparations, typically with multiple bands, was detected with sera from 17 (85%) of the 20 patients with scleroderma (33 bands) in contrast to only two sera from the 20 normal controls (two bands; $P<0{\cdot}01$) and three sera from the 11 patients with a recent myocardial infarction (four bands). In 11 of these 20 patients with scleroderma, the antibodies were specific for HUVEC membranes and did not cross-react with either fibroblast or HUT78 membranes.

Further analysis of patient subgroups revealed that IgG antibodies to HUVEC membranes were present in eight of the 11 patients with systemic sclerosis (Fig. 2) and in all nine patients with CREST syndrome (Fig. 3), being entirely specific for HUVEC membranes in five and six of these cases, respectively.

Although considerable heterogeneity in the antibody response to HUVEC membranes was seen, an antibody to what on further analysis on 12% gels proved to be an 18–19-kD membrane epitope was detected in 11 of the 20 patients with scleroderma but in none of the controls ($P<0{\cdot}01$). This antibody, which reacted primarily with both HUVEC ($n = 9$) and HUT78 ($n = 9$) membranes rather than fibroblasts ($n = 4$), was particularly detected in all nine patients with the CREST syndrome, but in only one of the 11 patients with systemic sclerosis ($P<0{\cdot}01$) (Figs 2 and 3).

In addition, a high incidence of antibodies reacting with both fibroblast and HUT78 membranes was also detected (Fig. 4). In the 20 patients with scleroderma, binding of IgG to fibroblast membranes was detected with 11 sera (18 bands) and to HUT78 membranes with 18 sera (42 bands). These antibodies were specific for either fibroblasts or HUT78 membranes in seven cases (nine bands) and 12 cases (27 bands), respectively.

There was no significant difference in the incidence of these fibroblast and HUT78 membrane antibodies in the two patient subgroups. In the 11 patients with systemic sclerosis, IgG binding to fibroblast and HUT78 membranes was detected in seven and nine sera, respectively, while in the nine patients with CREST syndrome, the corresponding figures were four and nine sera, respectively (data not shown).

Antibody to the 18-kD membrane epitope was found in all nine patients with CREST syndrome using HUVEC ($n = 9$), HUT78

