Lymphocyte-endothelium interaction in systemic sclerosis and Raynaud’s phenomenon

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Abstract

Objective
To investigate interactions of immune cells with vascular endothelium in patients with systemic sclerosis (SSc) and in patients with idiopathic or autoimmune Raynaud’s phenomenon (RP).

Methods
Lymphocytes obtained from 11 patients with SSc, 9 with RP and 14 control subjects were pre-stimulated in vitro with alloantigens and cultured together with human umbilical vein endothelial cells (HUVECs). Lymphocyte adhesion and induction of endothelial HLA-class II molecules were measured by flow cytometry. Lymphocyte cytotoxicity against HUVECs was also evaluated. In some cases cells were cultured under experimental conditions of hypoxia and reoxygenation.

Results
Lymphocyte adhesion and induction of endothelial cell expression of HLA-DR molecules were similar in controls and SSc patients, but significantly lower in RP (p < 0.05 and p < 0.03, respectively). Cytotoxic activity of lymphoblasts against endothelial cells was negligible in all patient groups. Under experimental conditions of hypoxia and reoxygenation lymphocyte adhesion was significantly greater than in normoxic conditions in SSc patients, while it was similar to normoxia in control subjects and RP patients.

Conclusion
These results suggest that in RP patients there may be regulatory mechanisms of lymphocyte response able to control the processes that lead to lymphocyte adhesion and endothelial HLA-DR molecule induction. These mechanisms could play an important role in RP, and might possibly be lost in clinically evident SSc.

Key words
Systemic sclerosis, Raynaud’s phenomenon, endothelial cells, lymphocytes, hypoxia.
Lymphocyte-HUVEC interaction in SSC and RP / S. Della Bella et al.

Introduction
Systemic sclerosis (SSc) is a multisystem disease involving microvascular injury, perivascular lymphocyte infiltration and fibrosis of the skin and internal organs (1). Interaction of immune cells with the vascular endothelium, through direct cell-to-cell contact and the effects of cytokines, is one of the earliest changes in the disease (2). Lymphocyte adhesion to endothelial cells is a vital step in cell migration to tissues, and is believed to be increased in SSC, as suggested by the common finding of perivascular T-cell infiltrates in the skin of these patients (3). Increased lymphocyte and endothelial expression of cell adhesion molecules has also been documented in SSC tissue biopsies, and in these patients high levels of soluble adhesion molecules in the peripheral circulation correlate with clinical disease activity (3-6). In vitro, in particular experimental conditions using cell preparations enriched in certain subpopulations (7, 8), lymphocytes from SSC patients show an enhanced interaction with vascular endothelial cells, compared with cells from healthy controls. Through the expression of class II MHC antigens and cellular adhesion molecules and through the production of cytokines endothelial cells participate in the triggering and perpetuation of the cell-mediated immune response in the vascular microenvironment. Class I MHC molecules are constitutively expressed on the endothelial cell surface, whereas in physiological conditions class II molecules are expressed only on the endothelium of some particular districts (9). In vivo, the appearance of class II MHC-positive endothelial cells in vascularized allografts is viewed as a sign and a major stimulus of rejection (10). In vitro, the presence of class II MHC molecules on endothelial cells correlates with an increase in the ability of these cells to stimulate T-cell proliferation and cytotoxicity (11-13), and is therefore of great importance in the study of SSC. Raynaud’s phenomenon (RP), an intense episodic vasospasm provoked by cold and emotion, is a major initial symptom of SSC, and may precede its development by several years (14). It has been suggested that autoimmune RP, defined as typical RP coupled with circulating antinuclear antibodies and dilated nailfold capillaries, might be a pre-SSc state (15, 16). Patients with idiopathic and autoimmune RP seem therefore to offer a useful model for investigating the early pathogenic stages in SSC.

It has been recently suggested that allogeneic cells play a pathogenic role in SSC (17), and we have in fact reported that alloantigens are a good trigger for lymphocyte proliferation and stimulation in vitro in SSC patients (18,19). Therefore in the present study we investigated whether alloantigen-stimulated lymphocytes interact in vitro with endothelial cells by comparing lymphocytes from SSC patients with those from RP patients and controls. Adhesion to endothelium and the ability to induce endothelial class II MHC molecules were evaluated. Because of the important role attributed to ischemia and reperfusion injury in the pathogenesis of SSC (20), in some experiments lymphocyte adhesion to endothelial cells was evaluated in vitro under conditions of hypoxia and reoxygenation.

