EXTENDED REPORT

Increased transendothelial migration of scleroderma lymphocytes

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Ann Rheum Dis 2004;**63**:569–574. doi: 10.1136/ard.2002.004838

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Accepted 4 June 2003

Systemic sclerosis (SSc) is a systemic inflammatory disease characterised by activation of circulating T lymphocytes, by increased levels of circulating mediators of inflammation, by the production of autoantibodies, and by typical local inflammatory infiltrates.^{1–5}

The excessive accumulation of collagen in the skin and in the inner organs of patients with SSc has been suggested to be a consequence of immune processes clustered within and around small vessels.⁶ In its early stages, SSc is characterised by both endothelial damage⁷ and perivascular infiltrations with an accumulation of lymphocytes.^{6, 8–10} In the skin, these lymphocytes predominantly consist of CD4⁺ T cells that express increased amounts of activation markers and adhesion molecules on their surfaces.^{6, 11, 12} Interestingly, endothelial cells (EC) in the skin exhibit corresponding surface alterations, suggestive of an activated and pro-adhesive phenotype.^{12–15}

In the peripheral blood of patients with SSc, T cells express markers of activation¹⁶ and are oligoclonally expanded.^{17, 18} Though the total number of CD3⁺ T cells is decreased, the CD4/CD8 ratio in peripheral blood is increased.^{16, 19–21} However, it is not clear whether these activated T cells are already systemically predetermined for migration or whether transendothelial migration is mediated by direct contact of SSc PBMC and SSc EC and local events only. To answer this question, we investigated the lymphocytic migration through monolayers of human umbilical vein endothelial cells (HUVEC) in patients with SSc and in HC, because HUVEC express the same adhesion molecules relevant for lymphocytic migration^{22, 23} as human arterial and human venous EC and show phenotypical homogeneity and stability during cultivation.^{24, 25} In addition, we evaluated the migrated CD4⁺

Background: CD4⁺ T lymphocytes play an important part in the pathogenesis of scleroderma (systemic sclerosis, SSc) and predominate in perivascular SSc skin lesions. Both soluble and membrane bound adhesion molecules are overexpressed in SSc, possibly influencing lymphocyte/endothelial cell (EC) contact.

Objective: To assess the transendothelial migration capacity of peripheral lymphocytes in vitro.

Patients and methods: Collagen was covered with human umbilical vein endothelial cells (HUVEC), and peripheral blood mononuclear cells (PBMC) of patients and matched healthy controls (HC) were added in parallel experiments. Before and after fractionated harvest of non-adherent, bound, and migrated lymphocytes, the CD4/CD8 ratio and the lymphocytic expression of activation markers and adhesion molecules were analysed by fluorocytometry.

Results: 13 (SD 12)% of the SSc PBMC migrated compared with only 5 (5)% HC PBMC ($p < 0.0002$); this increase was primarily due to the migration of CD3⁺ T lymphocytes and mainly to a larger proportion of CD4⁺ cells within this CD3⁺ fraction (71 (SD 14)% for SSc v 56 (14)% for HC, $p < 0.03$), leading to an increased CD4/CD8 ratio among migrated SSc lymphocytes in comparison with controls (3.3 (1.5) v 1.62 (0.93), $p < 0.006$). Among migrated SSc CD4⁺ T lymphocytes, the frequency of HLA-DR⁺ cells was increased; migrated lymphocytes highly expressed the adhesion molecules CD11a, CD49d, CD29, and CD44.

Conclusion: Transendothelial migration of CD4⁺ T lymphocytes is enhanced in SSc, and migrating cells exhibit an activated phenotype. The data suggest that activated CD3⁺CD4⁺ lymphocytes as found in SSc peripheral blood are prone to transvascular migration, thus contributing to the formation of typical perivascular lymphocytic infiltrates.

and CD8⁺ T cell subsets for possible differences in their expression of activation markers and adhesion molecules.

On the basis of the histopathological changes typical for SSc, we suggested that CD4⁺ lymphocytes would predominate among migrating T cells if the propensity to migrate was already a feature of circulating SSc lymphocytes.

