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*J Immunol* 2001; 166:544-551; doi: 10.4049/jimmunol.166.1.544

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Analysis of the Roles of ICAM-1 in Neutrophil Transmigration Using a Reconstituted Mammalian Cell Expression Model: Implication of ICAM-1 Cytoplasmic Domain and Rho-Dependent Signaling Pathway

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Interaction between ICAM-1 (CD54) and fibrinogen (fg) has been shown to enhance leukocyte adhesion, but its specific role in the process of migration across endothelial cell junctions remains unclear. To overcome the problem of multiple adhesion receptors found on endothelial cells, we have engineered stable Chinese hamster ovary cell lines expressing ICAM-1 (Chinese hamster ovary ICAM-1). The transfection of ICAM-1 alone in these cells is sufficient to recapitulate the entire process of neutrophil adhesion and transmigration. This phenomenon was mediated by fg-ICAM-1 interactions, as depletion of fg, as well as the use of an Ab that specifically inhibits ICAM-1-fg interaction (2D5), completely abolished the effect of ICAM-1 expression on PMN transmigration. In addition, this ICAM-1-mediated transmigration is clearly dependent on the occurrence of fg-ICAM-1 interactions on the monolayer, and not on neutrophils, as the preincubation of the PMN with the mAb was ineffective. Furthermore, PMN transmigration, but not adhesion, is totally abolished when the ICAM-1 cytoplasmic domain is deleted, indicating that signaling inside the cell is required to mediate the fg-ICAM-1 effect on transmigration. Using a specific inhibitor of the small GTP-binding protein Rho, we have obtained evidence that this signaling cascade is involved. Thus, our results clearly show that ICAM-1 plays a key role in the migration of leukocytes across cell junctions, and indicate that this phenomenon is not a direct consequence of the enhanced adhesion mediated by the expression of ICAM-1. The Journal of Immunology, 2001, 166: 544–551.

The regulated migration of leukocytes through the endothelial cell (EC) barrier forms the basis of host defense mechanisms and immune-inflammatory responses. Diapedesis (or extravasation) depends on a stepwise adhesion cascade coordinated by the sequential ligand recognition of cell adhesion molecules expressed on leukocytes and endothelium (1). At least three steps have been identified in this phenomenon: selectin-mediated rolling, β2 integrin-mediated leukocyte adhesion, and migration across EC junctions. This last step or transmigration consists in leukocyte migration across endothelial junctions. Little is known about the adhesion molecules and the intracellular mechanisms implicated in transendothelial migration. This process involves proteins expressed predominantly at intercellular junctions, such as platelet EC adhesion molecule-1 (PECAM-1) (2), a cell adhesion molecule of the Ig superfamily, which has been shown to play a major role in leukocyte transmigration (3). Proteins expressed at the apical surface of the cell may also be involved, either by transmitting a signal or by facilitating leukocyte migration toward the cell junctions (4).

ICAM-1 (5) is a key molecule in leukocyte-endothelium adhesion through its recognition of β2 integrin counterreceptors CD11a/CD18 (LFA-1) (6) and CD11b/CD18 (Mac-1) (7). In addition to its interaction with cell-associated counterreceptors, ICAM-1 recognizes fibrinogen (fg) (8), an abundant plasma glycoprotein of 340 kDa, which is also a ligand for the neutrophil β2 integrin Mac-1 (9). We have previously shown that fg interacts through its g-chain (γ1,2,3) (10) with the first Ig-like domain of ICAM-1 (11), and that fg enhances the attachment of leukocytes to HUVEC by acting as a molecular bridge between the two cell types (8). In addition, fg-ICAM-1 interactions have been shown to enhance neutrophil transmigration, stimulating this process by 20- to 30-fold (12), but it remained to be determined whether this enhancement was due to a direct effect from endothelial or leukocyte ICAM-1 or to a more complex pathway involving other adhesion molecules expressed by EC.

