Lymphocyte Migration Through Brain Endothelial Cell Monolayers Involves Signaling Through Endothelial ICAM-1 Via a Rho-Dependent Pathway

Peter Adamson, Sandrine Etienne, Pierre-Olivier Couraud, Virginia Calder and John Greenwood

*J Immunol* 1999; 162:2964-2973; ;
http://www.jimmunol.org/content/162/5/2964

**References**

This article cites 40 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/162/5/2964.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Lymphocyte Migration Through Brain Endothelial Cell Monolayers Involves Signaling Through Endothelial ICAM-1 Via a Rho-Dependent Pathway

Peter Adamson,*# Sandrine Etienne,† Pierre-Olivier Couraud,‡ Virginia Calder,* and John Greenwood*

Lymphocyte extravasation into the brain is mediated largely by the Ig superfamily molecule ICAM-1. Several lines of evidence indicate that at the tight vascular barriers of the central nervous system (CNS), endothelial cell (EC) ICAM-1 not only acts as a docking molecule for circulating lymphocytes, but is also involved in transducing signals to the EC. In this paper, we examine the signaling pathways in brain EC following Ab ligation of endothelial ICAM-1, which mimics adhesion of lymphocytes to CNS endothelia. ICAM-1 cross-linking results in a reorganization of the endothelial actin cytoskeleton to form stress fibers and activation of the small guanosine triphosphate (GTP)-binding protein Rho. ICAM-1-stimulated tyrosine phosphorylation of the actin-associated molecule cortactin and ICAM-1-mediated, Ag/IL-2-stimulated T lymphocyte migration through EC monolayers were inhibited following pretreatment of EC with cytochalasin D. Pretreatment of EC with C3 transferase, a specific inhibitor of Rho proteins, significantly inhibited the transmonolayer migration of T lymphocytes, endothelial Rho-GTP loading, and endothelial actin reorganization, without affecting either lymphocyte adhesion to EC or cortactin phosphorylation. These data show that brain vascular EC are actively involved in facilitating T lymphocyte migration through the tight blood-brain barrier of the CNS and that this process involves ICAM-1-stimulated rearrangement of the endothelial actin cytoskeleton and functional EC Rho proteins. *The Journal of Immunology, 1999, 162: 2964–2973.

To fulfill their role in tissue surveillance, lymphocytes must be able to leave the circulation and traffic through the solid tissues of the body. Thus, the migration of lymphocytes across the vascular endothelial cell (EC) wall is a prerequisite in the implementation of lymphocyte function. Lymphocyte transendothelial migration is dependent on lymphocyte activation via signaling through the T cell and IL-2 receptor (1). The in vivo correlate of these observations is that only Ag-specific, IL-2-dependent T cells are capable of trafficking to the central nervous system (CNS) (2). The vascular recruitment of circulating lymphocytes and their transvascular migration has been the subject of substantial investigation and has led to a greater understanding of the role of lymphocytes under normal and inflammatory conditions. Unlike most vascular endothelia, which are connected together by incomplete, permeable junctions, those of the brain and retina are joined together by impermeable tight junctions forming the blood-brain and inner blood-retinal barriers, respectively. Despite these barriers, a low level of leukocyte traffic into the CNS occurs (2), and this can be dramatically up-regulated during the development of immune-mediated diseases. To achieve entry, lymphocytes must either penetrate the tight junctions or migrate through the body of the CNS EC through the formation of pores. Whichever path is utilized during leukocyte diapedesis within the CNS, it is highly likely that this process can only operate with the active participation of the EC. Although it is clear that the activation state of lymphocytes is central in controlling transvascular migration (1, 3, 4), additional control may be provided by the CNS endothelia to further facilitate the passage of migratory cells across the impermeable vessel wall.

The mechanisms by which vascular EC capture circulating lymphocytes are now well characterized and many of the ligands that contribute to their adhesion and subsequent transvascular migration identified (5, 6). Previous studies have shown that initial capture of immune cells from the circulation occurs via endothelial selectin molecules that result in weak attachment and, under conditions of flow, result in leukocyte rolling along the vessel wall (6). Tighter binding and subsequent migration through the EC wall is mediated predominantly by the LFA-1/ICAM-1 (and possibly ICAM-2) pairing. However, on cytokine-activated endothelia, both the LFA-1/ICAM-1 and the very late Ag-4/VCAM-1 interaction appear to play a part in T lymphocyte migration (3, 7–9). Recent studies indicate that the molecules employed during lymphocyte adhesion to and migration through CNS endothelium are essentially the same as those governing recruitment at other vascular beds, with ICAM-1 being the predominant endothelial molecule involved in migration (1, 10, 11). However, the active participation...
of CNS EC in leukocyte extravasation, above that of the provision of adhesion molecules, has remained largely speculative.

If CNS endothelia play an active part in facilitating lymphocyte diapedesis, it is likely that they receive external signals from adherent lymphocytes. This has led to the possibility that molecules intimately involved in lymphocyte diapedesis, such as EC ICAM-1, may also be involved in the transduction of extracellular signals. Proteins of the Ig superfamily, including CD2, CD4, MHC molecules, and Fcγ are well-documented as signal transducers in both lymphocytes (12) and U937 cells (13). In addition, neural cell adhesion molecule has also been shown to be capable of signal transduction in PC12 cells (14). Although it has been demonstrated in leukocytes that ICAM-1 is capable of generating intracellular signals (15–17), and more recently, signaling via VCAM-1 and platelet endothelial cell adhesion molecule-1 has been demonstrated in platelets (18) and EC (19), the functional effects of signaling via Ig superfamily molecules in EC remain unresolved.

