British Journal of Pharmacology (1997) 122, 1315–1322

Effect of mycophenolic acid on TNFα-induced expression of cell adhesion molecules in human venous endothelial cells *in vitro*

[1]Ingeborg A. Hauser, #David R. Johnson, *Frank Thévenod & Margarete Goppelt-Strübe

Department of Medicine IV, University of Erlangen/Nürnberg, 90471 Nürnberg, F.R.G.; #Department of Pathology, Yale University School of Medicine, New Haven, CT 06510, U.S.A. and *II. Department of Physiology, Medical Faculty, University of Saarland, 66421 Homburg/Saar, F.R.G.

1 Mycophenolic acid (MPA) is an inhibitor of inosine-5′-monophosphate dehydrogenase and therefore interferes with cellular GTP biosynthesis. Recently, MPA has been used as an antiproliferative and immunosuppressive agent. In the present study, the effect of MPA on the expression of the endothelial cell adhesion molecules (CAMs), intercellular (I) CAM-1, vascular (V) CAM-1 and endothelial (E)-selectin, was investigated in tumour necrosis factor-α (TNFα)-activated cultured human venous endothelial cells (EC).

2 Surface expression of CAMs was measured by flow cytometry and mRNA expression by Northern blot analysis. Transcriptional activation of CAMs by the nuclear factor NF-κB was determined by an electromobility shift assay. The function of CAMs was studied by a static adhesion assay with human monocyte-like undifferentiated U937 cells.

3 Pretreatment of TNFα- (5 ng ml^{-1}, 12 h) activated EC with MPA (10 μM, 24 h) increased the binding of U937 cells, which had not been treated with MPA, by ≈2 fold. MPA-pretreatment of EC did not affect TNFα-induced surface expression of ICAM-1. However, VCAM-1 and E-selectin were increased 2–3 fold and remained elevated up to 24 h, by which time TNFα-activated control EC had returned to baseline levels of expression. The effect of MPA on the surface expression of CAMs was half-maximal at ≈1 μM and required ⩾12 h of pretreatment. Guanosine (0.3 mM), a precursor of GTP, did not prevent the effect of MPA on the expression of CAMs in TNFα-activated EC.

4 Kinetics of mRNA expression of CAMs mirrored protein expression: mRNA for ICAM-1 was unaffected, whereas TNFα-induced mRNA expression for E-selectin and VCAM-1 was prolonged and increased by MPA. This effect was not due to increased transcription mediated by the nuclear transcription factor NF-κB. However, half-life for E-selectin mRNA was increased 10 fold by MPA, whereas ICAM-1 mRNA half-life was unchanged.

5 The data demonstrate that apart from its antiproliferative effects on lymphocytes, MPA enhances TNFα-induced VCAM-1 and E-selectin surface expression on EC by selectively increasing the mRNA-stability of these cell adhesion molecules. This effect of MPA on EC appears to be independent from inhibition of inosine-5′-monophosphate dehydrogenase.

Keywords: Endothelial cell; VCAM-1; E-selectin; ICAM-1; mycophenolate mofetil; cytokines; mRNA stability; inosine-5′-monophosphate dehydrogenase; NF-κB

Introduction

Mycophenolate mofetil and its active metabolite mycophenolic acid (MPA) selectively inhibit lymphocyte proliferation (Allison & Eugui, 1993a). MPA is a potent uncompetitive and reversible inhibitor of inosine-5′-monophosphate dehydrogenase (IMPDH) (Sintchak *et al*., 1996), and thus interferes with the biosynthesis of guanosine and deoxyguanosine nucleotides. Since proliferating B and T lymphocytes are more dependent on this *de novo* pathway for purine biosynthesis rather than on the salvage pathway, MPA more effectively inhibits lymphocyte proliferation than proliferation of other cell types (Allison *et al*., 1977). One metabolic side-effect of guanosine depletion mediated by MPA is a decrease in the transfer of mannose and fucose to glycoproteins, such as the β1 integrin VLA-4 (Allison *et al*., 1993b). It has therefore been suggested that by this mechanism MPA could also inhibit recruitment of leukocytes to sites of inflammation (Laurent *et al*., 1996).

Leukocyte recruitment to sites of inflammation is regulated in part by specific endothelial cell-leukocyte adhesion molecules (Springer, 1994): the initial leukocyte rolling is mediated by the selectins, including E-selectin on endothelial cells (Bevilacqua *et al*., 1989), whereas firm adhesion and transmigration are mediated by the interaction of integrins on leukocytes with vascular cell adhesion molecule-1 (VCAM-1) (Rice & Bevilacqua, 1989) and intercellular adhesion molecule-1 (ICAM-1) (Dustin *et al*., 1986) expressed by endothelial cells. Transcription of VCAM-1 and E-selectin is induced and that of ICAM-1 is increased when endothelial cells are exposed to inflammatory cytokines, such as interleukin-1β (IL-1β) or tumour necrosis factor-α (TNFα) (Springer, 1990; Shimizu *et al*., 1991).

Cell adhesion molecules on both leukocytes and endothelial cells are critical for inflammatory processes. Since MPA inhibits the function of cell adhesion molecules on leukocytes (Allison *et al*., 1993; Laurent *et al*., 1996), we hypothesized that MPA might also interfere with the function of cell adhesion molecules on endothelial cells. We used an established *in vitro* assay to test directly the binding of untreated monocyte-like U937 cells to TNFα-activated confluent monolayers of human venous endothelial cells (EC), which had been pretreated with MPA. Contrary to expectations, we found an increased adhesion of U937 cells to EC and investigated the mechanisms underlying this effect of MPA.

Methods

Culture of cells

Human umbilical venous endothelial cells (HUVEC) were isolated according to standard procedures (Jaffe *et al*., 1973) and kindly provided by Dr W.H. Schmitt (Rheumaklinik Bad

[1] Author for correspondence.

Bramstedt, University of Lübeck, Germany). HUVEC were cultured on 1.5% gelatin in medium M199, 20% FCS, bovine hypothalamic growth factor (EC growth factor) and heparin. HUVEC were used between passages 3 and 10. Cultures were preincubated with mycophenolate mofetil, MPA or the solvent dimethylsulphoxide (DMSO) and additionally treated with or without TNFα. U937 cells, a monocyte-like cell line (Sundstrom & Nilsson, 1976), was obtained from American Type Culture Collection (Rockland, MD) and grown in RPMI 1640 with 10% FCS.