Although Abs against ICAM-1 have been shown to inhibit transmigration in in vitro systems (13–15), these experiments failed to demonstrate the direct implication of ICAM-1 in leukocyte transmigration, as these mAbs may, in fact, block the prerequisite step of tight adhesion to endothelium. Thus, the implication of ICAM-1 in leukocyte transmigration is not clearly demonstrated. To investigate the specific contribution of ICAM-1-fg interaction in neutrophil emigration, we have transfected the cDNA encoding ICAM-1, PECAM-1, or the two in combination into Chinese hamster ovary (CHO) cells, a cell type not normally expressing these molecules, to obtain stable CHO cell lines. Using cells expressing ICAM-1 alone, we have been able to specifically study the role of ICAM-1 in neutrophil transmigration without the interference of PECAM-1, which is implicated in this step. The role...
of fg-ICAM-1 interactions in PMN transmigration was investigated in adhesion and transmigration assays in the presence of a well-known neutrophil chemotactant, FMLP (16). In this paper, we show that ICAM-1 is able to support transmigration in the absence of other adhesion molecules expressed in EC. This enhancement of PMN migration across CHO ICAM-1 monolayers was fg dependent, as evidenced by the inhibition of transmigration obtained in the presence of fg-depleted plasma. This phenomenon was also blocked using function-blocking anti-ICAM-1 mAbs. These results demonstrate that ICAM-1, through its interaction with fg, is directly implicated in the process of neutrophil transmigration. Furthermore, a mutant of ICAM-1 lacking its cytoplasmic portion is not able to support PMN emigration, while PMN adhesion is not modified on these monolayers. This demonstrates that the enhanced transmigration induced by ICAM-1 is not a direct consequence of a facilitated adhesion. Finally, we show in this paper that ICAM-1-mediated transmigration involves the small GTP-binding protein Rho.

Materials and Methods

Materials

The following mAbs were used: mouse mAbs 3D6 and 2D5 to human ICAM-1 (12); mouse Ab 3F4-10 (Hemeris, Grenoble, France) to human PECAM-1. Mouse mAb OKM1 directed against β2 integrin Mac-1 was a kind gift from Dario Altieri (Boyer Center for Molecular Medicine, Yale University School of Medicine). Anti-LFA-1 mAb 25.3.1 was obtained from Immunotech (Marseille, France). Tetramethylrhodamine isothiocyanate and FITC-conjugated goat anti-mouse IgG F(ab’)2 secondary Abs were purchased from Jackson ImmunoResearch (Sassnieres, France). C3 transferase was kindly provided by M. Popoff (Institut Pasteur, Paris, France).

Cell culture

HUVEC were isolated from human umbilical cords by collagenase treatment as described by Jaffe et al. (17). Cells were maintained in medium 199 (M199; BioWhittaker, Gagny, France) containing 20% FBS (Life Technologies, Cergy Pontoise, France), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B (Life Technologies), 50 μg/ml EC growth supplement (prepared from bovine brain), and 100 μg/ml heparin (Sigma, St. Louis, MO). In some experiments, HUVEC were stimulated with 200 U/ml TNF-α (Boehringer Mannheim, Meylan, France) for 4 h at 37°C. PMN were isolated by differential centrifugation on Ficoll-Hypaque gradients (Sigma), followed by dextran sedimentation and hypotonic lysis of erythrocytes.

Transfection experiments

A full-length cDNA encoding human PECAM-1 (18) in the mammalian expression vector pcDNA3 (Invitrogen, Groningen, The Netherlands) was transfected in wild-type (WT) CHO cells by electroporation. Recombinant CHO cells were selected in DMEM (BioWhittaker) containing 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and nonessential amino acids (BioWhittaker) plus 1 mg/ml G418 (Geneticin; Life Technologies) for selection or 500 μg/ml for routine culture. CHO cells were transfected with a full-length cDNA clone encoding human ICAM-1 (19) in the mammalian expression vector pRC/CMV by electroporation (Invitrogen, San Diego, CA), with selection of stable transfectants in DMEM complemented with 10% FBS, 10 U/ml penicillin, 100 μg/ml streptomycin, 1 mM l-glutamine, and nonessential amino acids plus G418 as described previously (10).