PATIENTS AND METHODS

Patients

Heparinised peripheral blood was obtained from 12 white patients (10 female, 2 male, mean age 54.9 (SD 15.0) years) fulfilling the American College of Rheumatology criteria for SSc²⁶; six patients had diffuse SSc, six had the limited type of the disease. Among our patients, 11 of 12 had active skin disease: two patients were in the very early oedematous phase of skin involvement; eight had evidence for active cutaneous disease as indicated by a total skin score > 20 ²⁷ or a deterioration of the total skin score since the last observation²⁸; and one patient had stable cutaneous SSc but without showing signs of atrophy which are indicative for the late phase of cutaneous SSc.²⁹ Eight of our patients with SSc had a duration of skin involvement < 5 years (2.5 (SD 1.0) years), four patients had longer lasting cutaneous SSc (23.3 (10.1) years). These last four patients had evidence for still active cutaneous SSc.

Abbreviations: BND, bound; EC, endothelial cells; HC, healthy controls; HUVEC, human umbilical vein endothelial cells; MIG, migrated; NAD, non-adherent; PBS, phosphate buffered saline; SSc, systemic sclerosis; TEM, transendothelial migration

Table 1 Patients with SSc, clinical manifestations and treatment

Number of patients	12
Male/female	2/10
Age (years), mean (SD)	54.9 (15.0)
Disease duration (years), mean (SD)	12.4 (3.5)
Diffuse SSc	6 (50)
Limited SSc	6 (50)
Organ involvement	
Pulmonary fibrosis	8 (67)
Pulmonary hypertension	3 (25)
Oesophageal	9 (75)
Renal	2 (17)
Cardiac	1 (8)
Serological findings	
ANA positive	12 (100)
Anti-Scl-70 positive	5 (42)
Anticentromere positive	1 (8)
CRP >10 mg/l	1 (8)
Treatment	
Cyclosporin A	1 (8)
Prednisolone ≤ 10 mg	3 (25)
Extracorporeal photopheresis*	5 (42)

Results are shown as No (%) unless otherwise indicated.

*Blood was drawn before the respective photopheresis cycle after a treatment-free interval of 3 months.

Data on clinical manifestations (skin, pulmonary, cardiac, renal, oesophageal, gastrointestinal), serological findings (autoantibodies, C reactive protein), and treatment were recorded (table 1). Eleven healthy subjects (10 female, 1 male, mean age 55.6 (SD 15.25) years) served as a control group. In each experiment, patients and controls were tested in parallel. All patients and controls gave informed consent to venepuncture and analysis of 20 ml of heparinised venous blood.

Preparation and culture of PBMC and EC

PBMC were freshly isolated over a Ficoll-Hypaque gradient, washed, and counted on a haemocytometer.

Because HUVEC resemble human arterial and human venous EC in their expression of adhesion molecules and because, in striking contrast, human EC lines do not, HUVEC were regarded a good model for observing EC/lymphocyte interactions.^{22 30 31} We used EC harvested from human umbilical cord veins by collagenase digestion, as described.³² The culture medium of EC consisted of MCDB-M 104 (Gibco, Paisley, UK) supplemented with 20% fetal calf serum, 24 µg/ml endothelial cell growth supplement (TC Laevosan, Linz, Austria), 50 U/ml heparin, 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco), and 100 mg/ml streptomycin (Gibco). EC were removed from the culture flasks with trypsin-EDTA (Gibco). Because HUVEC retain phenotypical homogeneity and stability upon cultivation and in order to allow comparisons with previous studies^{22 24} EC of the third to fourth passage were used for the assay of transendothelial migration (TEM).

Assay of TEM

Monolayers of EC on collagen gels

To study TEM, an in vitro assay was used as previously described³³ (fig 1). Collagen gels were formed in 16 mm macrowell tissue culture plates of 50% bovine collagen (Collagen Biomaterials, Palo Alto, CA, USA), 7% 0.1 M NaOH, 10% highly concentrated phosphate buffered saline (PBS; 10× PBS by Gibco), and 33% distilled water. EC (5×10^5 per well) were incubated overnight to form a confluent monolayer on the collagen gels. Confluence was carefully controlled by two different investigators for each single well

Figure 1 Assay of transendothelial migration. HUVEC after the third to fourth passage formed confluent monolayers on collagen gels after overnight incubation. On the second day of the experiment, we added fresh PBMC, obtained from SSc and matched HC and processed them in parallel experiments. After 1 hour of migration we analysed the harvested cell fractions (NAD, BND, MIG) and ex vivo stained PBMC by fluorocytometry. NAD, non-adherent; BND, bound; MIG, migrated, EC, endothelial cells, PBMC peripheral blood mononuclear cells.

by phase contrast microscopy; wells with a disrupted EC monolayer were excluded.