Static adhesion assay

Adhesion of untreated U937 cells to untreated or TNFα-treated human EC was assessed as described elsewhere (Hauser *et al*., 1993). Briefly, HUVEC grown to confluency in gelatin-coated 24-well plates were pretreated for 24 h with 10 μM MPA or the solvent DMSO alone and incubated for an additional 12 h with or without TNFα (5 ng ml^{-1}). They were then incubated with 1×10^6 untreated U937 cells for 1 h, washed with warm medium, fixed in 10% buffered formalin/2% glutaraldehyde and stained with haematoxylin. Bound U937 cells were counted (five random microscopic fields per well, representing a total area of 0.3 mm^2) and results expressed as the number of adherent U937 cells per microscopic field.

Fluorescent flow cytometry (FACS)

Antibody labelling was carried out essentially, as described by Hauser *et al*. (1993). EC were treated with trypsin for 1 min at 37°C and then the trypsin was inactivated with 10% FCS. Cells were incubated for 60 min on ice with mouse monoclonal antibodies directed against ICAM-1 (1 : 10), VCAM-1 (1 : 25) and E-selectin (1 : 100), or with mouse IgG as a control. After washing twice with PBS/1% BSA, fluorescein-labelled sheep anti-mouse antibody (Boehringer Mannheim, Germany), diluted 1 : 40, was added for 30 min on ice. After washing cells were fixed with 1% paraformaldehyde/PBS and analysed in an EPICS Profile XL-MCL (Coulter Electronics Ltd., U.K.). The surface expression of cell adhesion molecules on human EC was represented by the fluorescence intensity expressed as mean channel number (MChN) compared to controls incubated with mouse IgG (see Table 1).

Northern blot analysis

Total cellular RNA was isolated by RNA-Clean (ASG GmbH, Heidelberg, Germany), according to the manufacturer's instructions. RNA 10 μg was size-fractionated by denaturating agarose formaldehyde gel electrophoresis and transferred to nylon membranes. Oligonucleotides (40-mer) specific for ICAM-1, VCAM-1 and E-selectin were endlabelled with [^{32}P]-γ-ATP and T4-kinase. Hybridization was performed in 50 mM Tris/HCl, 1 M NaCl, 1% SDS, 100 μg ml^{-1} salmon sperm DNA and 10% dextran sulphate at 65°C. A cDNA specific for 18S rRNA was labelled with an ECL nucleic acid labelling and detection kit obtained from Amersham, (Braunschweig, Germany). Autoradiographs were quantified by densitometry (Bioprofil, Fröbel, Germany). All values were corrected for differences of RNA loading by calculating the ratio of CAM expression to 18S rRNA expression. To determine mRNA stability confluent monolayers of human EC, which had been preincubated with or without MPA (10 μM) for 24 h, were treated with TNFα (5 ng ml^{-1}) for a further 3 h followed by addition of the transcription inhibitor actinomycin D (10 μg ml^{-1}) for the times indicated.

Nuclear extractions

Nuclear extracts were prepared by a modified mini-extraction protocol (Schreiber *et al*., 1989). Cells were pelleted (approximately $500 \times g$, 4 min, 4°C) and resuspended in hypotonic buffer (0.2 ml, 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$) supplemented with protease inhibitors (leupeptin and aprotinin each 1 μg ml^{-1}, PMSF 0.5 mM) and incubated for 15 min on ice. Cells were lyzed by the addition of 25 μl 2.5% Nonidet P-40 in hypotonic buffer and the nuclei pelleted ($500 \times g$, 4 min, 4°C). The nuclear pellet was resuspended in extraction buffer (20 mM HEPES pH 7.9, 0.45 M NaCl, 1 mM EDTA supplemented with the protease inhibitors) and incubated for 15 min at 4°C on a rocking platform, then the tubes were centrifuged (10 min at $14{,}000 \times g$, 4°C). Extracts were diluted 1 : 1 in 20 mM HEPES, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 20% glycerol (Dignam *et al*., 1983), yielding 1.5–2.0 μg ml^{-1} against a BSA standard in a modified Bradford protein assay (Bradford, 1976), frozen in liquid nitrogen and stored at −20°C.

DNA binding assay (EMSA, electrophoretic mobility shift assay) for NF-κB

DNA oligonucleotides were synthesized (E-selectin κB: 5′-CATTGGGGATTTCCTCTTTA, Sp-1: 5′-CGAGAGCCC CGCCCTCGTTC, Keck Biotechnology, Yale Medical School) and annealed. Probe DNA (blunt-end) was labelled with [^{32}P]-γ-ATP and polynucleotide kinase and separated from unincorporated nucleotides over a Sephadex G-25 spun column. To 1 μl double-stranded non-specific DNA (1 μg poly-dI:poly-dC) was added 4 μl nuclear extract (6–8 μg) and 5 μl diluted probe DNA (2 fmol, approximately 4,000 c.p.m.). The reaction was mixed by gentle rocking, incubated at room temperature for 20 min, then 5 μl were loaded on a 3.5% polyacrylamide gel/0.25 × TBE buffer and separated at 220V/18 cm for 2 h. Gels were dried under vacuum and exposed to a storage phosphor screen for quantification and documentation (Phosphor Imager, Molecular Dynamics, Sunnyvale, CA). Competition experiments were performed as above except that 100 fold excess competitor DNA was added to the incubations in 1 μl immediately before the addition of probe DNA in 4 μl.

Materials

The following reagents were obtained from the listed sources and used at the concentrations indicated in the text. Human recombinant TNFα (2×10^8 u mg^{-1}) was generously provided by Knoll AG (Ludwigshafen, Germany) and used at 5 ng ml^{-1} medium. Mouse monoclonal antibodies directed against ICAM-1 (R6.5 and R6.1 supernatants) were kind gifts of Dr Robert Rothlein (Boehringer Ingelheim, Ridgefield, CT). Mouse monoclonal antibodies against VCAM-1 (BBIG-V1, IgG1) and E-selectin (BBIG-E4, IgG1) were from H. Biermann AG (Bad Nauheim, Germany). Mycophenolate mofetil was obtained from Syntex Discovery Research (Palo Alto, CA), mycophenolic acid, guanosine, actinomycin D and pyrrolidine dithiocarbamate from Sigma (Deissenhofen, Germany). Specific DNA probes (40-mer) for ICAM-1, VCAM-1 and E-selectin were from H. Biermann AG (Bad Nauheim, Germany). Polynucleotide kinase was from New England Biolabs (Beverly, MA). Sephadex G-25 and double-stranded non-specific DNA (poly-dl:poly-dC) were from Pharmacia (Piscataway, NJ). All other reagents were of the highest analytical grade available.