Studies aimed at exploring the signal transduction pathways in CNS EC that may lead to lymphocyte extravasation have been greatly expedited by the recent development of two immortalized Lewis rat microvascular EC lines that have been immortalized with the E1A adenovirus protein (RBE4 cell line; 20, 21) and SV40 large T Ag (GP8.3 cell line; 22) and that retain in culture the differentiated phenotype of brain endothelia. Previous studies with RBE4 cells have shown that Ab cross-linking of endothelial ICAM-1 molecules (used to mimic lymphocyte binding to EC via LFA-1) or coculture of EC with lymphocytes triggers p60<sup>src</sup> activity, which appears to be responsible for the enhanced tyrosine phosphorylation of the actin-binding protein cortactin (20). The enhanced tyrosine phosphorylation of cortactin following cross-linking of EC with ICAM-1 or coculture with encephalitigenic T lymphocytes was the first indication of an active endothelial involvement in controlling transvascular lymphocyte migration following T cell binding. Furthermore, it also served to demonstrate that T lymphocyte binding can be mimicked by ligation of EC ICAM-1. However, more recently, additional actin cytoskeletal-associated proteins, such as focal adhesion kinase (FAK), paxillin, and p130<sup>cas</sup>, have also been shown to be tyrosine phosphorylated in response to ICAM-1 cross-linking (23). In this paper, we have described (above and incubated with 10% normal goat serum/PBS. Cells were subsequently exposed to 1 μg/ml of anti-rat ICAM-1 (I2A9) for 1 h at room temperature, exhaustively washed in PBS, and incubated with anti-rat ICAM-1-FITC or anti-mouse-Cy3 (1:50; Jackson ImmunoResearch, West Grove, PA) for 1 h. Cells were exhaustively washed in PBS and viewed on a Leica confocal fluorescence microscope.

**Fluorescence microscopy**

**Actin localization.** Cells were fixed in 3.7% paraformaldehyde in PBS for 10 min, followed by 50 mM Tris-HCl (pH 7.5)/PBS for 10 min. Cells were subsequently permeabilized in 0.5% Triton X-100/PBS and incubated with 0.1 μg/ml of FITC-phalloidin for 1 h. Cells were exhaustively washed in PBS and viewed on a Leica (Mannheim, Keynes, U.K.) confocal fluorescence microscope.

**ICAM-1 localization.** For ICAM-1 localization, cells were fixed as described above and incubated with 10% normal goat serum/PBS. Cells were subsequently exposed to 1 μg/ml of anti-rat ICAM-1 (1A29) for 1 h at 4°C. Cell lysates were then prepared using the ECL system (Amersham) and exposed to x-ray film. Immunoprecipitation of p80/85 cortactin was achieved following lysis of cells for 30 min in buffer (10 mM Tris-HCl [pH 7.5], 140 mM NaCl, 1% digitonin, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 50 μM of aprotinin, 1 mM EDTA, 2 μg/ml of pepstatin, and 2 μg/ml of leupeptin), followed by removal of nuclei (13,000 × g for 30 min) and incubation with 1 μg/ml of anti-cortactin mAb for 1 h at 4°C. Cell lysates were preincubated with 50 μl of RAM-coated pansorbin (Calbiochem, Natick, MA) for an additional 30 min at 4°C, and then immune complexes were collected by centrifugation. Immune complexes were extensively washed in lysis buffer and further analyzed by Western blot analysis. p80/85 cortactin phosphorylation was quantitated by densitometry and normalized to levels of immunoprecipitated cortactin.

**Soluble (S)-Ag-specific CD4<sup>+</sup> T cell lines**

Lewis rat T lymphocyte cell lines specific for purified bovine retinal S-Ag were prepared as previously described (26). Briefly, lymph nodes were removed from bovine S-Ag immunized rats, and the lymphocytes were propagated by periodically alternating Ag activation with IL-2 stimulation. The cell lines express the marker of the CD4<sup>+</sup> T cell subset, are well-documented as signal transducers in the molecular context of MHC class II determinants (26). These cell lines have previously been shown to be highly migratory across monolayers of primary cultured brain and retinal endothelia (1, 4, 11) and represent Ag-stimulated lymphocytes.

**Adhesion of peripheral lymph node cells (PLNC) to endothelia**

Adhesion assays were conducted as previously described (27, 28) using cells harvested from Lewis rat peripheral lymph nodes. Briefly, PLNC were isolated, and T lymphocytes were obtained after purification on nylon wool columns. These cells that represent non-Ag-activated T lymphocytes are therefore nonmigratory but highly adhesive when activated with the mitogen Con A (1, 4, 11). PLNC were activated for 24 h with type V Con A, washed twice in HBSS, and cells labeled with 3 μCi [35S]Cr per 10<sup>6</sup> cells in HBSS for 90 min at 37°C. After washing the cells three times with HBSS, they were resuspended in RPMI 1640 medium containing 10%
LYMPHOCYTE MIGRATION THROUGH BRAIN EC REQUIRES Rho

FCs. Endothelial monolayers grown on 96-well plates were prepared by removing the culture medium and washing the cells four times with HBSS. [35S]Cr-labeled PLNC (200 μl) at a concentration of 1 × 10⁶/ml was then added to each well and incubated at 37°C for 1.5 h. In each assay, γ emissions from each of six replicate blank wells were determined to provide a value for the total amount of radioactivity added per well and to allow calculation of the specific activity of the cells. After incubation, nonadherent cells were removed with four separate washes from the four poles of the well at 37°C HBSS as previously described (27, 29). Adherent PLNC were lysed with 2% SDS, the lysate removed, and γ emissions quantitated by spectrometry. Results are expressed as the fractional differences between groups, Student’s t test.