Lymphocyte migration

PBMC (3×10^6) resuspended in fresh EC culture medium were added onto the EC monolayers and incubated at 37°C. Because antibody dependent cellular cytotoxicity^{7 34} could lead to defects in the HUVEC monolayer after longer periods of incubation, we kept the system free of SSc serum and the migration time to 1 hour, which had previously been determined to give optimal results.^{34a}

Collection of lymphocyte fractions

After migration, PBMC were recovered in three fractions: (a) cells that were non-adherent (NAD) to the endothelial cells were removed by gently washing three times with culture medium; (b) cells that bound (BND) to the EC monolayer were then recovered by rinsing each well twice with warm (37°C) Puck's EDTA, twice with warm (37°C) EGTA (0.5×10^{-3} M EGTA in PBS), and once with cold (5°C) Puck's EDTA; (c) finally, cells that had migrated (MIG) into the collagen gels were recovered. The collagen gels were gently stirred with a pipette tip, 0.7 ml collagenase solution (0.1% collagenase (Sigma, St Louis, MI, USA), 1% fetal calf serum, 50 mM Hepes buffer (Sigma) in Hanks's balanced salt solution) was added, and the culture plate was incubated at 37°C for 40 minutes. Subsequently, the migrating population was recovered by washing the wells twice with PBS.

The PBMC recovered in each population (that is, NAD, BND, MIG) were centrifuged, resuspended in culture medium, and counted with a haemocytometer. In SSc, we recovered $11.71 (7.24) \times 10^6$ cells in NAD (mean (SD)), $2.84 (5.00)$ in BND, and $2.46 (3.39)$ in MIG, respectively; in HC, we recovered $11.71 (7.24)$ in NAD, $1.53 (2.10)$ in BND, and $0.88 (1.12)$ in MIG, respectively. The intra-assay variability was 8 (1)%. Additional analyses by fluorocytometry were performed as described below.

Preparation and labelling of peripheral blood cells

Freshly prepared PBMC and the three fractions obtained after the incubation (NAD, BND, MIG) were stained in parallel according to standard procedures using antibodies against CD3, CD4, CD8, CD11a, CD25, CD69, HLA-DR (Becton Dickinson, San Jose, CA, USA), CD29, CD49d (Immunotech, Marseille, France), CD44 (Pharmingen, San Diego, CA, USA), CD45RO, CD54 (Serotec, Raleigh, NC, USA) directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin chlorophyll protein

Figure 2 Increased transendothelial migration of SSc PBMC. Testing PBMC of SSc and HC in parallel experiments. Filled triangles indicate diffuse SSc, open triangles indicate limited disease, and the small circle indicates that the respective negative control had highly activated lymphocytes and developed influenza the next day. This single sample was therefore excluded from all analyses.

(PerCP). Isotype matched antibodies to irrelevant antigens conjugated to FITC (Dako, Glostrup, Denmark), PE, or PerCP (both from Becton Dickinson) served as negative controls.

Fluorocytometry

Immediately after staining, cells were analysed on a Becton-Dickinson FACScan fluorocytometer. Gates were carefully set for lymphocytes, excluding the monocyte population.³⁵ Cells brighter than the respective isotype control were defined as positive in surface immunofluorescence.

Detection of autoantibodies

In our patients with SSc, antinuclear antibodies and anti-Scl-70 antibodies were detected by indirect immunofluorescence and Ouchterlony double immunodiffusion, respectively, according to standard procedures. All patient sera were additionally analysed for anticentromere antibodies by line immunoassay, which has been considered a reliable alternative to conventional techniques with an, at least, similar sensitivity and specificity.³⁶

Statistics

Numeric data are expressed as mean (SD). Student's *t* tests and paired *t* tests were employed for the comparison of groups and paired samples, respectively. Fisher's exact test

and χ^2 test were used to compare non-parametric variables in 2×2 and 2×4 tables, respectively. Pearson correlation coefficients were calculated for investigating possible associations between variables. The predefined primary variables were the percentage of migrated PBMC and the CD4/CD8 ratio, for which *p* values <0.025 (two comparisons) were considered significant. For all other variables, the *p* values are regarded as descriptive and are thus not corrected for.