Statistics

Statistical analyses were carried out with the Statgraphics programme by use of unpaired Student's t test. Results with levels of $P < 0.05$ were considered significant.

Results

Increased adhesion of U937 cells to TNFα-treated human EC by mycophenolate mofetil and MPA

Previous studies have demonstrated that the adhesion of U937 monocyte-like cells to cultured venous endothelial cells con-

stitutes a useful *in vitro* assay to assess the role of adhesion molecules in endothelial cell-leukocyte interaction (e.g. Hauser *et al*., 1993). Confluent monolayers of HUVEC that had been treated for 12 h with 5 ng ml^{-1} of TNFα bound 93±23 undifferentiated U937 cells per microscopic field (mean± s.e.mean of 3 different experiments; Figures 1a and 1c) within 1 h. When HUVEC were preincubated with the immunosuppressant mycophenolate mofetil or its active metabolite mycophenolic acid (MPA) (both agents 10 μM) for 24 h before addition of TNFα, adhesion of U937 cells was increased approximately 2 fold. For mycophenolate mofetil 185±40 cells were counted ($P<0.001$; $n=3$; Figures 1b and c), but similar results were obtained with MPA (data not shown). Mycophenolate mofetil (MM) or MPA did not affect viability or morphology of HUVEC. In the absence of TNFα no binding of U937 cells to HUVEC was detected in the absence or presence of the immunosuppressive compounds (not shown). In a previous study (Hauser *et al*., 1993), we had demonstrated that the binding of untreated U937 cells to TNFα-activated EC is mediated by the ligand pairs E-selectin/sLex and VCAM-1/VLA-4. For that reason we investigated the effect of mycophenolate mofetil and MPA on the endothelial cell expression of VCAM-1 and E-selectin in the absence and presence of TNFα. In addition, we also determined the surface expression of ICAM-1, an endothelial cell adhesion molecule, which is also upregulated by TNFα (Shimizu *et al*., 1991).

Increased surface expression of VCAM-1 and E-selectin on TNFα-treated human EC by mycophenolate mofetil and MPA

Expression of the cellular adhesion molecules ICAM-1, VCAM-1 and E-selectin on EC was assessed by FACS analysis. As shown in the representative FACS analysis of Figure 2, EC constitutively expressed ICAM-1, but not VCAM-1 or E-selectin (Shimizu *et al*., 1991; Collins *et al*., 1995). Since undifferentiated U937 cells do not express the ICAM-1 ligand LFA-1 in an active form (Cavender *et al*., 1991), no binding to EC occurred (see above). Preincubation of EC with 10 μM mycophenolate mofetil induced a small increase of ICAM-1 surface expression. Incubation of EC with 5 ng ml^{-1} for 12 h significantly increased ICAM-1 protein expression. In addition, TNFα-induced *de novo* protein expression of VCAM-1. E-selectin was also induced by TNFα on EC. Mycophenolate mofetil did not increase ICAM-1 surface expression on TNFα-treated EC (Figure 2). However, mycophenolate mofetil significantly increased TNFα-induced surface expression of VCAM-1 and E-selectin (Figure 2). Since VLA-4 and sLex, the ligands for VCAM-1 and E-selectin, are active on undifferentiated U937 cells (Hauser *et al*., 1993), this could explain the increased adhesion of undifferentiated U937 cells to TNFα-treated EC induced by pretreatment with mycophenolate mofetil (Figure 1b). MPA had similar effects on the expression of cell adhesion molecules in TNFα-treated EC (data not shown). All subsequent experiments were therefore carried out with MPA alone. The results of 6–10 independent FACS analyses of HUVEC performed in the presence or absence of MPA are shown in Table 1.

MPA alters the magnitude and kinetics of surface expression of VCAM-1 and E-selectin in TNFα-treated human EC

Following TNFα-treatment, ICAM-1 expression on human EC was increased and a transient expression of VCAM-1 and E-selectin was induced *de novo* (Figure 3). E-selectin protein expression was maximal between 3–6 h after TNFα and VCAM-1 protein expression between 12–24 h after TNFα. In contrast, ICAM-1 remained elevated for more than 48 h (data not shown). Preincubation of human EC with 10 μM MPA for 24 h did not change the magnitude or kinetics of TNFα-induced surface expression of ICAM-1 up to 24 h (Figure 3). However, TNFα-induced surface expression of VCAM-1, was