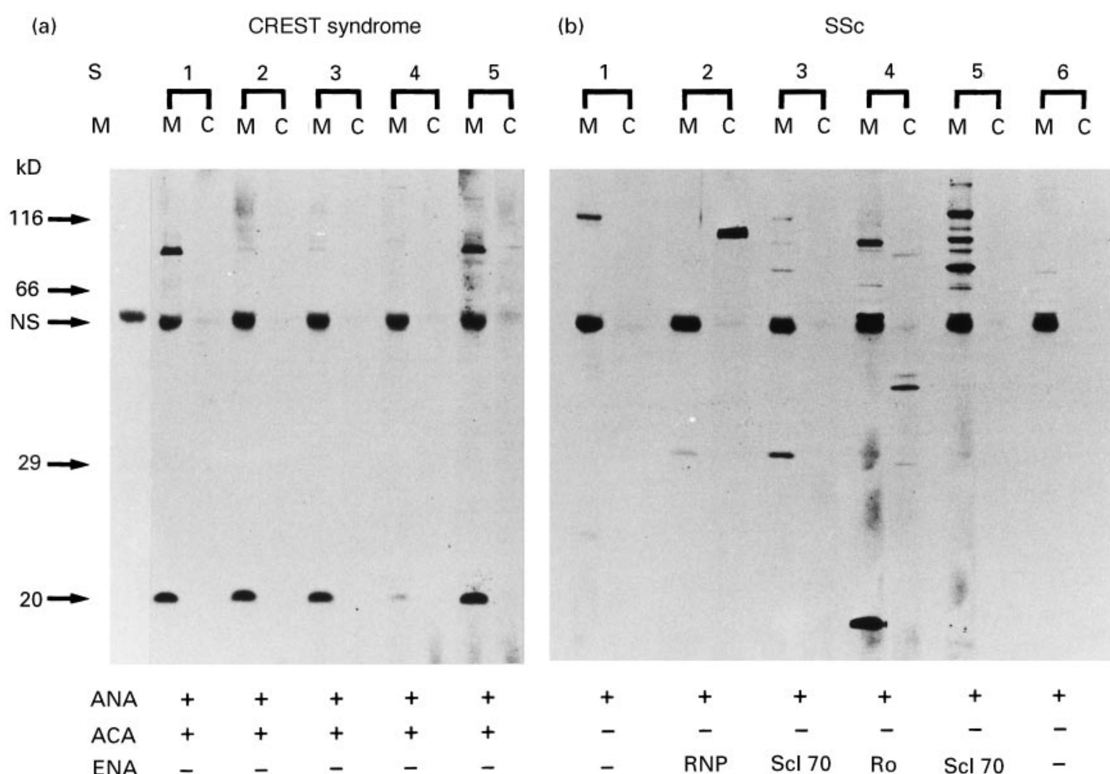

Fig. 3. Immunoblot of sera from patients with systemic sclerosis (SSc) and CREST syndrome with membrane (M) and cytosolic (C) preparations of human umbilical vein endothelial cells (HUVEC), illustrating (a) the association of antibodies to an 18–19-kD membrane epitope with anticentromere antibodies (ACA) and the CREST syndrome, and (b) the lack of correlation between antibodies to other HUVEC membrane epitopes and the presence of either antinuclear antibodies (ANA) or antibodies to extractable nuclear antigens (ENA), e.g. anti-Scl 70, anti-RNP, and anti-Ro. The preparations show non-specific binding of IgG fragments derived from the culture media (NS) as indicated in the saline control (S).

($n = 9$) and fibroblast ($n = 3$) membranes. In systemic sclerosis, only one of the 11 patients had antibodies to the 18–19-kD epitope detected by HUT78 membranes in one case and by HUVEC, fibroblast and HUT78 membranes in the other case.

Correlation of autoantibodies with antimembrane antibodies
Antinuclear antibodies, detected by immunofluorescence on both rat liver and HEp2 cells, were present in 17 of the 20 patients. Anti-DNA antibodies, however, were not detected. Anticentromere antibodies were present in eight of the nine patients with CREST syndrome and in two of the 11 patients with systemic sclerosis. Antibodies to extractable nuclear antigens were not detected in the patients with CREST syndrome but were present in four of the 11 patients with systemic sclerosis, and were characterized as anti-Scl 70 ($n = 2$), anti-RNP ($n = 1$) and anti-Ro ($n = 1$). The known reactivities of these antibodies with nuclear peptides of 95–100 kD [27], 68 kD and 33 kD [28], and 60 kD [29], respectively, make it unlikely that the immunoblotting profiles of these four patients, illustrated among others in Fig. 2, have resulted from nuclear and cytoplasmic contamination of the EN4-defined membrane preparations. However, anticentromere antibodies, which are known to react with interrelated centromeric antigens of 17–19 kD, 80 kD and 140 kD [30], showed a strong association with antibodies to an 18–19-kD membrane antigen, being present in 10 of the 11 patients with this membrane antibody. In three cases associated reactivity with an 80-kD antigen was seen, but no reactivity with a 140-kD membrane antigen was detected in any of these cases.

Antinuclear reactivity of eluted anti-endothelial membrane antibodies
Positive staining for anticentromere antibody, as defined by a speckled immunofluorescence pattern thoughout the interphase nuclei, localized especially in the condensed nuclear chromatin during mitosis, was observed in the original patient sera, and in antibody eluted from nitrocellulose strips containing proteins at 18–19 kD and 80 kD (Fig. 5). The eluate from the 40-kD control strip was negative in this assay.

DISCUSSION

Scleroderma, whose pathogenesis is likely to involve multiple autoimmune mechanisms, is characterized by a distinctive vascular pathology which is associated with increased collagen formation by fibroblasts, often in a perivascular distribution in the early stages of the disorder [11]. The vascular lesions, which consist of both endothelial cell damage and proliferation of myointimal cells, result in elevated levels of von Willebrand factor antigen [31] and endothelin [32], and are associated with raised levels of β-thromboglobulin as an indicator of platelet activation [33].

Various mechanisms capable of producing endothelial cell damage have been described in the disorder. A cytotoxic protease-like serum factor, quite distinct from IgG, has been described [34], but has been attributed by others to a storage artefact arising from the oxidation of lipoproteins [35]. While circulating immune complexes are a well recognized feature of the disease [4,5],