RESULTS

Increased migration of PBMC and T lymphocytes in SSc compared with controls

Testing PBMC of SSc and HC in parallel experiments, we found that 13 (12)% of the SSc PBMC migrated compared with only 5 (5)% HC PBMC, with the percentage MIG of patients with SSc always higher than that of the healthy subjects tested on the same day (*p*<0.0002, χ^2 test; fig 2). Moreover, the proportion of SSc T lymphocytes among the migrated cells was higher than that of HC T lymphocytes (67 (14)% *v* 58 (13)%). There was a significant correlation between CD3+ cell migration and total PBMC migration (*r*² = 0.55, *p* = 0.006). Therefore, the increased migration of SSc PBMC is, at least in part, due to an increased propensity of CD3+ T lymphocytes towards transendothelial migration.

Increased migration of CD3+CD4+ lymphocytes in SSc

Fluorocytometry further showed that the increased migration of T lymphocytes of patients with SSc was mainly due to an increase in CD4+ cells within the migrated CD3+ fraction (71 (14)% in SSc *v* 56 (14)% in HC, *p*<0.03). Interestingly, the proportions of CD3+CD4+ cells of HC, but not of SSc, were lower in the MIG (56 (14)%) fraction than in PBMC before migration (68 (11)%, *p*<0.05; table 2).

Vice versa, and in accordance with the literature on cell migration, the frequency of CD3+CD8+ lymphocytes was increased in the MIG fraction of HC cells,³³ but not in the MIG fraction of SSc lymphocytes (43 (14) *v* 29 (15), respectively, *p*<0.04; table 2), indicating that SSc CD8+ lymphocytes undergo TEM, but to a lesser degree than SSc CD4+ or HC CD8+ T cells.

Rise in the CD4/CD8 ratio in migrated SSc lymphocytes

In SSc, reports on changes in the distribution of T cell subsets suggested a decreased number of CD3+ T cells and an increased CD4/CD8 ratio in peripheral blood which is, at least in part, due to a greater resistance of CD4+ lymphocytes to apoptotic stimuli.^{16 19-21} In the present study, we observed only a slightly increased CD4/CD8 ratio when staining lymphocytes immediately after isolation (ex vivo fraction, 3.18 (1.99) for SSc *v* 2.56 (1.13) for HC, *p* = NS). After TEM, the CD4/CD8 ratio increased in 8/12 patients with SSc (to 3.27 (1.54)) but decreased in 10/11HC (to 1.62 (0.93), *p*<0.002 calculated by Fisher's exact test; *p*<0.006 in unpaired *t* test; table 2, fig 3).

Table 2 Distribution of CD3+CD4+ cells, CD3+CD8+ cells, and the CD4/CD8 ratio

	% CD3+CD4+		% CD3+CD8+		CD4/CD8	
	HC	SSc	HC	SSc	HC	SSc
Ex vivo	68 (11)	69 (16)	28 (9)	30 (15)	2.56 (1.13)	3.18 (1.99)
NAD	71 (8)	69 (15)	28 (9)	28 (18)	2.76 (0.89)	3.26 (1.91)
BND	58 (15)	62 (16)	42 (18)	38 (17)	1.66 (1.04)	2.68 (1.78)
MIG	56 (14)*	71 (15)*	43 (14)*	29 (15)*	1.62 (0.93)*	3.27 (1.54)*

Results are shown as mean(SD).

NAD, non-adherent; BND, bound; MIG=migrated.

*Denotes *p*<0.05 (unpaired *t* test) between SSc and HC lymphocyte fractions.

Figure 3 Rise in the CD4/CD8 ratio in migrated SSc lymphocytes. The CD4/CD8 ratio in patients with SSc was only slightly raised ex vivo ($p = \text{NS}$), but increased in 8/12 patients with SSc and decreased in 10/11 HC, leading to a significantly raised CD4/CD8 ratio in migrated lymphocytes of patients with SSc when compared with HC (for details see table 2).