CHO cells expressing ICAM-1 cytoplasmic deletion mutant (CHO ICAM-1 Δcyt) were generated. A PCR procedure was performed to delete the cytoplasmic portion of ICAM-1 using the pcDNA3 plasmid containing the human ICAM-1 coding sequence. The following primers were used: a sense primer (5’-931 TGTGCTGGAATTCCTCAGCC 3’), containing the EcoRI restriction site and an antisense primer (5’-GCTCAGATCA240 GTTATAGGA245), containing a stop codon (underlined) and the XhoI restriction site to facilitate subsequent cloning. The PCR product was digested by EcoRI and XhoI and ligated into the pcDNA3 vector containing native human ICAM-1 that was also cut by the same enzymes. CHO cells were then transfected with the resulting construction and recombinant cells were selected in DMEM (BioWhittaker) containing 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and nonessential amino acids (BioWhittaker) plus 1 mg/ml G418 (Genetecin; Life Technologies) for selection or 500 μg/ml for routine culture.

CHO ICAM-1/PECAM-1 were obtained by transfection of CHO ICAM-1 cells with a full-length cDNA clone encoding human PECAM-1 in the mammalian expression pUT-SV1 (Eurogentec, Seraing, Belgium). Selection of stable transfectants was performed in DMEM complemented with 10% FBS, 1 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 500 μg/ml G418, and nonessential amino acids plus 50 μg/ml phenol red (Eurogentec) for selection or 25 μg/ml for routine culture.

Phenotypic characterization of WT, ICAM-1, ICAM-1 Δcyt, PECAM-1, or ICAM-1/PECAM-1 transfectants was conducted by flow cytometry and immunofluorescence staining on adherent cells with anti-ICAM-1 (3D6) or anti-PECAM-1 (5F4-10) mAbs.

Flow cytometry analysis

Single-cell suspensions obtained after trypsinization were centrifuged (400 × g, 5 min) and resuspended in medium containing 5% FBS. After incubation with 20 μg/ml primary mAbs for 30 min at 4°C, cell suspensions were washed and mixed with a FITC-conjugated secondary Ab (20 μg/ml) (Jackson ImmunoResearch) for an additional 30 min at 4°C. After washing, cells were analyzed on a Becton Dickinson FACScan (Mountain View, CA).

Immunofluorescence staining and confocal imaging

CHO cells were seeded on glass coverslips and grown to confluency before immunofluorescence staining. Glass coverslips were coated with 25 μg/ml human plasma fibronectin (prepared from human plasma, as described by Boehringer and Rusnati (20)). Cells were fixed with 2% paraformaldehyde, permeabilized with 0.5% Triton X-100, and processed for indirect immunofluorescence microscopy. Incubation with the primary Ab was followed by incubation with tetramethylrhodamine isothiocyanate-conjugated secondary Ab (Jackson ImmunoResearch), with three washes in 1× PBS, Ca2+/-Mg2+, 1% BSA, and 0.05% Tween 20 between the various steps. Coverslips were then mounted in Aquamount (BDH Laboratory Supplies, Dorset, U.K.) before microscopic observation. Cells were imaged using a Zeiss confocal microscope (Zeiss, Oberkochen, Germany). Cross-sectional reconstruction was accomplished with a Metamorph program (Princeton Instruments, Evry, France) to combine the entire Z series into a stack projection and to overlay and process the final image.