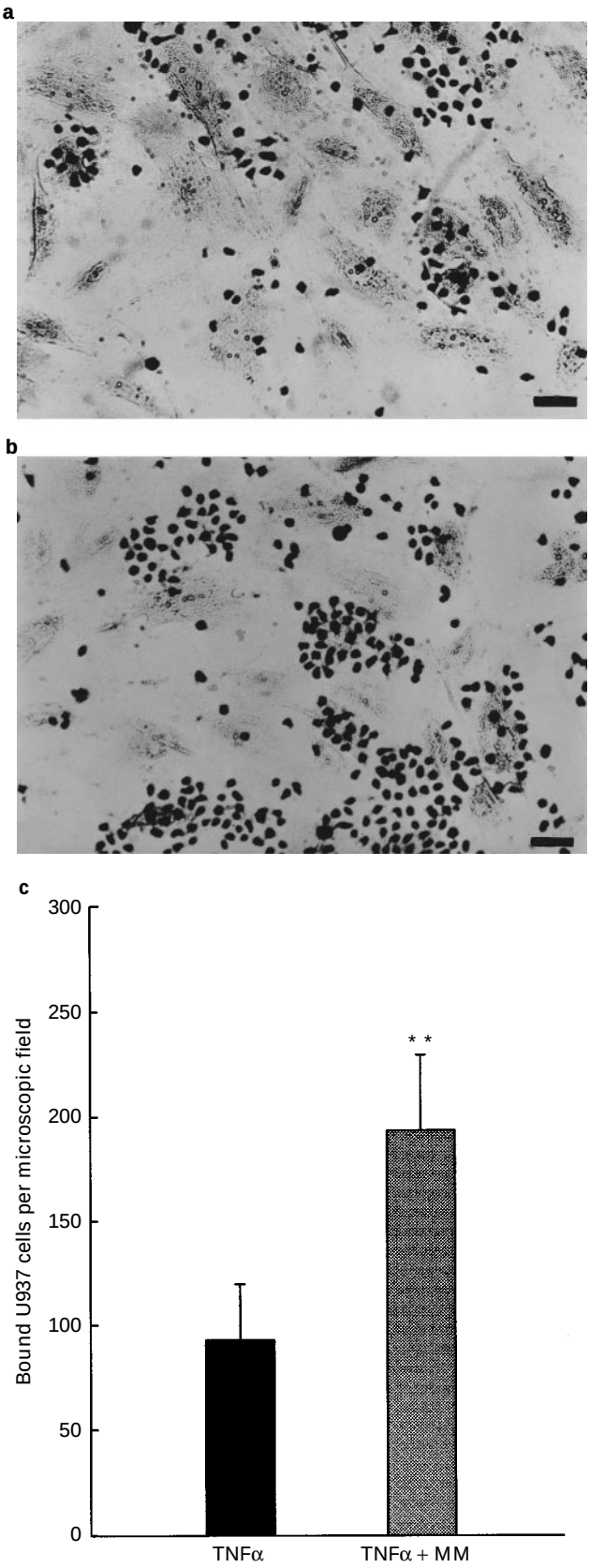

Figure 1 Effect of mycophenolate mofetil (MM) on adhesion of untreated U937 cells to TNFα-activated monolayers of EC. (a and b). Representative microscopic fields of the adhesion of untreated U937 cells to TNFα-activated EC (12 h incubation with 5 ng ml^{-1} TNFα), which had been preincubated for 24 h with the solvent DMSO (a) or with 10 μM mycophenolate mofetil (MM) (b). (c) The means ±s.e.mean number of bound U937 cells from 3 different experiments (**$P<0.01$). Cell numbers in each experiment were obtained by counting 5 random microscopic fields in triplicate wells. Scale bars in (a) and (b), 25 μm.

significantly increased 6–24 h after addition of TNFα in MPA-treated EC. TNFα-induced surface expression of E-selectin was also significantly increased by MPA-treatment and remained elevated throughout the time course of the experiment up to 24 h (Figure 3).

Increased surface expression of adhesion molecules on TNFα-treated human EC by MPA is concentration- and preincubation time-dependent

A dose-response analysis of MPA was performed between 0.1 and 50 μM (preincubation for 24 h before addition of 5 ng ml^{-1} TNFα for 12 h). The concentration-dependence of increased surface expression of adhesion molecules by MPA was similar for both VCAM-1 and E-selectin: an increase was detectable at 0.5 μM, the half-maximal effective concentration of MPA, which increases the expression of the cell adhesion molecules VCAM-1 and E-selectin on TNFα-activated EC, was estimated to ≈1 μM, and a plateau was reached at 10 μM (Figure 4). ICAM-1 surface expression remained unchanged, but marked increases of VCAM-1 and E-selectin surface expression were observed at the clinically relevant concentrations of 0.5–10 μM (Langman *et al*., 1996).

Application times beween 15 min and 36 h of the maximally effective concentration of 10 μM MPA were also tested. At least 12 h preincubation with MPA were necessary for the increase of TNFα-induced VCAM-1 and E-selectin surface expression to develop. Maximal effects were observed with a preincubation time of ≈24 hours (data not shown).

Preincubation with guanosine does not prevent the increased expression of VCAM-1 and E-selectin induced by MPA in TNFα-activated EC

To determine whether the inhibition of inosine-5′-monophosphate dehydrogenase (IMPDH) is responsible for the increased expression of VCAM-1 and E-selectin induced by MPA in TNFα-activated EC, EC were preincubated in the presence or absence of 10 μM MPA with 300 μM guanosine for 24 h and cells were activated with TNFα (5 ng ml^{-1}) for an additional 12 h. In a recent study, guanosine treatment has been shown to prevent MPA-mediated suppression of cytokine-induced nitric oxide production in rodent vascular endothelial cells (Senda *et al*., 1995). The effect of guanosine was explained by its action as a salvage pathway precursor for GTP biosynthesis. When EC were preincubated with guanosine with or without MPA, constitutive expression of CAMs was not different from that of controls (data not shown). TNFα-induced activation of ICAM-1, VCAM-1 and E-selectin on EC was not influenced by 300 μM guanosine (Figure 5). Preincubation with 10 μM MPA increased VCAM-1 and E-selectin expression induced by TNFα, as shown above. This effect of MPA was not reversed by concomitant preincubation with 300 μM guanosine (Figure 5). Higher concentrations of guanosine (up to 500 μM) were also without effect (data not shown).

MPA causes a sustained increase of mRNA encoding E-selectin and VCAM-1 in TNFα-activated EC

To determine whether the increased surface expression of VCAM-1 and E-selectin by MPA was mediated by an increased transcription of mRNAs encoding VCAM-1 and E-selectin, Northern blots were performed with RNA from confluent EC that were left untreated or preincubated with MPA (10 μM) for 24 h and then stimulated with TNFα for the times indicated (Figure 6). In untreated EC, mRNAs for ICAM-1, VCAM-1 and E-selectin were not detectable (Figure 6a). After stimulation with TNFα, rapid increases in ICAM-1, VCAM-1 and E-selectin mRNAs were observed that lasted for more than 20 h for ICAM-1 but declined within 12 h for VCAM-1 and E-selectin, in accordance with published data (Collins *et al*., 1995) (Figure 6a). Pretreatment of the cells with MPA did not change constitutive mRNA expression. In MPA-treated and control cells mRNA levels of ICAM-1 were equal after 12 h of TNFα-activation. In contrast, the decline in TNFα-induced E-selectin mRNA was slowed in MPA-pretreated EC, leading to 3 fold higher levels of E-selectin mRNA after 12 and 24 h of TNFα-ac-

Table 1 Effect of mycophenolic acid (MPA) on the surface expression of ICAM-1, VCAM-1 and E-selectin in EC treated with or without TNFα

	Mouse IgG	*ICAM-1*	*VCAM-1*	*E-selectin*
Control	59±10	110±21	61±8	63±8
MPA	63±7	131±13*	64±7	64±6
TNFα	59±6	160±18^{c}	79±12^{b}	84±14^{b}
MPA+TNFα	61±7	172±11^{c}	100±18*^{c}	119±16***^{c}

The results of 6–10 different experiments with human venous endothelial cells are summarized. Surface expression of cell adhesion molecules is represented by the mean channel number of cells (MChN). Numbers are mean±s.d. *$^{a}P\leqslant0.01$, **$^{b}P\leqslant0.001$, ***$^{c}P\leqslant0.0001$ calculated by use of unpaired Student's *t* test. Asterisks represent comparison of conditions±MPA and letters comparison of conditions±TNFα.