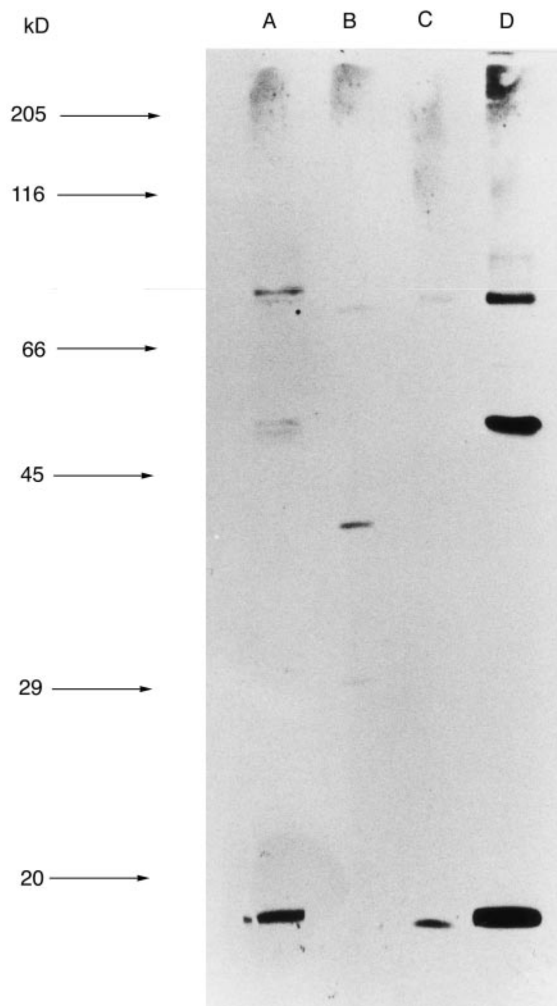

Fig. 4. Immunoblot of systemic sclerosis serum with membrane (A) and cytosol (B) preparations of human umbilical vein endothelial cells (HUVEC) and membrane preparations of human dermal fibroblasts (C) and lymphoma HUT78 (D) illustrating IgG binding that is specific for HUVEC membrane epitopes in addition to reactivity/cross-reactivity with fibroblast and HUT78 membranes.

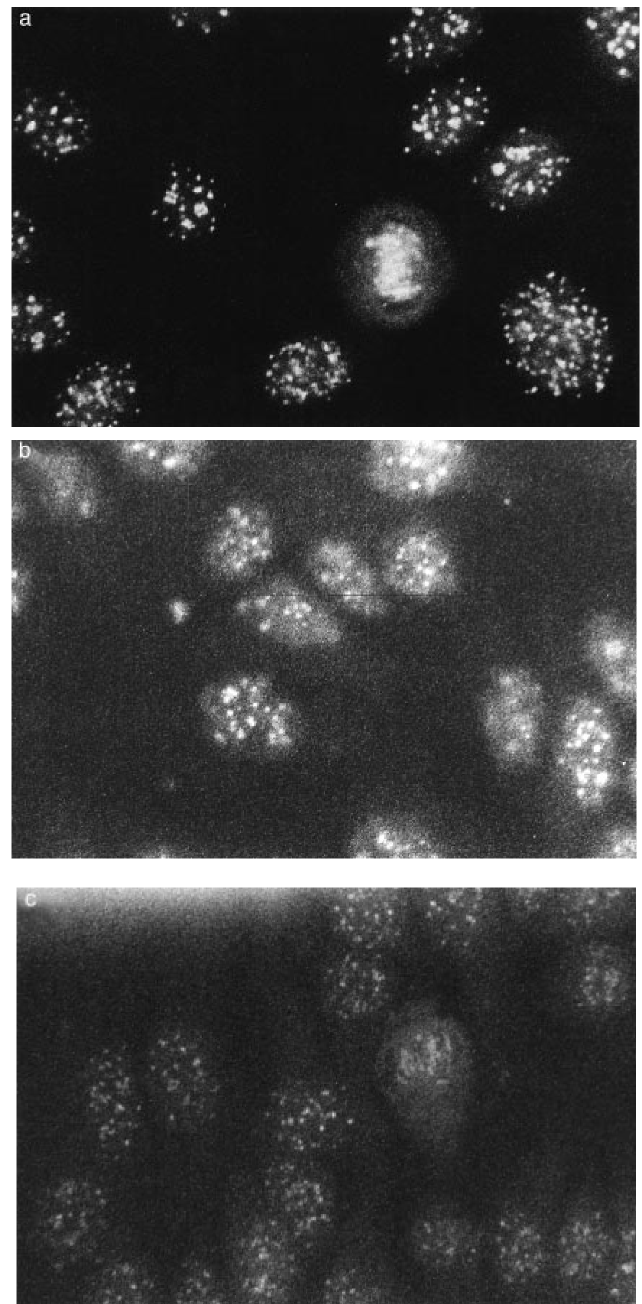

Fig. 5. Indirect immunofluorescence on HEp2 cells (× 800) of (a) anticentromere antibody-positive serum from a patient with CREST syndrome, together with eluted antibody derived from the same serum and reacting with (b) 18–19-kD and (c) 80-kD endothelial cell membrane epitopes.