Materials and methods
Patients
Eleven patients with SSC (all women, mean age 52.1 ± 16.8 years) followed at our out-patient clinic were studied. Nine patients had limited SSC and 2 had diffuse SSC according to the classification criteria proposed by LeRoy et al. (21). Four patients (1 with diffuse SSC and 3 with limited SSC) had early onset disease, defined as cutaneous involvement of 3 years or less (22); the mean disease duration of the others was 13.8 ± 9.2 years. One patient with diffuse and one patient with limited SSC had active disease, judged on the basis of a 15% increase in the total skin thickness score and a worsening of lung function with a 15% decrease in forced vital capacity within the six months preceding the study (23). We also studied 9 patients with RP (1 male and 8 females, mean age 38.4 ± 11.6 years, mean duration of RP 9.5 ± 11.6 years).
9.4 years); 3 had idiopathic RP diagnosed on the basis of the biphasic/triphasic digital color changes characteristic of RP (24). Six patients had the autoimmune form of RP based on the presence of circulating antinuclear antibodies and dilated nailfold capillaries in addition to RP (15). Three patients had anti-nuclear, 2 had anti-nucleolar, and 1 had anti-centromere antibodies, assessed by indirect immunofluorescence on rat liver and on the Hep-2 cell line (25). No patient had positive Scl70 antibodies, as assessed by counterimmunoelectrophoresis using rabbit thymus extract and human spleen extract as antigens (26).

The severity of vasospasm, assessed by attack intensity and frequency and by capillaroscopic features, was not different between the RP and SSc patients. Seven patients with SSc (5 calcium channel blockers, 2 ketanserin) and 4 patients with RP (4 calcium channel blockers) were receiving vasoactive agents. Patients were required to discontinue aspirin and any other non-steroidal anti-inflammatory drugs for at least 14 days before entering the study. Treatment with corticosteroids within one month or with cytotoxic drugs within six months before venopuncture was a criterion for exclusion.

All patients gave their informed consent to participate in the study, in accordance with the standards of the responsible local ethics committee. The control group consisted of 14 healthy volunteers (5 males and 9 females, mean age 36.0 ± 8.5 years). Cigarette smoking and diabetes mellitus were criteria for exclusion in both the patient and control groups.

Cell isolation and culture

Single-donor HUVEC lines were isolated by collagenase digestion of umbilical cord veins, grown in standard medium containing 20% heat-inactivated fetal bovine serum, 100 g/ml crude extract of endothelial cell growth factor (ECGF), 50 g/ml porcine intestinal heparin (Sigma Chemical, St. Louis, MO, USA), and passaged as previously described (27, 28). For the experiments, confluent endothelial cells used. Lymphocyte-enriched allo-stimulated cells were obtained from primary bulk mixed cells used lymphocyte cultures. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density centrifugation on Lymphoprep (Nycomed AS, Oslo, Norway), as previously described (29). The cells were then suspended in culture medium containing 10% heat-inactivated AB human serum. Primary bulk mixed lymphocyte cultures were established with equal quantities of donor PBMC and irradiated (50 Gy) pooled allogeneic stimulator lymphocytes obtained from 20 different healthy individuals (30). After 5 days of incubation at 37°C in 5% CO₂ the blasts were harvested, assessed for vitality, and suspended in culture medium for further assays.

Lymphocyte-endothelial cell adhesion assay

Confluent endothelial cells in 96-well plates were washed and 5 x 10⁴ lymphoblasts were added to each well in complete culture medium containing human serum. Multiple wells for each condition were cultured. To minimize the effects of alloreactivity, lymphocytes obtained from several individuals were cultured with the same HUVEC line, and subjects belonging to the three groups (SSc, RP, controls) were represented in each experimental setting. One subject was evaluated twice, and similar adhesion values were obtained with lymphocytes cultured in the presence of different HUVEC lines (data not shown). Cells were incubated at 37°C in 5% CO₂ for 60 minutes (7, 31, 32). To investigate a possible correlation between lymphocyte adhesion and the induction of endothelial cell HLA-DR expression, duplicate cultures were incubated for 24 hours. Lymphocyte adhesion after 2 h and 4 h incubation did not significantly differ from adhesion after 60 min incubation. At the end of the incubation time the plates were gently washed twice, the remaining cells were detached by treatment with trypsin-EDTA, and labelled with FITC-conjugated anti-CD45 antibodies (Becton Dickinson, San Jose, CA, USA), according to Korlipara (33).