Increased HLA-DR positive cells among migrated SSc CD4+ lymphocytes

Among freshly isolated lymphocytes and the NAD fraction, we found low frequencies of HLA-DR positive CD4+ lymphocytes in both HC (5 (3)%) and 4 (2)%, respectively) and SSc (7 (6)%) and 6 (5)%, respectively; table 3).

In HC the frequencies of CD4+DR+ lymphocytes were higher in the BND fraction (10 (7)%), and in the MIG fraction (14 (10)%). In SSc, we found an even more pronounced increase of CD4+DR+ lymphocytes in the BND fraction (14 (12)%) and a further increase in the MIG fraction (31 (23)%), $p < 0.04$ when compared with HC).

The frequencies of CD4+CD25+ were similar for SSc and HC: 19 (13)% and 13 (6)%, respectively ($p = \text{NS}$) in the PBMC analysed ex vivo before TEM and 31 (38)% and 36 (37)%, respectively ($p = \text{NS}$) in the MIG population. In addition, also CD4+CD69+ cell frequencies tended to be higher among migrated lymphocytes than ex vivo in SSc (table 3).

CD45Ro is highly expressed among migrated CD4+

In HC and SSc, the proportions of CD45Ro+ cells among the CD4+ population in the MIG fraction amounted to 74 (9)% in HC and 77 (10)% in SSc. This was higher than in the NAD

(57 (12)% and 59 (16)%, respectively, $p < 0.02$ for both) and slightly higher than in the BND fraction (65 (16)% and 70 (15)%, respectively, $p = \text{NS}$ for both).

CD54 (ICAM-1) is increased on migrated CD4+ lymphocytes

Similar frequencies of CD4+CD54+ lymphocytes were present ex vivo and in the NAD fraction of both HC (7 (6)% and 6 (5)%, respectively) and SSc (6 (7)% and 4 (4)%, respectively). However, the proportion of CD4+CD54+ cells increased in the BND fractions of HC and SSc (15 (11)% ν 16 (12)%, $p < 0.02$ and $p < 0.04$, respectively) and in the MIG fraction, with a more pronounced increase among SSc cells (26 (20)% ν 33 (23)%, $p < 0.02$ and $p < 0.003$, respectively), consistent with previous findings in migrated lymphocytes.^{37 38}

Adhesion molecules are expressed on migrated lymphocytes

It has been shown that infiltrating lymphocytes in vivo¹² and migrated lymphocytes in vitro highly express various adhesion molecules.³⁹ In accordance with these findings, we found in both HC and SSc a high expression of CD11a (LFA-1; 86 (23)% and 79 (32)%, respectively), CD44 (H-CAM; 96 (10)% and 97 (8)%, respectively), CD29 (VLA β chain; 89 (8)% and 93 (6)%, respectively), and CD49d (integrin α_4 ; 73 (47)% and 75 (14)%, respectively) among migrated CD3+CD4+ cells. Differences between HC and SSc or within the different lymphocyte fractions were not significant.³⁸

Comparison of different patient subgroups

We found no relevant differences in the TEM or the distribution of lymphocyte subsets between patients with diffuse or limited disease. We also found no significant differences when analysing patients with cutaneous disease < 5 years or > 5 years, nor when comparing patients undergoing photopheresis with those receiving other forms of treatment.

DISCUSSION

The data presented show an increased transendothelial migration of SSc T lymphocytes and a particular propensity of CD4+T cells towards migration. These observations contribute to the understanding of how the increased CD4/CD8 ratio in perivascular infiltrates of patients with SSc may arise. Moreover, we investigated the lymphocytic expression of activation markers and adhesion molecules in these patients.

Indeed, the migration of PBMC through an human endothelial monolayer was increased in SSc in comparison with healthy people. Moreover, this increased percentage of migrated PBMC in SSc was mainly due to a surprisingly high proportion of CD4+ cells within the migrated CD3+ fraction. In contrast with SSc, we found an increased migratory capacity of CD8+ lymphocytes from HC, corresponding to previous findings.^{33 39} Thus, while the ratio of CD4/CD8 cells