deposition of IgG and complement components in diseased vessels and tissues is not well described. Immune complexes, however, could react with both macrophage and platelet Fc receptors with release of cytokines such as IL-1, platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) with resulting stimulatory and proliferative effects on both myointimal cells and perivascular fibroblasts. Finally, anti-endothelial cell antibodies have been shown repeatedly to be present in scleroderma [13–15], as is the case in other related connective tissue diseases. Endothelial cell ELISAs have revealed these antibodies in 30% of patients, while the technique of ADCC has shown antibodies capable of causing cytotoxicity of endothelial cells by this mechanism in 20% of patients [14,15], thereby also establishing a pathogenic capability for these antibodies.

The present investigation has established that patients with scleroderma have a very high incidence of antibodies to the cell membranes of not only endothelial cells, as defined by MoAb EN4, but also fibroblasts and other cell lines. These findings were partially predicted by a previous report that sera from patients with the disease were capable of causing ADCC against a wide range of established cell lines [36] in addition to endothelial cells, and suggest that further investigation of the functional effects of these membrane-reactive antibodies would be of interest. Although the sera of patients with scleroderma contain antibodies to a wide range of nuclear and cytoplasmic antigens [2,3], we think it is unlikely that possible contamination of the EN4-defined membrane preparations by these antigens has been responsible

for the immunoblotting profiles obtained. While the range of nuclear epitopes defined by anti-Scl 70 antibody in various reports has ranged from 66 kD and 86 kD to 95–100 kD [27], probably as a result of proteolytic degradation when extractions were performed without adequate protease inhibition, it is quite clear that membrane epitopes quite distinct from these were detected by the two sera shown to contain this antibody. Similarly, the two sera with either anti-RNP or anti-Ro antibodies detected HUVEC membrane epitopes quite distinct from the 68 kD, 33 kD, occasionally 22 kD and 60 kD nuclear peptides typically defined by these two antibodies [28,29]. Although further efforts to compare the immunoblotting profiles of sera with nuclear, in addition to membrane and cytoplasmic fractions, were made, these proved inconclusive as the resultant nuclear fractions were not free of membrane contamination as defined by EN4 MoAb binding.

The finding of IgG antibodies to an 18–19-kD membrane epitope of HUVEC, fibroblast and HUT78 cells which associated strongly with both anticentromere antibodies and the CREST syndrome, is a particularly interesting aspect of this investigation in view of the ability of anticentromere antibodies to recognize a 17–19-kD centromeric peptide, CNP-A. This peptide, however, has been shown to be part of a family of three interrelated peptides in which CNP-B, with a molecular weight of 80 kD, has been regarded as the central antigen, sharing epitopes with both CNP-A and the 140-kD CNP-C [30]. In this context, our finding that antibodies eluted from immobilized endothelial cell membrane proteins at 18–19 kD and 80 kD react on immunofluorescence with centromeric antigens, suggests that membrane expression of these antigens can occur. Certainly Earnshaw *et al.* [30], in their paper defining the three centromeric antigens, considered that such a proposition was feasible. Moreover, there is some evidence that membrane expression of nuclear and cytoplasmic antigens may take place as, for example, studies with anti-DNA MoAbs have shown that binding can occur to DNA that is presented at cell membranes (37]. In addition, some forms of cell damage have been shown to result in the display of antigens such as Ro, RNP and Sm at the cell surface where they may react with their appropriate antibodies [38]. This possibility of a limited membrane expression of centromeric antigens would include anticentromere antibodies in the heterogeneous group of anti-endothelial cell antibodies that this and other studies have shown to be present in scleroderma. Further investigation of the functional effects of all of these anti-endothelial cell antibodies, especially in relation to their membrane specificities, is clearly indicated. Characterization of the specificities responsible for the potentially pathogenic ADCC of endothelial cells that is found in some 20% of patients [14,15] would be of considerable interest, as would further studies to define which of the antibodies might have inhibitory effects on endothelial prostacyclin release, as has been suggested might be one of their effects in the primary antiphospholipid syndrome [39]. Investigation of the effects on endothelial cell-mediated fibrinolytic mechanisms, adhesion molecule expression and endothelin and cytokine release might also provide further evidence that these endothelial membrane antibodies may have, in various ways, a central role in the pathogenesis of scleroderma.

ACKNOWLEDGMENTS

This investigation was supported by a grant from the Arthritis and Rheumatism Council.

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