Ten thousand cells per sample were analyzed by flow cytometry on a Cytoron Absolute (Ortho Diagnostics, Raritan, NJ, USA). Each assay was run in duplicate. Lymphocyte adhesion was calculated as the percentage of CD45 positive cells.

Cytotoxicity assay

The specific cytotoxicity of lymphoblasts against endothelial cells was estimated by a commercial kit based on the measurement of lactate dehydrogenase (LDH) released from the cytosol of damaged cells into supernatants (Boehringer Mannheim - Roche, Italy). Lymphoblasts (1 x 10⁴ to 1 x 10⁵) were co-cultured with confluent HUVECs in a final volume of 200 1 in 96-well plates at 37°C in 5% CO₂ for 4 hrs. After centrifugation, 100 μl of each supernatant was harvested to measure the levels of LDH, according to manufacturer’s instructions.

HLA class II DR induction assay

Cell cultures were prepared as for the lymphocyte binding assay. Control cultures consisted of endothelial cells incubated in the absence of lymphocytes. After 24 h incubation, established on the basis of preliminary experiments (data not shown) and of data from literature (9, 10), cells were processed for flow cytometric analysis. Endothelial cells are generally easily distinguishable from lymphocytes by light scatter, allowing gating only on endothelial cells during analysis. However, to ensure that detectable HLA-DR was endothelial in origin, two-color anti-CD45 FITC/anti-DR PE (Becton Dickinson, San Jose, CA, USA) staining was always done, excluding CD45 positive cells (lymphocytes) from the analysis. Each assay was run in duplicate. Expression of HLA-DR molecules on the endothelial cell surface was calculated as a percentage of the HLA-DR positive endothelial cells.

Exposure of cell cultures to hypoxia and reoxygenation

To assess the effects of hypoxia on lymphocyte-endothelial cell interactions, in some experiments the culture plates were incubated in anaerobic con-
ditions, with less than 2% oxygen (Gas-Pak Pouch System, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA). After anaerobic conditions had been achieved, cell cultures were incubated for 2 h and then processed for lymphocyte-endothelial cell adhesion assay. Control cell cultures were run in normoxic conditions for the same incubation time. The effects of reoxygenation were evaluated on cultures incubated in normoxia for 30 min after 2 h hypoxia.

**Statistical analysis**

Student’s t-test was used for comparison of lymphocyte adhesion and HLA-DR induction between groups. The t-test for paired data were used for analysis of lymphocyte adhesion in cultures prolonged from 60 min to 24 h and for analysis of cultures exposed to hypoxia and reoxygenation. The significance level was set at 0.05.

**Results**

**Lymphocyte adhesion to endothelial cells in normoxic conditions**

As shown in Figure 1A, the *in vitro* adhesion of alloantigen stimulated lymphocytes to endothelial cells after 60 min co-incubation was similar in healthy controls (mean adhesion 37.4% ± SE 2.3, n=12), patients with SSc (36.3% ± 2.7, n=9) and patients with RP (34.9% ± 2.9, n=8). When the lymphocytes were incubated with endothelial cells for 24 hours (Fig. 1B), lymphocytes from SSc patients presented adhesion levels similar to controls (SSc: 53.5% ± 3.4; controls: 51.9% ± 4.7, n=12; p=n.s.), while lymphocytes from RP patients showed significantly lower adhesion to endothelial cells than control lymphocytes (RP: 37.1% ± 4.6, n=9, controls: 51.9% ± 4.7, n=12; p < 0.05).

Figure 2 presents lymphocyte adhesion at 60 min and 24 h in each individual, divided in the three study groups. In the control group lymphocyte adhesion to endothelial cells was significantly greater at 24 h than 60 min (24 h: 53.0% ± 3.8, 60 min: 36.3% ± 2.7, n=9, p < 0.001). In contrast, lymphocytes from RP patients presented similar adhesion levels at 60 min and 24 h (24 h: 35.9% ± 5.0, 60 min: 34.9% ± 2.9, n=8, p=n.s.). As illustrated in Figure 2, only two subjects had increased adhesion at 24 h (one patient with antinucleolar antibodies and RP duration of 16 years; one patient with anti-centromere antibodies and RP duration of three years).

In two cases of limited SSc, two with RP (one idiopathic, one autoimmune RP), and two controls we analyzed the CD45 positive cells adhering to endothelial cells after 24 h co-incubation. SSc patients had CD4/CD8 ratios similar to controls (SSc: 0.93 and 1.19; controls: 1.04 and 1.42), while RP patients had higher relative levels of CD8 positive cells (CD4/CD8 ratios: 0.32 and 0.59) than controls.