T lymphocyte transendothelial migration

The ability of the immortalized cells to support the transendothelial migration of Ag-specific T lymphocytes was determined using a well-characterized assay as previously described (1, 4, 11). Briefly, T lymphocytes were added (2 × 10⁶ cells/well) to 24-well plates containing EC monolayers. Lymphocytes were allowed to settle and migrate over a 4-h period. To evaluate the level of migration, cocultures were placed on the stage of a phase-contrast inverted microscope housed in a temperature-controlled (37°C), 5% CO₂, gassed chamber (Zeiss, Herts, U.K.). A 200 × 200-μm field was randomly chosen and recorded for 10 min spanning the 4-h time point using a camera linked to a time-lapse video recorder. Recordings were replayed at 160× normal speed, and lymphocytes were identified and counted that had either adhered to the surface of the monolayer or had migrated through the monolayer. Lymphocytes on the surface of the monolayer were identified by their highly refractive morphology (phase-bright) and rounded or partially spread appearance. In contrast, cells that had migrated through the monolayer were phase-dark, highly attenuated, and were seen to probe under the EC in a distinctive manner (1, 4, 11). Treatment of EC with cytochalasin D or C3 transference was conducted before addition of lymphocytes, following extensive washing and replacement into new medium. Control data were expressed as the percentage of total lymphocytes within a field that had migrated through the monolayer. All other data were expressed as a percentage of the control migrations. A minimum of three independent experiments using a minimum of wells per assay was performed. The results are expressed as the means ± SEM, and significant differences between groups are determined by Student’s t test.

Expression and purification of C3 transference

Glutathione S-transferase-C3 was expressed in Escherichia Coli for 5 h using 1 mM isopropyl β-D-thiogalactoside (Life Technologies/BRL). Cells were harvested by centrifugation at 4000 × g for 15 min and sonicated three times for 5 min in lysis buffer (50 mM Tris-HCl [pH 8], 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, and 1 mM 4-[2-aminoethyl] benzenesulfonyl fluoride (AEBSF)). Bacterial lysates were then centrifuged at 10,000 × g for 30 min, and the supernatant was chromatographed over glutathione-agarose. The column was washed with 5 vol of AEBSF-free lysis buffer and 3 vol of thiorbin cleavage buffer (50 mM Tris-HCl [pH 8], 50 mM NaCl, 5 mM MgCl₂, 2.5 mM CaCl₂, and 1 mM DTT). Bovine plasma thrombin (10 U/ml gel) was then added to the column for 16 h at 4°C. The eluate from the column was collected and subsequently washed with 3 vol of PBS. Thrombin was removed from C3 transference protein released from the column by chromatography over p-aminobezamidine-agarose and dialysed into PBS. C3 transference was then concentrated in ultrafiltration units (Amicon, Beverley, MA). This procedure produced pure C3 transference protein as assessed by SDS-PAGE. Protein concentration was assessed using bicinchoninic acid (BCA) reagent (Pierce, Chester, U.K.).

[^125]P-ladenosine 5′-diphosphate (ADP)-ribosylation of endothelial and lymphocyte lysates with C3 transference

Cells were lysed in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 2 mM MgCl₂, and 1 mM AEBSF, and protein concentrations were determined using BCA reagent (Pierce). Lysate protein (25 μg) was then added to reactions containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 2 mM MgCl₂, 0.5 mM ATP, 0.3 mM guanosine triphosphate (GTP), 1 mM AEBSF, 5 μCi/ml of [α-^32]P]NAD, and 250 ng/ml of recombinant C3 transference and incubated at 37°C for 1 h. Reactions were stopped by the addition of 2 vol of 30% w/v trichloroacetic acid on ice for 30 min. Precipitated proteins were separated by centrifugation at 14,000 × g for 10 min, and pellets were washed three times with ethanol at −20°C. Pellets were dried, solubilized in 10 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.4% dithiothreitol, and 0.1% bromophenol blue, and proteins resolved on 15% SDS-PAGE.

Guanine-nucleotide, immunoprecipitation, and immunoblot analysis of endothelial Rho proteins

Serum-starved GP8.3 (5 × 10⁶) cells were incubated in phosphate-free DMEM overnight in the presence of 0.2 mM/cell of [α-^32]P]orthophosphate. Cells were washed in HBSS and recultured in serum-free DMEM. After addition of stimuli, cells were lysed in 100 mM HEPES (pH 7.4), 2% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 300 mM NaCl, 10 mM MgCl₂, 2 mM EDTA, 2 mg/ml of BSA, and protease inhibitors (20 mM benzamidine, 20 μg/ml of leupeptin, 20 μg/ml of pepstatin, 20 μg/ml of aprotinin, 20 μg/ml of soybean trypsin inhibitor, and 2 mM A2B5). Lysates were centrifuged at 14,000 × g for 5 min to sediment nuclei, and supernatants were adjusted to 500 mM NaCl. Lysates were immunoprecipitated by incubation with 4 μg rabbit polyclonal anti-Rho (Autogenbioclear) for 2 h at 4°C followed by incubation with 50 μl of 50% protein G-Sepharose (Pharmacia, Oxon, U.K.) for 2 h at 4°C. Immunoprecipitates were washed eight times with 1 ml of 50 mM HEPES (pH 7.4), 0.005% SDS, 500 mM NaCl, and 0.1% Triton X-100. Immunoprecipitates were subsequently heated to 68°C for 20 min in the presence of 5 mM EDTA, 2 mM DTT, 0.2% SDS, 0.5 mM GTP, and 0.5 mM guanosine diphosphate (GDP) to elute [α-^32]P-phosphate-labeled nucleotides. Nucleotides were separated by polyethyleneimine-cellulose plates (Machery-Nagel Beds, U.K.) in 1.2 M ammonium formate/0.8 M HCl (30) and autoradiographed at ~70°C using Fuji-RX film. Autoradiographs were subsequently used as templates and radioactive areas that corresponded with GDP and GTP standards, scraped, and radioactivity determined by β-scintillation spectrometry. EC lysates were also resolved on 15% SDS-PAGE gels and electrotransferred to nitrocellulose. Membranes were blocked with 10% dried milk protein for 1 h at room temperature and incubated in 0.1 μg/ml of polyclonal anti-Rho Ab (Autogenbioclear) for 1 h at room temperature. Endothelial Rho proteins were visualized following incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:15,000; Promega, Hants, U.K.) and subsequent development by ECL (Amersham). [α-^32]P-nucleotides were normalized to levels of immunoprecipitated Rho proteins.