Permeability assay

Cells (104 at seeding in 100 μl) were cultured for 4 days on 6.5-mm diameter polycarbonate membranes of 0.4-μm pore size (Costar; Dominique Dutcher, Brumath, France), previously coated with human fibronectin (25 μg/ml) for 2 h at 37°C and rinsed with 1× PBS. Six hundred microliters of culture medium filled the lower compartment. Before the experiment, the culture medium of both the upper and lower compartments was replaced, without washing, with DMEM supplemented with 5% FBS (0.1 ml, upper chamber and 0.6 ml, lower chamber). Special culture medium for HUVEC (described above) was used for assay of HUVEC permeability. HRP (35 nM; VI-A type, 44,000 m.w.; Sigma) was added in the upper compartment. After 1 h at 37°C, 10 μl of the upper compartment was collected and kept on ice until enzymatic activity of HRP was assayed using a Sigma Fast kit (Sigma). The reaction was allowed to proceed for 20 min at room temperature (RT) before measuring the absorbance at 490 nm. To control monolayer permeability in the same condition as in the transmigration assay, CHO monolayers were assessed for cellular integrity 3 days after seeding on 0.4-μm polycarbonate membranes. Medium from the lower compartments was replaced by DMEM/HEPES (20 mM, pH 7.4). The upper compartments were filled with the same medium plus plasma (1:5 dilution) and 100 μM N-phenylalanine-1-prolyl-L-arginine chloromethyl ketone (Boehringer Mannheim). After 3 h at 37°C in 5% CO2, 7 μl of HRP (35 μM) was added in the upper compartment, followed by gentle agitation and a 30-min incubation at 37°C in 5% CO2. Quantification of HRP presence in the lower compartment was determined as described above.

Adhesion assay

WT or recombinant CHO cells were grown to confluency on 48-well plates (Falcon; Becton Dickinson). In some cases, CHO cell monolayers were pretreated with a specific inhibitor of Rho proteins, C3 transferase (50 μg/ml in complete medium). After a 4-h incubation at 37°C, cells were extensively washed before addition of labeled PMN. PMN (at 5 × 106/ml) were fluorescently labeled with 7.5 μM calcein acetoxymethyl (Molecular Probes, Leiden, The Netherlands) in RPMI 1640/HEPES (20 mM, pH 7.4)
Recombinant CHO cell lines

We have developed stable CHO cell lines expressing ICAM-1 or PECAM-1 (CHO ICAM-1 and CHO PECAM-1, respectively) or the two adhesion molecules in combination (CHO ICAM-1/PECAM-1). Expression levels of ICAM-1 and PECAM-1 in the three recombinant cell lines were quantified by FACS analysis.

As expected, WT CHO did not express ICAM-1 or PECAM-1 (Fig. 1A), whereas CHO ICAM-1 expressed ICAM-1 at a high expression level comparable to TNF-α-stimulated HUVEC (Fig. 1B). In CHO PECAM-1, PECAM-1 is expressed at nearly the same level as in HUVEC, where its expression is constitutive. Similar expression levels of both proteins were found for the cell line coexpressing ICAM-1 and PECAM-1 (Fig. 1A).

On HUVEC, ICAM-1 is distributed over the whole cell surface whereas PECAM-1 is almost exclusively expressed at interendothelial junctions. To verify the correct localization of ICAM-1 and PECAM-1 at the cell surface after transfection, confluent CHO ICAM-1 and CHO PECAM-1 monolayers were examined by immunofluorescence staining, followed by confocal analysis. The localization of ICAM-1 and PECAM-1 was determined using confocal microscopy with reconstruction in an x-z cross-sectional plane to directly visualize cell surface and cell-cell junctions. Confocal analysis clearly shows that ICAM-1 was uniformly distributed on the upper cell surface, including the cell junctions of the recombinant cell line (Fig. 1C) while PECAM-1 was concentrated at the intercellular junctions (Fig. 1D), indicating a correct localization of these recombinant proteins in CHO cells. Similar results were found for the cell line coexpressing ICAM-1 and PECAM-1 (data not shown).

Since we intended to use these CHO cell lines for transmigration assays instead of HUVEC monolayers, we assessed their capacity to form confluent monolayers in comparison to resting or TNF-α-stimulated HUVEC. The integrity of the monolayers and their function as barriers were studied by a permeability assay using HRP (21).