**Lymphocyte cytotoxicity against endothelial cells**

Lymphoblasts obtained from all groups showed negligible cytotoxic activity directed against HUVEC cells (SSc patients: 1.55% ± 2.86; RP patients: 1.37% ± 2.50; control subjects: 0.13% ± 0.35).

**Lymphocyte induction of endothelial cell HLA class II DR molecules**

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**Fig. 1.** Adhesion of alloantigen-stimulated lymphocytes to endothelial cells after 60 min (A) or 24 h (B) co-culture. Data are expressed as mean ± SE of the percentage of CD45 positive cells as measured by flow cytometry. *RP vs controls p < 0.05.
Fig. 2. Adhesion of alloantigen-stimulated lymphocytes to endothelial cells after 60 min to 24 h co-incubation. Data are expressed as mean ± SE of the percentage of CD45 positive cells, as measured by flow cytometry. *24h vs 60 min p = 0.007; ** 24h vs 60 min p < 0.001.

Fig. 3. Endothelial cell expression of HLA-DR molecules after 24 h co-incubation with alloantigen-stimulated lymphocytes. Data are expressed as mean ± SE of the percentage of HLA-DR positive endothelial cells (CD45 negative) as measured by flow cytometry. *RP vs controls p < 0.03.

HLA-DR molecules were not expressed on the endothelial cell surface of HUVECs grown in culture media alone, but their expression was significantly induced when the cells were incubated for 24 h with alloantigen pre-stimulated lymphocytes, in all groups. As shown in Figure 3, lymphocytes from SSc patients induced levels of endothelial HLA-DR expression similar to controls (SSc: 38.9% ± 6.2, n=11; controls: 47.5% ± 4.9, n=11; p < 0.03). No correlation was found between lymphocyte adhesion and the induction of endothelial HLA-DR molecules.

In hypoxia and reoxygenation, lymphocytes from 3 RP patients (1 idiopathic, 2 autoimmune), 4 patients with SSc (3 limited, 1 diffuse SSc) and 5 controls were alloantigen pre-stimulated in vitro and then cultured with HUVECs under either normoxic conditions or in hypoxia, followed or not by reoxygenation. Both in control subjects and RP patients lymphocyte adhesion to endothelial cells was similar in the three experimental conditions, while lymphocytes from SSc patients showed significantly increased adhesion to endothelial cells when exposed to hypoxia (hypoxia: 38.0% ± 2.4, normoxia: 33.5% ± 2.6, n=4, p < 0.04) and to reoxygenation (reoxygenation: 44.5% ± 2.6, normoxia: 33.5% ± 2.6, n=4, p = 0.005) (Fig. 4).

Discussion
The present study investigated the interactions between endothelial cells and in vitro alloantigen-prestimulated lymphocytes from patients with SSc and patients with RP. As we reported previously (18, 19), lymphocyte stimulation with oligoclonal alloantigens offers an ideal in vitro model for studying SSc lymphocyte functions, for several reasons. Alloantigen-specific helper, inducer, suppressor, and cytolytic functions are all activated in allogeneic mixed lymphocyte cultures (34-37). SSc shares several analogies and clinical similarities with chronic graft-versus-host disease (GVHD), so it has been suggested that the mechanisms involved in the immunological dysregulations of GVHD may also play a role in the induction and persistence of SSc (38). Finally, since SSc has a strong predilection for women, with a peak incidence after the childbearing years, and since fetal progenitor cells can persist in maternal peripheral blood for decades after childbirth, it has recently been suggested that low levels of chimerism may contribute to the pathogenesis of SSc (17).

Our results indicate that in normoxic conditions lymphocytes from SSc patients do not differ from control lymphocytes in their ability to adhere to endothelial cells. This result was unexpected, since the histological finding in SSc skin biopsies of perivascular lymphocyte infiltration and of increased adhesion molecule expression on lym-
phocytes and endothelial cells (3-5) strongly suggested that circulating lymphocytes from these patients should show an increased ability to adhere to endothelial cells. When the incubation time of lymphocyte-HUVEC co-cultures was prolonged from 60 min to 24 h, lymphocyte adhesion increased to the same extent in SSc and controls, so that adhesion levels were similar in the two groups at 24 h. It is possible that this result was due to the patients selected for this study, since most of them had stable limited SSc, while immunological alterations are likely to be more evident in the early stages of the diffuse form of the disease. Nevertheless, the few patients in our study with diffuse or active early stage disease presented adhesion levels similar to those observed in the larger group of stable limited SSc patients. In particular, one patient with early stage diffuse SSc and evident progression of skin fibrosis in the two months before entry to the study presented levels of lymphocyte adhesion both at 60 min and 24 h similar to patients with stable limited disease. Nor did the lack of increased adhesivity of SSc lymphocytes seem to be related to the older age of the patients, since lymphocyte adhesion and age did not correlate.