Protein determination

Protein concentration in cell lysates was determined using BCA reagent (Pierce) with BSA as standard.

Results

Cross-linking of endothelial ICAM-1 results in clustering of ICAM-1 and actin stress-fiber formation

Ligation of the external domain of endothelial ICAM-1 molecules with mouse anti-rat ICAM-1 (1A29; 20 μg/ml for 30 min) followed by cross-linking with RAM IgG (60 μg/ml for 15 min) led to clustering of endothelial ICAM-1 molecules and a redistribution of the endothelial actin cytoskeleton in both serum-starved RBE4 (Fig. 1, c and d) and GP8.3 cells (data not shown), which had previously been incubated with 50 U/ml of IFN-γ for 48 h to increase ICAM-1 expression. In EC, under identical conditions but in the absence of ICAM-1 cross-linking, FITC-phalloidin staining showed the actin cytoskeleton to be diffuse with few stress fibers and a cortical concentration of actin in both RBE4 (Fig. 1b), GP8.3 (data not shown), and primary culture cells (data not shown). ICAM-1 staining was uniformly distributed within the plasma membrane (Fig. 1a). However, following ICAM-1 cross-linking for 15 min, there was a dramatic increase in the number of actin stress fibers that was not observed after treatment of cells with either RAM (Fig. 1b) or anti-ICAM-1 alone (data not shown). Identical results were observed in both RBE4 and GP8.3 cells (data not shown). Exposure of RBE4 (Fig. 1c and j) or GP8.3 (data not shown) cells to 10 μM lysophosphatidic acid (LPA) also resulted in a similar induction of actin stress fibers (Fig. 1j). Cross-linking of the transferin receptor did not induce stress fibers in either GP8.3 or RBE4 cells and were identical to control or RAM-treated.
FIGURE 1. Effect of ICAM-1 cross-linking Abs on ICAM-1 localization and actin distribution: effect of cytochalasin D and C3 transferase. RBE4 cells were preincubated for 48 h with 50 U/ml of rat IFN-γ and exposed to 20 μg/ml of mouse anti-rat ICAM-1 (1A29) for 30 min, followed by RAM (60 μg/ml) for 15 min. Cells were then subsequently fixed in 3.7% paraformaldehyde for 10 min, followed by 50 mM Tris-HCl (pH 7.5) for 10 min and stained for actin and ICAM-1 localization. Cells were either incubated with 1 μg/ml of anti-rat ICAM-1 (1A29) or permeabilized in 0.5% Triton X-100 and incubated in 0.1 μg/ml of FITC-phalloidin for 1 h. For ICAM-1 staining, cells were washed six times in phosphate buffered saline A and incubated with anti-mouse-Cy3 (1:50) for 1 h. Cells were then exhaustively washed in phosphate buffered saline A and visualized by confocal fluorescence microscopy. Cells were serum- and growth factor-deprived 48 h before addition of cross-linking Abs. Identical fields were stained for ICAM-1 and actin. a and b, RAM (60 μg/ml) only. c and d, ICAM-1 (20 μg/ml) followed by RAM (60 μg/ml). e and f, ICAM-1 (20 μg/ml) followed by RAM (60 μg/ml) in presence of 2 μM cytochalasin D. g and h, ICAM-1 (20 μg/ml) followed by RAM (60 μg/ml) following 8 h pretreatment with 50 μg/ml of C3 transferase. i and j, 10 μM LPA. a, c, e, g, and i, stained for ICAM-1. b, d, f, h, and j, stained for actin. Scale bar = 25 μm. ICAM-1 stains are shown as projections of all optical sections. A single identical optical section is shown for phalloidin staining.

cells (data not shown). Similar effects on actin stress-fiber formation were also observed in both RBE4 and GP8.3 cells following ICAM-1 cross-linking to basal levels of ICAM-1 (data not shown).

Cross-linking of endothelial ICAM-1 or coculture with T lymphocytes results in GTP-loaded endothelial Rho

Serum-starved GP8.3 cells induced with 50 U/ml of IFN-γ for 48 h were labeled with 0.2 mCi/ml of [32P]-orthophosphate in phosphate-free growth medium for 20 h. Cells were washed in phosphate containing serum-free growth media and stimulated with RAM (60 μg/ml) alone, anti-ICAM-1 (20 μg/ml) followed by RAM (60 μg/ml), anti-transferin receptor (20 μg/ml) followed by RAM (60 μg/ml), or a 10:1 ratio of syngeneic peripheral node lymphocytes. Rho proteins were subsequently immunoprecipitated from cell lysates, bound nucleotides eluted, and analyzed by TLC. Analysis of GTP-GDP ratios of Rho proteins immunoprecipitated from GP8.3 EC showed that following cross-linking of EC ICAM-1 for 10 min, or coculture with T lymphocytes for 10 min
to allow the majority of the T lymphocytes to settle on the EC monolayer.