Table 3 Expression of activation markers on CD3+CD4+ lymphocytes

	% CD4+HLA-DR+		% CD4+CD25+		% CD4+CD69+		% CD4+CD45Ro+	
	HC	SSc	HC	SSc	HC	SSc	HC	SSc
Ex vivo	5 (3)	7 (6)	13 (6)	19 (13)	7 (9)	6 (5)	61 (17)	66 (15)
NAD	4 (2)	6 (5)	13 (3)	21 (12)	3 (3)	6 (6)	57 (12)	59 (16)
BND	10 (7)	14 (12)	16 (9)	28 (19)	8 (10)	3 (3)	65 (16)	70 (15)
MIG	14 (10)*	31 (23)*	36 (37)	31 (38)	23 (24)	4 (6)	74 (9)	77 (10)

Results are shown as mean (SD).
NAD, non-adherent; BND, bound; MIG, migrated.
*Denotes $p < 0.05$ (unpaired t test) between SSc and HC lymphocyte fractions.

declined among migrated T cells of HC, it even increased among migrated cells of patients with SSc. This finding is therefore apparently similar to the situation in scleroderma skin lesions, and, indeed, practically all our patients had signs of active skin involvement. In contrast, the processes responsible for the accumulation of CD8+ lymphocytes in SSc alveolitis are probably based on other mechanisms.¹ These may include tumour necrosis factor α induced effects of primary human alveolar epithelial cells on lymphocyte migration⁴⁰ and differential effects of lung endothelial cells or, more likely, chemokines, which are all outside the scope of the present investigations.

Interestingly, many of the migrating CD4+ cells in SSc exhibited an activated, HLA-DR positive phenotype and highly expressed adhesion molecules. Although we cannot completely exclude the possibility that antigenic interactions between T cells and EC activate the T cell and lead to a concomitant increase in migratory capacity, we regard this as unlikely for several reasons: (a) T cells show signs of activation in peripheral blood; (b) if local activation were occurring, the total number of T cells with activated phenotypes ought to increase. This, however, was not the case. Rather, while proportions of activated T cells increase among the migrating populations, they decrease among the NAD population (table 3); (c) in human T cells, HLA-DR is a late marker of activation and its expression becomes detectable 72 hours after activation with the potent activator phytohaemagglutinin^{41, 42} and exceeds pre-activation levels 6 days after activation with tetanus toxoid⁴²; (d) we observed higher frequencies of HLA-DR+ lymphocytes mainly among migrated SSc lymphocytes and to a much lesser degree among HC lymphocytes.

Our data thus suggest that activated CD3+CD4+ lymphocytes as found in SSc peripheral blood are prone to migrate through vessel walls. This increased migratory capacity is likely to contribute to the perivascular accumulation of CD4+ lymphocytes, which is typical for the cutaneous manifestations in SSc. The data further suggest that T cells from patients with SSc are activated systemically, rather than locally, and migrate to the skin after such activation. Because the EC were derived from the umbilical cord, primary disease related EC changes could not account for the migratory behaviour of T cells.

In the context of these T cell abnormalities, it is of interest, that fibroblasts actively producing pro α 1 (I) and pro α 1 (III) collagen mRNAs are located next to the area of T cell infiltration, at least in localised SSc.⁴³ Therefore, conceivably, these activated T cells may in turn have a causal relationship with fibrosis.⁴⁴

In vivo, EC apoptosis mediated by antibodies has been seen in SSc⁷ and might facilitate the diapedesis of immune cells. To avoid endothelial damage and subsequent EC monolayer leakage in vitro, we kept the system serum-free and the migration time short. The confluence of the EC monolayer was carefully observed in each migration experiment. Moreover, perivascular lymphocytic infiltrates are found in SSc even in areas where endothelial damage is not evident,⁶ suggesting that lymphocytes also actively migrate through vessel walls.

Taken together, our observations suggest that, in addition to locally derived pro-migratory signals from fibroblasts⁴⁵ and an increased permeability of damaged endothelium, an increased migratory capacity of CD4+ lymphocytes may promote the formation of cutaneous infiltrates in scleroderma. Our findings therefore provide a further step towards a more profound understanding of these intertwined processes in SSc. Targeting cell migration and thus preventing T cells from interfering with local cells may contribute to an interesting therapeutic approach.

ACKNOWLEDGEMENTS

We thank Mrs Daniela Eselsböck and Mrs Irene Radda for their help in the preparation and culture of EC, and Dr Günter Steiner for performing the line immunoassay.

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