Three days after seeding, resting and TNF-α-stimulated HUVEC presented a low permeability level with 19.4 ± 0.1% and 21.6 ± 0.1% of controls, respectively (Fig. 2). CHO ICAM-1,
CHO PECAM-1, and CHO ICAM-1/PECAM-1 formed monolayers that were nearly impermeable to HRP, with 12.9 ± 3.1%, 17.2 ± 4.1%, and 15.7 ± 4.4% of controls, respectively; these values are similar to those obtained with WT CHO monolayers (11.9 ± 1.1%) (Fig. 2). Permeability across WT CHO or CHO ICAM-1 was also investigated in the conditions established for transmigration assays. Monolayers were incubated for 3 h at 37°C with a 1:5 dilution of plasma (in the medium used in transmigration) before the addition of HRP in the upper compartment to verify if plasma could have any effect on monolayer integrity. In these conditions, similar results were obtained with no significant difference between the two cell lines (data not shown). These results show that recombinant CHO cells are able to form correct, continuous monolayers with a high cohesion of cell–cell contacts and a permeability level comparable to that observed with EC. Finally, the cellular location of recombinant proteins was identical to that observed for ICAM-1 and PECAM-1 in EC.

**Human plasma enhances leukocyte transmigration across CHO ICAM-1 monolayers**

To study the role of ICAM-1 by itself in neutrophil transmigration, we used CHO ICAM-1 monolayers in an in vitro transmigration assay. The implication of ICAM-1-fg interactions in our model was first studied in a protein-free medium with or without fg. In these conditions, few PMN were able to migrate across CHO monolayers after a 3-h incubation time period and most of migrated PMN were either dead or adherent to the well surface. To maintain the viability of human neutrophils, all subsequent experiments were conducted in the presence of autologous human plasma.

In preliminary experiments, we tested PMN transmigration across WT CHO and CHO cells expressing either ICAM-1 or PECAM-1 or the two in combination toward fMLP or IL-8 gradients, which are well-known neutrophil chemoattractants (16). It has previously been shown that PECAM-1 plays a crucial role in neutrophil transendothelial migration during inflammation (3). Fig. 3 clearly shows that PECAM-1 expresses on CHO cells can support transmigration, with a 4-fold increase in the number of migrated cells in comparison to WT CHO (29.8 ± 4.5% vs 6.7 ± 3.9%), demonstrating that the function of PECAM-1 is maintained in these transfected cells.

Surprisingly, CHO cells transfected with ICAM-1 alone were also able to promote transmigration of human PMN in the presence of a fMLP gradient at a comparable level to CHO PECAM-1 (31.6 ± 8.8% vs 29.8 ± 4.5% of transmigrated PMN, respectively) (Fig. 3). Similar results were obtained when IL-8 was used instead of fMLP. All subsequent experiments were conducted in the presence of a fMLP gradient. It can be noted that, in our model, co-expression of the two proteins did not provide any additional effect in comparison to CHO cells expressing ICAM-1 or PECAM-1 alone (Fig. 3). These data clearly show that ICAM-1 is directly implicated in PMN transmigration.

We next investigated the effect of the absence of fg on PMN transmigration. fg was depleted from human plasma by action of thrombin, which is known to specifically degrade fg. Interestingly, results in Fig. 4 show that the addition of fg-depleted plasma reduced PMN transmigration across CHO ICAM-1 to only 22 ± 3%, while no effect was observed on PECAM-1-dependent transmigration or across WT CHO monolayers. This demonstrates that ICAM-1-fg interactions are implicated in this process.

To confirm that fg plays a direct role in PMN emigration, we performed experiments using purified fg added to fg-depleted plasma. As shown in Fig. 4, addition of purified fg in fg-depleted plasma completely restored PMN migration across CHO ICAM-1 monolayers, demonstrating that fg depletion of plasma has no side effects and that the observed inhibition was due to the lack of fg.
Furthermore, depletion of fg had the same effect when the transmigration assay was performed toward a gradient of IL-8 (data not shown). This demonstrates that fg via its interaction with ICAM-1 is directly involved in the passage of PMN across the cell barrier, independently of the chemoattractant used.

Fg-dependent transmigration is blocked by anti-ICAM-1 mAbs

To better understand this mechanism, we used anti-ICAM-1 mAbs in transmigration assays. A panel of mAbs recognizing different ICAM-1 domains is available in our laboratory (12). Among these, 2D5 mAb binds to domain 1 of ICAM-1 and is known to completely suppress ICAM-1-fg interactions, while 3D6, which interacts with the second domain, has no effect on these interactions (11). The effect of 2D5 and 3D6 mAbs was tested on transmigration of PMN across CHO ICAM-1 and CHO PECAM-1 monolayers in the presence of human plasma.