Since ICAM-1 cross-linking induced the formation of actin stress fibers in EC, the potential role of the actin cytoskeleton in regulating ICAM-1-mediated signals was assessed. Therefore, ICAM-1 was cross-linked on RBE4 EC as described above in the presence of 2 μM cytochalasin D or vehicle (0.5% DMSO). p80/85 cortactin was immunoprecipitated from EC lysates obtained from either control or treated cells. Immunoprecipitated proteins were resolved on 7.5% SDS-PAGE and immunoblotted with anti- phosphotyrosine Ab. p80/85 cortactin (20) showed a 2.7-fold increase in tyrosine phosphorylation following cross-linking of ICAM-1, which was abolished (inhibited by 100%) in the presence of 2 μM cytochalasin D (Fig. 3). Reprobing of immunoblots with mouse anti-cortactin mAb revealed that equal amounts of p85 cortactin were immunoprecipitated following each treatment. Treatment of RBE4 cells with 2 μM cytochalasin D for 1 h resulted in the disruption of all polymerized actin that exhibited a punctate distribution (Fig. 1f). Under these conditions, cross-linking ICAM-1

Cytochalasin D inhibits ICAM-1-mediated stress-fiber formation and cortactin phosphorylation in EC

FIGURE 2. ICAM-1 cross-linking induces an increase in GTP-loaded Rho proteins. A. Serum-starved GP8.3 cells were cultured in the presence of 50 U/ml of rat IFN-γ for 48 h and in phosphate-free growth medium supplemented with 0.2 mM orthophosphate for 20 h. Cells were washed in normal growth medium and were stimulated with either RAM (60 μg/ml) alone for 10 min, anti-ICAM-1 (20 μg/ml) for 30 min followed by RAM (60 μg/ml), or a 10:1 ratio of lymphocytes. In C3 transerse experiments, cells were treated for 4 h with 50 μg/ml of C3 transerse before incubation with Abs or coculture with PLNC. Cells were lysed on ice in 0.5 ml of 100 mM HEPES (pH 7.4), 2% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 300 mM NaCl, 10 mM MgCl2, 2 mM EGTA, 2 mg/ml of BSA, and protease inhibitors. Nuclei were removed and lysates adjusted to 500 mM NaCl. Anti-Rho Ab (4 μg) (+) or anti-transferin receptor (control) (+) was added and incubated with lysates for 2 h at 4°C followed by 50 μl of 50% protein-G Sepharose. Immunoprecipitates were washed with 50 mM HEPES (pH 7.4), 0.005% SDS, 500 mM NaCl, 0.1% Triton X-100 and heated to 68°C for 20 min in the presence of 3 mM EDTA, 2 mM DTT, 0.5 mM GTP, and 0.5 mM GDP. Labeled nucleotides were separated by TLC on polyethyleneimine-cellulose plates in 1.2 M ammonium formate/0.8 M HCl and autoradiographed. Areas containing GTP and GDP as assessed by comigration with GTP and GDP standards were scraped and radioactivity determined by β-scintillation spectrometry. +, presence of ICAM-1 mAb, RAM transferin-receptor mAb, C3 transerse, or lymphocytes. −, no mAb, C3 transerse, or lymphocytes added. Time points involving the addition of lymphocytes were taken following an initial coculture period of 30 min that was necessary to allow the majority of the T lymphocytes to settle on the EC monolayer.

FIGURE 3. Effect of cytochalasin D on ICAM-1-stimulated cortactin phosphorylation. Serum-starved RBE4 cells cultured in the presence of 50 U/ml of rat IFN-γ for 48 h were stimulated with either RAM (60 μg/ml) alone (+) or anti-ICAM-1 (20 μg/ml) (+) in the presence of 2 μM cytochalasin D (0.5% DMSO) alone or vehicle (DMSO alone). Cells were lysed and immunoprecipitated with anti-cortactin Ab, and immunoprecipitates were resolved on 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine Ab (A), followed by stripping and reprobing with anti-cortactin Ab (B). Immunoblots were exposed to anti-mouse HRP-conjugated secondary Ab and developed using ECL.
Abs were unable to induce the appearance of actin stress fibers. Pretreatment of EC with cytochalasin D also appeared to alter the cellular localization of ICAM-1 (Fig. 1e).

ICAM-1-stimulated stress-fiber formation, but not cortactin phosphorylation, is inhibited by pretreatment of EC with C3 transferase