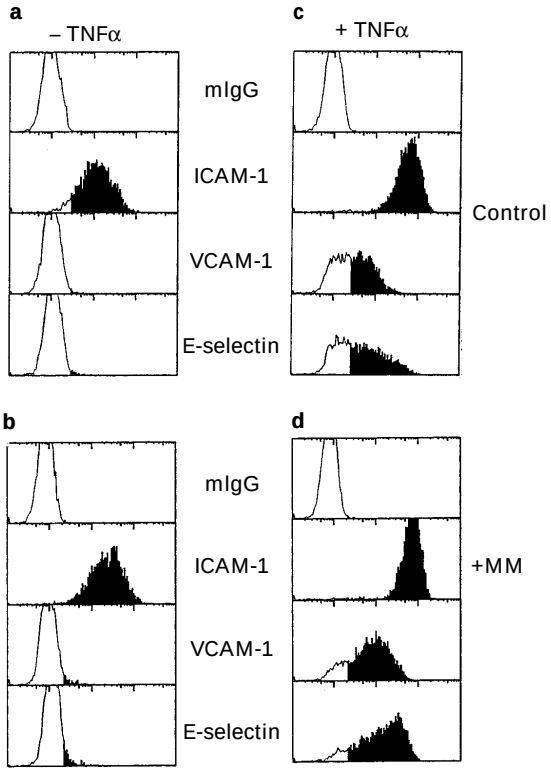

Figure 2 Effect of mycophenolate mofetil (MM) on TNFα-induced surface expression of ICAM-1, VCAM-1 and E-selectin on EC determined by FACS analysis. The X-axis of the histograms shows the fluorescence intensity of the cells on a logarithmic scale and the Y-axis the cell number; 5000 cells were analysed per histogram. The top histogram in each panel is background staining with mouse IgG. EC were preincubated for 24 h with the immunosuppressant mycophenolate mofetil (10 μM (b and d)) or the solvent DMSO (a and c) and incubated for an additional 12 h in the absence (a and b) and presence (c and d) of TNFα 5 ng ml^{-1}. The shaded portions of the histograms contain the cells that stained brighter than background.

tivation. Similar data were also obtained for VCAM-1 expression, although the effects of MPA were more variable (Figure 6b). In addition, the rapid (3–4 h) TNFα-mediated increase in mRNA expression of CAMs was slightly delayed by MPA-pretreatment. This effect was most obvious for ICAM-1 (see Figure 6a).

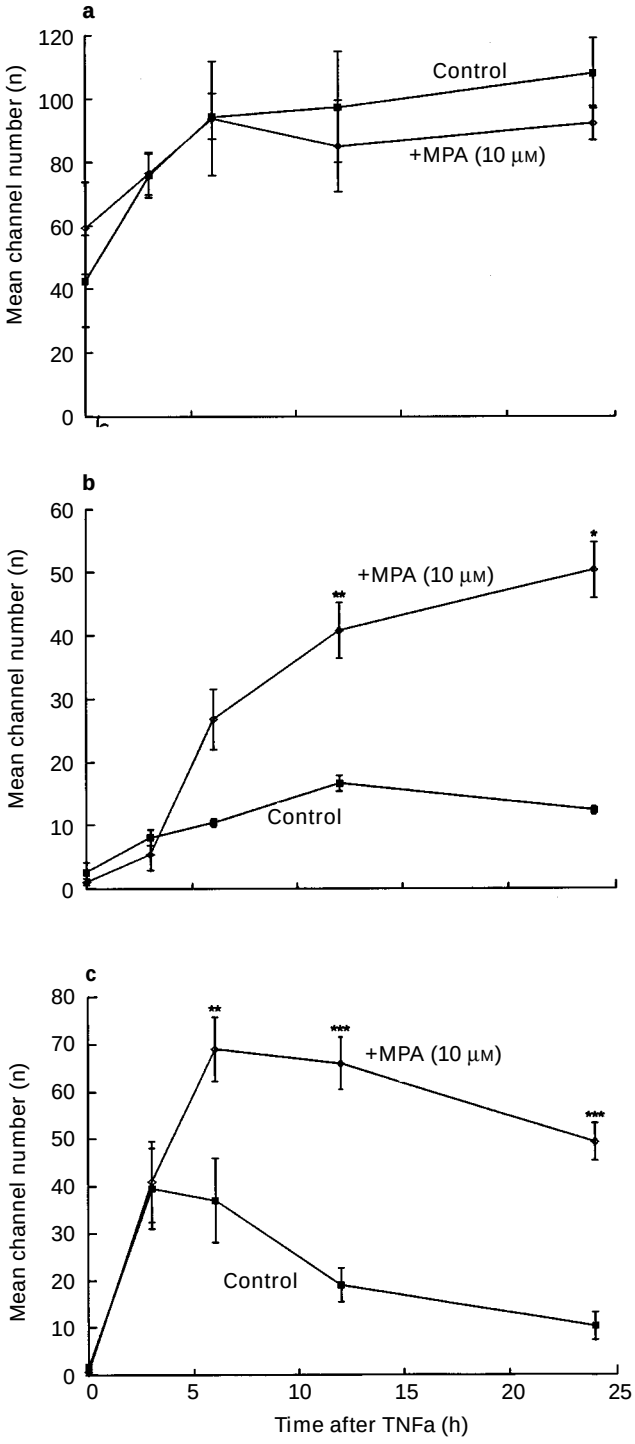

Figure 3 Effect of mycophenolic acid (MPA) on the kinetics of surface expression of the cell adhesion molecules (a) ICAM-1, (b) VCAM-1 and (c) E-selectin on TNFα-activated EC. Cells were pretreated for 24 h without or with 10 μM MPA followed by stimulation with 5 ng ml^{-1} TNFα for 3, 6, 12 or 24 h. Zero time-points represent expression of cell adhesion molecules in the absence of TNFα. Mean values of 6–10 different experiments are plotted and vertical lines show s.d. where applicable. Background was subtracted in all cases. $*P<0.01$; $**P<0.005$; $***P<0.0005$.