As reported by other authors, the supposed increased adhesivity of SSc lymphocytes is difficult to document in vitro, possibly because of the in vitro migration of activated lymphocytes to the perivascular space, so that they can only be demonstrated in cell preparations enriched in particular subpopulations (7, 8). It is possible, therefore, that our results are simply referable to the experimental model used. The behaviour of lymphocytes from the RP patients was different. After 24 h of lymphocyte-HUVEC co-culture, they presented significantly lower adhesion to endothelial cells than control lymphocytes. This was because lymphocyte adhesion did not progress on prolonging the incubation time of co-cultures, and the levels of adhesion were similar at 60 min and 24 h. The different behaviour between lymphocytes from SSc and RP patients was not ascribable to different degrees of vasospasm in the two groups, since vascular symptom severity was matched in SSc and RP subjects. It is worth noting that the behaviour of lymphocytes from autoimmune RP was similar to that of lymphocytes from primary RP patients. Only two patients with autoimmune RP lasting for 16 and 3 years respectively presented increased adhesion levels with the longer incubation time, and it will certainly be interesting to follow their clinical course to see whether they progress towards clinically evident SSc. In a previous study of 26 patients with RP (16 idiopathic RP, 10 autoimmune RP) we found that interleukin-2 (IL-2) in vitro production by alloantigen-stimulated lymphocytes was significantly lower in RP patients than in age- and sex-matched controls, with no differences between idiopathic and autoimmune RP (unpublished observation). Since pre-treatment of lymphocytes with IL-2 stimulates their adhesion to endothelial cells (7,31), the lower cell adhesion presented by RP lymphocytes might be ascribable to reduced IL-2 production in these patients. Results similar to lymphocyte adhesion at 24 h were obtained for lymphocyte induction of endothelial cell HLA-DR molecule expression. Lymphocytes from SSc patients induced similar endothelial HLA-DR expression to control lymphocytes, presumably for the same reasons as lymphocyte adhesion. Interestingly, lymphocytes from RP patients induced significantly lower endothelial expression of HLA-DR molecules than control lymphocytes. Resting vascular endothelial cells do not express HLA class II molecules, but they can be stimulated to do so by IFN- and lymphocyte adhesion (39-41).

We recently reported that RP lymphocytes, in the same alloantigen-stimulation model used in the present study, produced higher levels of IFN- than control lymphocytes (19). Furthermore, we did not find any correlation in the present study between lymphocyte adhesion and endothelial HLA-DR induction. It is possible, therefore, that soluble factors or cell populations with suppressor activity are involved in patients with RP, causing a reduced activation of the immune response with consequent inhibition of the mechanisms that lead to lymphocyte adhesion and HLA-DR molecule induction. This seems to be supported by our finding in RP patients of higher relative levels of CD8 positive cells adhering to HUVECs. These CD8 positive cells may reasonably be supposed to be suppressor lymphocytes, since the cytotoxic activity of lymphocytes against HUVECs was negligible. As for lymphocyte adhesion, endothelial HLA-DR induction as well was similar in
primary and autoimmune RP. Although evaluated in only a small number of patients, the lack of differences between groups could reflect the homogeneous level of immunological activation that characterized RP patients in our above mentioned previous studies on IL-2 and IFN- production.

In the cultures exposed to hypoxia and reoxygenation, lymphocytes from controls and RP patients did not differ in their adhesion to endothelial cells compared with normoxic conditions whereas lymphocytes from SSc patients presented a significant increase in their ability to adhere to HUVECs, both under hypoxic conditions and during reoxygenation. This suggests that hypoxia and reoxygenation may lead to endothelial cell changes able to trigger the adhesion of SSc lymphocytes. Another possibility is that during hypoxia and reoxygenation SSc lymphocytes themselves undergo changes which increase their ability to adhere to endothelium.

The molecular mechanisms mediating lymphocyte adhesion were not investigated in the present study. Several reports in the literature indicate that following hypoxia and reoxygenation HUVECs express increased levels of VCAM-1 and other adhesion molecules.

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Lymphocyte-HUVEC interaction in SSc and RP / S. Della Bella et al.

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