In a variety of other cell types, the regulation of actin stress fibers is regulated by members of the Rho family of small GTP-binding proteins (31). Therefore, to assess the potential role of these proteins in ICAM-1-mediated signaling, EC were treated with the bacterial toxin C3 transferase, which is capable of entering cells and inactivating Rho proteins. Treatment of EC with 50 μg/ml of C3 transferase followed by in vitro ribosylation of EC lysates with [32P]NAD showed a time-dependent inactivation of EC Rho proteins (Fig. 4). Inactivation of EC Rho proteins is apparent as a reduction in the availability of substrate for subsequent in vitro [32P]ADP ribosylation in EC lysates. Only one major C3 transferase substrate was observed in EC or lymphocyte lysates with an apparent m.w. of ~25 kDa, which is consistent with Rho proteins that have been correctly posttranslationally modified (32). C3 transferase treatment of both RBE4 (Fig. 1h) and GP8.3 cells (data not shown) significantly reduced the number of actin stress fibers and abolished the induction of stress-fiber formation following subsequent ICAM-1 cross-linking. In a similar manner to cytochalasin D, C3 transferase also resulted in a redistribution of EC ICAM-1 (Fig. 1g). Immunoprecipitation and Western blot analysis demonstrated that preincubation of either RBE4 (Fig. 4) or GP8.3 (data not shown) EC for 8 h with 50 μg/ml of C3 transferase, under which conditions all EC Rho protein was inactivated, was ineffective in inhibiting the ICAM-1-mediated enhanced tyrosine phosphorylation of p80/85 cortactin (Fig. 5). Cross-linking of RBE4 cells with ICAM-1/RAM led to a 3.7-fold increase in tyrosine phosphorylated cortactin, which compared with a 3.6-fold induction following preincubation with C3 transferase.

Cytochalasin D inhibits lymphocyte migration through, but not adhesion to, EC monolayers

Rat brain EC monolayers were able to support the ICAM-1-dependent transendothelial migration of Ag-specific lymphocytes over a 4-h period with 43 ± 5.5% (n = 22) of the lymphocytes migrating through the EC monolayer. The ICAM-1-dependency of Ag-specific T cell migration through CNS EC has previously been demonstrated in Ab blockade studies (1, 11). Pretreatment of GP8.3 monolayers with 2 μM cytochalasin D for 1 h following removal, enzymatic washing, and replacement with fresh medium before coculture with lymphocytes dramatically reduced transendothelial migration of Ag-specific lymphocytes to 61.8 ± 14.3% (p < 0.0001; n = 6) of the control value (Fig. 6A). Increasing the pretreatment time of GP8.3 monolayers with 2 μM cytochalasin D to either 3 h or 24 h brought about a further decrease in migration to 39.7 ± 12.3% and 35.8 ± 14.8% of control values, respectively. Similar results were obtained from identical experiments using both primary cultures (data not shown) and RBE4 cells (data not shown). Increased pretreatment times of EC with cytochalasin D were found to be necessary since cytochalasin D was not present during the 4-h lymphocyte coculture, and the effects of cytochalasin D treatment on EC stress-fiber formation was observed to be partially reversible under these conditions. Basal adhesion of Con A-stimulated PLNC, which adhere in an identical manner to Ag-specific T cell lines, but are not capable of transendothelial migration, (4) to GP8.3 EC was 18.0 ± 0.7%. Basal adhesion of PLNC to primary cultures and RBE4 cells was similar to that observed for GP8.3. Pretreatment of EC with either 2–20 μM cytochalasin D did not affect the adhesion of PLNC to GP8.3 (Fig. 6B), RBE4, or primary cultures of EC (data not shown). These findings demonstrate that the effects of cytochalasin D in inhibiting lymphocyte migration is due to an effect on the endothelial support of lymphocyte migration and not to prevention of lymphocyte binding to endothelia. In addition, it was also observed that treatment of EC with cytochalasin D did not affect the adhesion of cocultured Ag-specific lymphocytes since, during time-lapse video microscopy, lymphocytes displayed normal spreading and motile behavior on the EC surface. All EC monolayers used were able to exclude trypan blue following pretreatment with cytochalasin D and coculture with lymphocytes.

FIGURE 4. Time course of C3-mediated ADP ribosylation of Rho proteins in EC and lymphocytes. Confluent cultures of EC were pretreated for specified times (0–8 h) with 50 μg/ml of recombinant C3 transferase. In separate experiments, GP8.3 cells, RBE4 cells, or lymphocytes cocultured with C3-treated GP8.3 EC were washed, lysed, and ADP ribosylated with 250 ng/ml of C3 transferase in the presence of 5 μCi/ml of [α-32P]NAD, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM MgCl2, 1 mM AEBSF, 300 μM GTP, and 0.5 mM ATP. Lysate proteins were then resolved on 15% SDS-PAGE and autoradiographed.
groups were determined by Student’s t test. *, p < 0.02. Identical data was also obtained with both primary cultures and RBE4 cells.

**FIGURE 6.** Effect of cytochalasin D on lymphocyte adhesion to and migration through brain endothelial monolayers. GP8.3 cells were treated with either vehicle (0.5% DMSO) or 2 μM cytochalasin D for various times. After removal and vigorous washing, 51Cr-labeled Con A-activated (5 μg/ml) rat PLNC (adhesion) or Ag-specific T cells (migration) were added and allowed to adhere for 90 min or migrate over a 4-h period, respectively. A, Migration of Ag-specific lymphocytes through GP8.3 cells. B, Adhesion of Con A-stimulated PLNC to GP8.3 cells. Observations are a minimum of three independent experiments using a minimum of four wells per assay. Data is expressed as mean ± SEM percent of control migration. Significant differences between groups were determined by Student’s t test. *, p < 0.02. Identical data was also obtained with both primary cultures and RBE4 cells.

Transendothelial lymphocyte migration but not adhesion is inhibited by pretreatment of EC with C3 transferase

Pretreatment of both GP8.3 and RBE4 EC with 50 μg/ml of C3 transferase resulted in the inhibition of transendothelial lymphocyte migration (Fig. 7, A and B). Lymphocyte migration through monolayers of GP8.3 cells after incubation with C3 transferase for 4 h and 8 h was reduced to 60.1 ± 4.2% (p < 0.005) and 18.4 ± 4.1% (p < 0.005) of control values, respectively. Lymphocyte migration across cultures of RBE4 cells was only significantly inhibited after preincubation with C3 transferase for 12 h before coculture with lymphocytes, which resulted in a reduction to 23.4 ± 4.6% (p < 0.0001) of control lymphocyte migration. The time of C3 transferase incubation necessary for significant inhibition of lymphocyte migration corresponded to total inactivation of RBE4 cell Rho protein as assessed by in vitro ADP ribosylation (Fig. 4).