TNFα-activation of the transcription factor NF-κB is not increased by MPA in EC

The transcription factor NF-κB is involved in the transcriptional activation of the genes for ICAM-1, VCAM-1 and E-selectin by TNFα (Collins *et al*., 1995). To investigate whether the increased expression of VCAM-1 and E-selectin mRNAs induced by MPA in TNFα-activated EC is caused by persistent activation of NF-κB (Johnson *et al*., 1996), we measured the TNFα-activation of NF-κB in MPA-treated cells by EMSA of DNA-binding proteins (Figure 7). In four separate experiments, TNFα-activation of NF-κB was decreased by MPA-preincubation, on average approximately by 50% at 3h (Figure 7a). In contrast, the transcription factor Sp-1, which binds to its DNA recognition element constitutively and is not activated by TNFα was only slightly reduced by combined treatment with MPA and

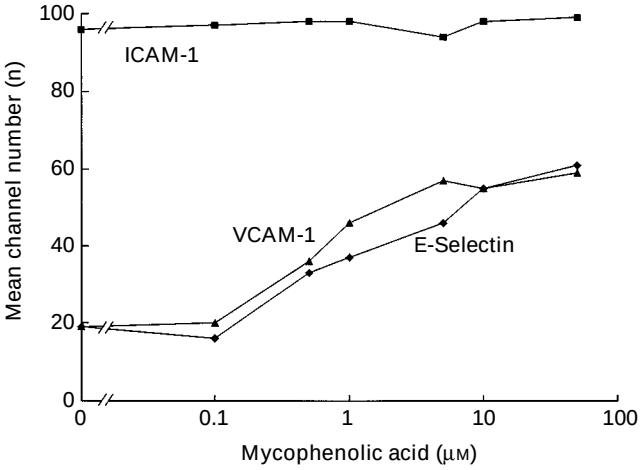

Figure 4 Concentration-dependence of the effect of mycophenolic acid (MPA) on TNFα induced expression of cell adhesion molecules in EC. Different concentrations of MPA were tested under identical conditions (24 h preincubation with MPA and additional 12 h incubation with TNFα (5 ng ml^{-1}). The results are representative of 3 independent experiments.

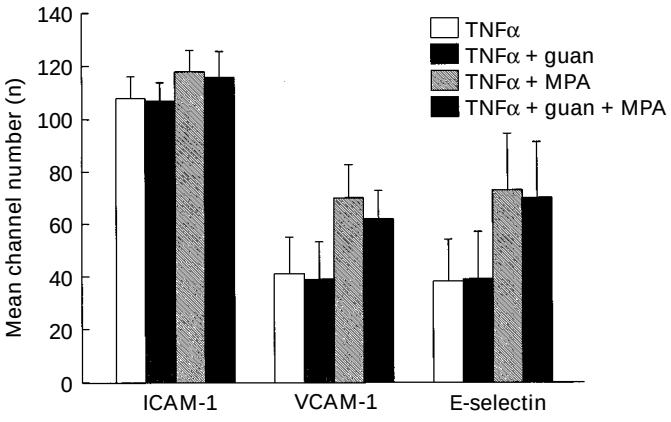

Figure 5 Effect of mycophenolic acid (MPA) on the expression of ICAM-1, VCAM-1 and E-selectin in TNFα-activated EC in the absence or presence of guanosine (guan.). EC were preincubated without or with 300 μM guanosine and in the absence or presence of MPA (10 μM) for 24 h. Thereafter, the cells were activated with TNFα (5 ng ml^{-1}) for 12 h. The expression of cell adhesion molecules was determined by FACS analysis. Background fluorescence intensity (mouse IgG) was not affected by the experimental conditions tested. Data are means±s.e.mean of 4 different experiments.

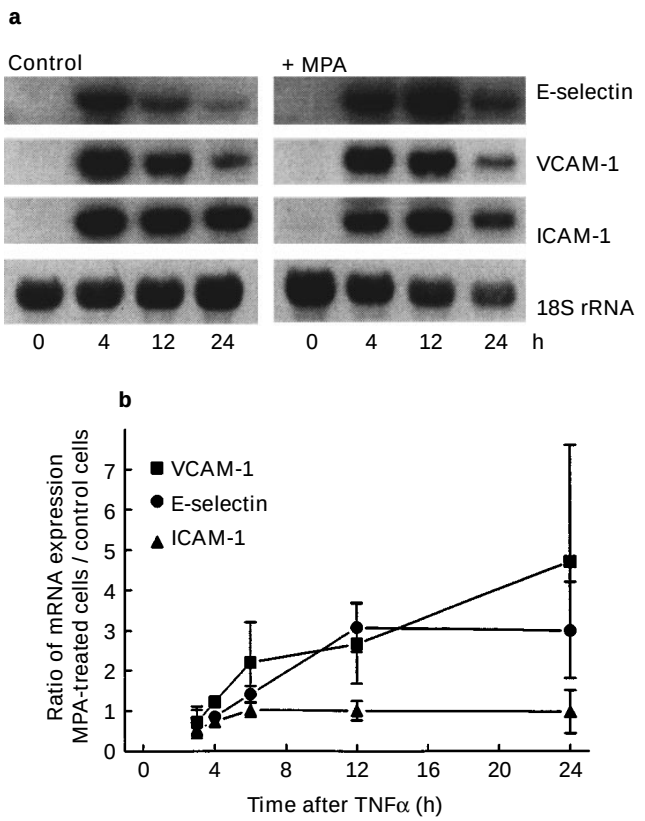

Figure 6 Effect of mycophenolic acid (MPA) on the kinetics of mRNA expression of ICAM-1, VCAM-1 and E-selectin in TNFα-activated EC. EC were left untreated or preincubated with MPA (10 μM) for 24 h. Thereafter, the cells were stimulated with TNFα for the times indicated and mRNA expression was determined by Northern blot analysis (a). Data shown in (b) represent the ratio of mRNA in the presence of MPA to mRNA expression in the absence of MPA. All values are corrected for differences of RNA loading by calculating the ratio of mRNA encoding cell adhesion molecules to 18S rRNA. ($n=3-4$ independent preparations; means $\pm$ s.d. (vertical lines) are shown).