ADP ribosylation of lymphocyte lysates obtained from cells previously cocultured for 4 h with C3 treated EC showed no inactivation of lymphocyte Rho proteins (Fig. 4). The ability of lymphocytes to migrate across untreated endothelial monolayers was also not affected by their pre-exposure to C3-treated EC, since lymphocytes that had previously been cocultured with C3-treated endothelial monolayers were found to migrate normally when subsequently cocultured with untreated EC (data not shown). The binding of Con A-activated PLNC to GP8.3 or RBE4 cells was unaffected by preincubation of EC with 50 μg/ml of C3 transferase for up to 24 h (Fig. 7, C and D). In addition, treatments that are known to activate Rho function, such as exposure of cells to LPA (10 μM) (31) or epidermal growth factor (100 nM), which is capable of up-regulating RhoB mRNA in GP8.3 cells (P.A. and J.G., unpublished observations) and rat fibroblasts (33), did not affect the extent of lymphocyte/EC adhesion in either GP8.3 or RBE4 cells (Fig. 7, C and D). Ag-specific lymphocytes cocultured with C3 transferase-treated EC displayed normal surface spreading and motility on the EC surface. C3 treatment of EC had no affect on the integrity of the EC monolayer, and the EC retained their ability to exclude trypan blue. Con A-stimulated PLNC were used in adhesion assays to demonstrate that the effects of both cytochalasin D and C3 transferase were due to effects on EC mechanisms responsible for facilitating T lymphocyte migration, and not due to inhibition of lymphocyte binding to EC. Con A-stimulated PLNC and Ag-specific T lymphocytes have the same adhesive capacity, but PLNC do not have the ability to migrate due to the absence of Ag and IL-2 stimulation (1). This approach allows independent assessment of factors affecting the separate steps of adhesion and migration. Using time-lapse video microscopy, no differences were observed in the adhesion of Ag-specific T lymphocytes to EC following treatment of cells with either cytochalasin D or C3 transferase (data not shown).

**Discussion**

The activation state of a circulating T lymphocyte is of paramount importance in determining its ability to migrate out of the circulatory system (2–4, 7). Nevertheless, using functional assays, our studies have shown for the first time that brain EC are intimately and actively involved in facilitating lymphocyte diapedesis. It has previously been demonstrated that the expression of the adhesion molecule ICAM-1 on EC is pivotal in supporting lymphocyte migration across vascular endothelium (1, 3, 7, 11). To date, its role in this process has been generally interpreted as the provision of an extracellular docking molecule for leukocyte attachment and locomotion via ligation with receptor LFA-1. However, despite a lack of direct evidence, it has been proposed that the efficient transvascular migration of lymphocytes requires intracellular signals to be generated within the EC. This paper demonstrates that the migration of lymphocytes through CNS vascular barriers can only
proceed through the active involvement of intracellular processes within the EC, in addition to those elicited within the lymphocyte following Ag stimulation. In support of this thesis, we have recently shown that brain EC ICAM-1 is capable of eliciting signal transduction events following ICAM-1 cross-linking or coculture with T lymphocytes, demonstrating that EC actively respond to leukocyte adhesion (20). Mimicking lymphocyte attachment through cross-linking EC ICAM-1 molecules in vitro, we have confirmed in both GP8.3 and RBE4 rat brain EC lines that there is an enhanced tyrosine phosphorylation of cortactin (20), FAK, paxillin, and p130\(^{Cas}\) (23). Following ligation of EC ICAM-1, which was up-regulated by prior exposure to IFN-\(\gamma\), there is a reorganization of the EC actin cytoskeleton that results in stress-fiber formation. However, similar effects were also observed following ligation to basal ICAM-1 expressed on EC, suggesting that pretreatment of cells with IFN-\(\gamma\) was not involved in ICAM-1-induced signals. Pretreatment of EC with cytochalasin D inhibited both ICAM-1-stimulated cortactin phosphorylation and lymphocyte transendothelial migration, which supports the proposal that the endothelial actin cytoskeleton plays a vital role in orchestrating these events.

The observation that both ICAM-1 cross-linking and coculture of EC with Con A-stimulated PLNC results in increased levels of GTP-loaded endothelial Rho proteins strongly suggests that endothelial Rho proteins are involved in ICAM-1-mediated signaling events. As previously reported (34), Rho itself may be activated through cell-surface signals propagated through the actin cytoskeleton. In support of this, pretreatment of EC with C3 transferase, which is able to specifically ADP ribosylate (35) and inhibit Rho proteins, resulted in a substantial inhibition of transendothelial lymphocyte migration, inhibition of both ICAM-1 and lymphocyte-stimulated Rho-GTP loading, and inhibition of ICAM-1-stimulated stress-fiber formation, but was unable to inhibit ICAM-1-stimulated cortactin phosphorylation. Other studies from these laboratories have also demonstrated that ICAM-1-induced increases in the tyrosine phosphorylation of FAK, paxillin, and p130\(^{Cas}\) and increases in the activity of Jun-kinase, are all effectively inhibited following pretreatment of GP8.3 and RBE4 cells with C3 transferase (23). Treatment of EC monolayers with 50 \(\mu\)g/ml of recombinant C3 transferase for up to 8 h resulted in the complete inactivation of EC Rho proteins, as assessed by the inability of C3 transferase to incorporate \([\alpha-\text{32P}]\)ADP-ribose into lysates of EC monolayers previously exposed to C3 transferase in culture (Fig. 4). It was also observed that the inactivation of Rho proteins with C3 transferase was more rapid in GP8.3 cells than in RBE4 cells, which may reflect either a reduced uptake of C3 transferase into RBE4 cells or, as suggested in Fig. 4, increased levels of

![FIGURE 7. Effect of C3 transferase on lymphocyte adhesion to and migration through EC monolayers. Endothelial monolayers were pretreated with 50 \(\mu\)g/ml of recombinant C3 transferase for specified times between 0–12 h. After removal and vigorous washing, \(^{59}\text{Cr}-\)labeled Con A-activated (5 \(\mu\)g/ml) rat PLNC (adhesion) or Ag-specific T cells (migration) were added and allowed to adhere for 90 min or migrate over a 4-h period, respectively. A, Migration of Ag-specific lymphocytes through GP8.3 cells. B, Migration of Ag-specific lymphocytes through RBE4 cells. C, Adhesion of Con A-stimulated PLNC to GP8.3 cells. D, Adhesion of Con A-stimulated PLNC to RBE4 cells. Observations are a minimum of three independent experiments using a minimum of four wells per assay. Data is expressed as mean \pm SEM percent of control migration. Significant differences between groups were determined by Student’s \(t\) test. *; \(p < 0.02\).]
of C3 transferase substrate. The reduced efficiency of C3 transferase in ribosylating Rho proteins in RBE4 cells, compared with GP9.3 cells, correlated with the reduced effectiveness of C3 transferase in inhibiting transendothelial lymphocyte migration through monolayers of RBE4 cells. These studies have also demonstrated that the reduction in the ability of lymphocytes to migrate through EC monolayers is not due to C3-mediated inactivation of lymphocyte Rho proteins, since cells cocultured with C3-treated EC monolayers are able to migrate normally when transferred to untreated EC monolayers (data not shown). In addition, previous studies have shown that pretreatment of leukocytes with C3 transferase prevents their adhesion to vascular endothelia (36), which was not observed following treatment of EC with C3 transferase. Taken together, these data suggest the effect of C3 transferase treatment is mediated through inactivation of EC Rho proteins.

The inability of C3 transferase to inhibit tyrosine phosphorylation of cortactin, which is effectively inhibited following pretreatment of cells with cytochalasin D, suggests that the role of both the actin cytoskeleton and cortactin phosphorylation may be upstream of endothelial Rho proteins. Thus, it would appear that ICAM-1-mediated signals within EC are propagated via the actin cytoskeleton, which results in the subsequent activation of Rho proteins. This scheme has previously been suggested in studies on fibroblasts, in which constitutively activated Rho, when introduced directly into cells, was able to restore Rho mediated focal adhesions in the presence of cytochalasin D (33). Recent studies have demonstrated that tyrosine phosphorylation of cortactin, which we have shown to be phosphorylated by p60csrc (20), is responsible for an increase in organized actin (37). The observation that increased cortactin phosphorylation following ICAM-1 cross-linking is insensitive to pretreatment of EC with C3 transferase implies that cortactin may be intimately associated with the propagation of ICAM-1-induced signals within EC, which results in the subsequent activation of Rho proteins and is therefore upstream of Rho activation. Alternatively, tyrosine phosphorylation of cortactin may lie on an independent ICAM-1-stimulated pathway.

The ability of Con A-stimulated PLNC to stimulate endothelial Rho-GTP loading also demonstrates that the transduction of signaling events within the EC is not dependent on the ability of the lymphocytes to migrate through the EC monolayer and is therefore likely to occur through cell-cell adhesion. As previously suggested, the ability of T lymphocytes to migrate through EC monolayers therefore resides in an ability to effectively signal to EC coupled with signal transduction events elicited within T lymphocytes via Ag stimulation (1). The finding that preincubation of T lymphocytes with anti-LFA-1 Abs is able to effectively inhibit both T lymphocyte adhesion and transendothelial migration (11) also supports the view that signals induced following coculture of T cells with EC are mediated through endothelial ICAM molecules.

The physiological mechanisms within EC that allow lymphocytes to migrate through the physical barrier afforded by CNS EC, and that lead to either pore formation or disaggregation of tight junctions, remains unresolved. However, in polarized epithelial cells, RhoA has been shown to be present in the cytosol of Madin Darby canine kidney (MDCK) cells where cell-cell contacts are disrupted, but translocate to cell margins when cell-cell contacts are restored (38). In addition, Rho has been reported to regulate both perijunctional actin organization and tight junctions in both T84 and Caco2 epithelial cells (39). Alternatively, Rho proteins have been shown to regulate cell membrane invaginations in Xenopus oocytes (40). A similar role for Rho proteins in regulating junctional organization or pore formation within CNS EC may therefore provide a mechanism by which lymphocytes are able to penetrate the tight vascular barriers of the CNS. In conclusion, we propose that T lymphocyte diapedesis through CNS microvessels is actively facilitated by mechanisms involving adhesion-dependent signaling within the endothelium by a process that requires an intact endothelial actin cytoskeleton and functional endothelial Rho proteins.

Acknowledgments

We thank L. Fieg for the pGEX-2T-C3 construct, J. T. Parsons for anti-cortactin mAb, and O. Durieu-Trautmann for her contribution to the initial phase of this study.

References