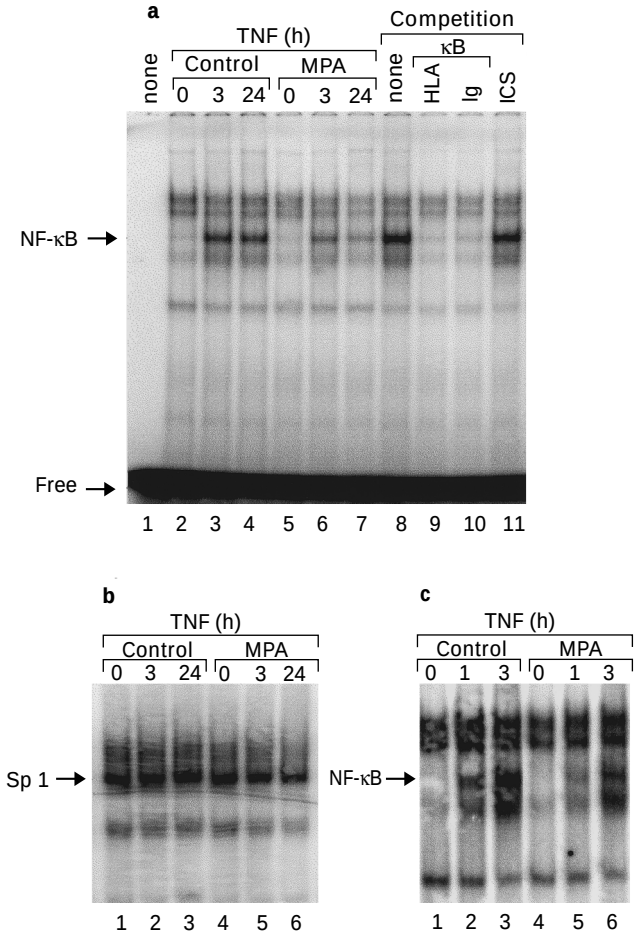

Figure 7 Effect of mycophenolic acid (MPA) on TNFα-activation of NF-κB in EC. (a) EMSA of NF-κB in the nuclear extracts of EC that were treated with DMSO (control) or MPA for 12 h, then left untreated (0) or treated with TNFα for 3 h or 24 h. Locations of the specific NF-κB-DNA complexes and the free κB probe are marked. Binding specificity was tested by competition with unlabelled κB DNAs from the immunoglobulin κ gene (Ig) or HLA class 1 (HLA) or else with an irrelevant control DNA, HLA class 1 interferon consensus sequence (ICS). (b) The same extracts used to measure NFκB (a) were used to measure Sp-1. Details of the gels are shown in (b) and (c). (c) Activation of NF-κB was reduced in MPA-pretreated EC at early times after TNFα-treatment. HUVEC were pretreated with MPA/DMSO or DMSO alone for 12 h, then treated with TNFα for the times indicated.

TNFα (Figure 7b). MPA inhibition of NF-κB activation was particularly marked at early (1 h) and late (24 h) times of TNFα-activation (Figures 7a and c).

MPA increases the stability of E-selectin mRNA in TNFα-treated EC

The increased E-selectin mRNA levels could be attributed to an mRNA stabilizing effect of MPA-preincubation: in the presence of 10 μg ml^{-1} actinomycin D, the half-life of E-selectin mRNA after TNFα-activation was determined to be in the order of $\approx$10 h. However, after pretreatment with MPA the half-life of E-selectin mRNA was calculated by extrapolation to about 80 h, since more than 90% of the E-selectin mRNA were still detected after 9 h (Figure 8b). ICAM-1 mRNA proved to be very stable with a decrease of only about 20% within 9 h. Additional effects of MPA-preincubation were not detectable (Figure 8a).

Discussion

The process of leukocyte migration from the blood to the tissue during inflammation involves the interaction of granulocytes, lymphocytes and monocytes with EC. This leukocyte-endothelial cell interaction is mediated by cell adhesion molecules the expression of which is increased by cytokines. The present study demonstrates that pretreatment of cultured EC with the immunosuppressant mycophenolate mofetil (MM) and its active metabolite mycophenolic acid (MPA) increases magnitude and duration of TNFα-induced expression of VCAM-1 and E-selectin, but not of ICAM-1. These increases appear to result from the increased stability of mRNAs encoding E-selectin and VCAM-1. Furthermore increased VCAM-1 and E-selectin on EC were functionally active by mediating increased adhesion of monocytic U937 cells which have previously been used as a model system to study the interaction of VLA-4 and sLex on leukocytes with VCAM-1 and E-selectin on TNFα-activated EC (Hauser *et al*., 1993).

The molecular mechanisms underlying the antiproliferative effect of MM on lymphocytes have been partially characterized (Allison & Eugui, 1993). MPA specifically inhibits inosine monophosphate dehydrogenase (Sintchak *et al*., 1996), thus inhibiting *de novo* GTP formation and depleting cells of guanosine and deoxyguanosine nucleotides required for lymphocyte proliferation. The GTP depleting effect is less pronounced in other cell types, such as EC, that have a salvage pathway for purine synthesis (Allison & Eugui, 1993). MPA-mediated GTP depletion in activated

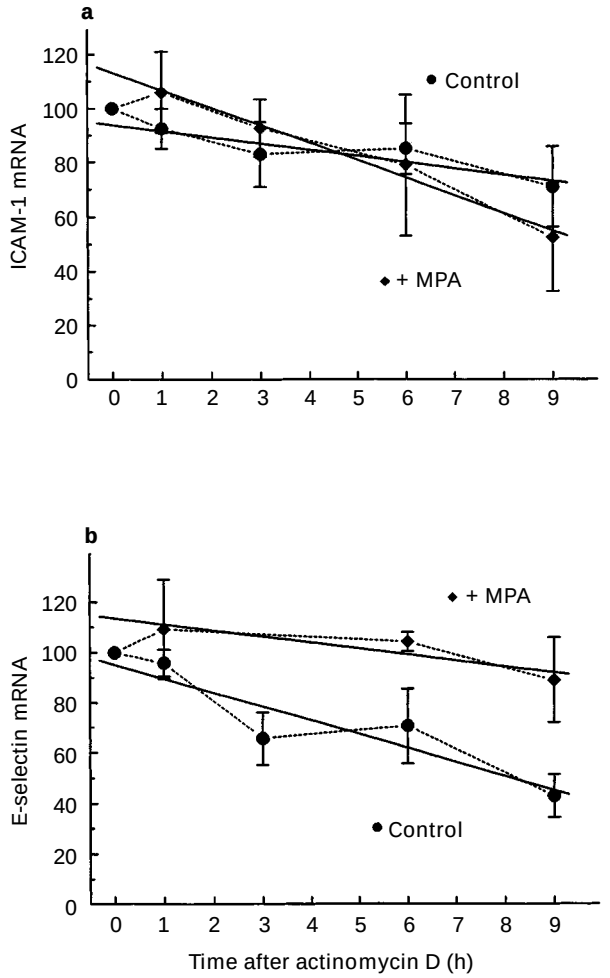

Figure 8 Effect of mycophenolic acid (MPA) on mRNA stability of ICAM-1 (a) and E-selectin (b) in TNFα-activated EC. EC were preincubated with or without MPA (10 μM) for 24 h. Thereafter, the cells were stimulated with TNFα for 3 h. Actinomycin D (10 $\mu g\,ml^{-1}$) was added for the times indicated and mRNA expression was determined by Northern blot analysis. All values were corrected for differences of RNA loading by calculating the ratio of mRNA encoding adhesion molecules to 18S rRNA. To compare different experiments, expression after 3 h incubation with TNFα was set to 100%. Data are means of 3 different experiments; vertical lines show s.d. Solid lines represent exponential curve-fits of the data.

lymphocytes has also been shown to decrease glycosylation of the β1 integrin VLA-4, resulting in decreased adhesion to endothelial cells (Allison *et al*., 1993). In contrast, the present study demonstrates that adhesion of U937 cells to TNFα-treated human EC is increased in MM-pretreated EC (Figure 1), suggesting that if inhibition of glycosylation also occurs in MM-pretreated EC it has a negligible effect on U937 cell adhesion to EC. To get closer to the *in vivo* situation it will be interesting to study the binding of peripheral mononuclear cells and granulocytes to MM-pretreated TNFα-activated EC. Furthermore, we demonstrated, that GTP depletion induced by inhibition of IMPDH cannot account for MPA-mediated increase of VCAM-1 and E-selectin expression in TNFα-activated EC, since addition of guanosine, which acts as a salvage pathway precursor for GTP synthesis (Senda *et al*., 1995), did not prevent the effect of MPA on CAMs (see Figure 5).

MPA-inhibition of TNFα-activated NF-κB (Figure 7) did not block the induction of ICAM-1, VCAM-1 or E-selectin, suggesting that sufficient NF-κB is activated to promote transcription of these genes. We (data not shown) and others (Ferran *et al*., 1995) have observed that pretreatment with the proteasome inhibitor pyrrolidine dithiocarbamate (PDTC, 100 μM for 2 h) abolishes the increased surface expression of cell adhesion molecules induced by TNFα alone or with MPA, demonstrating that initial activation of NF-κB is necessary for the MPA effect in TNFα-treated EC. Proteasome inhibitors are thought to reduce NF-κB activation by stabilizing inhibitor κB-α (IκB-α), an inhibitor of NF-κB, against TNFα-induced degradation by the proteasome. The effect of PDTC on CAM expression in TNFα-activated EC is therefore consistent with residual IkB-α in proteasome-inhibited EC inhibiting transcriptional activation by NF-κB more than its DNA-binding and suggests, moreover, that MPA-inhibition of TNFα-activated NF-κB does not occur through inhibition of the proteasome.

The data shown in Figure 8 indicate that the main mechanism of MPA action on cell adhesion molecule expression in TNFα-activated EC is an increase in the mRNA stability of VCAM-1 and E-selectin. Among the *cis*-acting determinants of E-selectin mRNA stability, the role of destabilizing AU-rich sequence elements has been best characterized (Chu *et al*., 1994). However, AU-rich sequence elements are also present in ICAM-1 mRNA (Ohh *et al*., 1994), the half-life of which was not increased by MPA (Figure 8), suggesting that sequence elements specific to VCAM-1 and E-selectin are responsible for the increased stability of their mRNAs found in the presence of MPA. *Trans*-acting regulatory proteins bind to RNA sequences shared by many mRNAs or to sequences that are specific to the mRNA of interest (for review, see Ross, 1995). For instance, treatment of endothelial cells with protein synthesis inhibitors increases resting and interleukin-1 stimulated E-selectin mRNA steady-state levels by preventing synthesis of labile proteins involved in destabilization of E-selectin mRNA (Ghersa *et al*., 1994). The effect of MPA on VCAM-1 and E-selectin surface expression required at least 12 h preincubation, suggesting that MPA could regulate one or more protein factors that control the stability of E-selectin and VCAM-1 mRNAs.

The concentrations of MPA used in this *in vitro* study are in the range of blood concentrations obtained *in vivo*. It is conceivable that increased adhesion of leukocytes, especially granulocytes, to EC would increase leukocyte accumulation at sites of inflammation, which could contribute to a major side-effect of MM therapy, i.e. gastrointestinal disorders with diarrhoea and eosinophilic infiltration (Sollinger, 1995). It has been suggested that cell adhesion molecules expressed in venules of the intestinal walls, such as E-selectin, are involved in the mucosal injury induced by anti-inflammatory drugs and which is characterized by neutrophil infiltration and inflammation of the intestinal wall (Asako *et al*., 1992; Wallace *et al*., 1993). It would therefore be additionally important studying the adhesion of granulocytes to EC *in vitro* to mimic the natural interaction between E-selectin and its ligand sLex on granulocytes, which in contrast to U937 cells do not express the VCAM-1 ligand VLA-4 on their cell surface.

I.A.H. thanks Professor Dr R.B. Sterzel for valuable discussions. The authors thank Mr H. Thüringer for excellent technical assistance and Prof. Dr J. Kalden and Dr P. Rohwer (Medical Clinic III, Department of Immunology, University Erlangen) for permission to use the FACS Analyzer. This research was supported by Grant DFG Ha 2-1/2.

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(Received June 17, 1997
Revised August 28, 1997
Accepted September 2, 1997)