As shown in Fig. 5, neither 2D5 nor 3D6 had inhibitory effects on PMN transmigration across WT CHO or CHO PECAM-1 monolayers. However, 2D5 mAb completely inhibited ICAM-1-dependent PMN transmigration across CHO ICAM-1 monolayers, resulting in only 19.2 ± 6.1% of transmigrated PMN. Interestingly, 3D6 did not have any effect on ICAM-1-mediated transmigration. These inhibition experiments with 2D5 mAb provide evidence for a direct role of fg-ICAM-1 interactions in neutrophil transmigration.

Because 2D5 mAb can recognize ICAM-1 on both CHO cells and PMN, it was important to determine which cell type was targeted by this inhibitory effect. For this, either PMN or CHO monolayers were pretreated with 2D5 mAb.

PMN transmigration was almost totally abolished when 2D5 was added on both PMN and CHO cells with only 4.7 ± 1.1% of transmigrated PMN (Fig. 6). The inhibition was similar when 2D5 was added on CHO ICAM-1 alone (3.6 ± 2% of transmigrated PMN), whereas 3D6 used in the same conditions, as a control, had no effect. In contrast, preincubation of 2D5 with PMN alone did not inhibit their transmigration (24.3 ± 3.2% of transmigrated PMN) (Fig. 6). These results demonstrate that the enhancement of PMN emigration is due to interaction of fg with ICAM-1 expressed on CHO cells.

We have investigated which receptor on PMN was implicated in this interaction. Although anti-LFA-1 mAb did not have any effect on PMN transmigration across CHO ICAM-1 monolayers, OKM1, an anti-Mac-1 that specifically blocks fg-Mac-1 interaction, inhibited this extravasation by 62% (data not shown). These results demonstrate that fg interacts with ICAM-1 on CHO monolayers and Mac-1 on PMN.

Because we wanted to understand the mechanism that regulates ICAM-1-mediated transmigration, we evaluated the implication of ICAM-1 in a signaling cascade.

Fg-dependent transmigration requires the presence of ICAM-1 cytoplasmic sequence

The ability of ICAM-1 to function as a signaling molecule has been demonstrated in previous studies. In brain EC, ICAM-1 cross-linking leads to tyrosine phosphorylation of cytoskeleton proteins and enhanced lymphocyte migration across brain EC (22, 23). To assess whether ICAM-1 might be implicated in a signaling cascade in our system, we have produced a recombinant cell line expressing ICAM-1 without its cytoplasmic portion (CHO ICAM-1 Δcyt). Expression level of this mutated protein was comparable to native ICAM-1 expressed in CHO ICAM-1 (Fig. 7A). Furthermore, this mutation did not have any effect on ICAM-1 localization on the cell (Fig. 7B) since we found the protein expressed uniformly on the whole cell surface.

We have investigated PMN adhesion and transmigration across CHO cells expressing ICAM-1 with or without its cytoplasmic portion. Results in Fig. 8 clearly show that ICAM-1 expressed on CHO cells are able to support PMN adhesion in the presence of purified fg, with a 2-fold increase of adherent cells in comparison to WT CHO (63.3 ± 4.8% and 30 ± 3.3%, respectively). Furthermore, the absence of fg led to a decrease in adherent PMN, whereas no effect was observed on WT CHO monolayers. Interestingly, CHO ICAM-1 Δcyt are able to support PMN adhesion in the presence of fg (62 ± 2.7%) to the same level as that of native protein (Fig. 8A), while ICAM-1-dependent adhesion was inhibited in the absence of fg (40.2 ± 5.3%). Surprisingly, PMN transmigration across CHO ICAM-1 Δcyt monolayers was totally abolished in the presence or absence of fg (2.3 ± 3.1% vs 0.2 ± 2.3%, respectively) (Fig. 8B). This defect in PMN transmigration is not due to an impaired interaction between fg and ICAM-1 Δcyt protein. First, adhesion of PMN is enhanced in the presence of purified fg, as it does on CHO ICAM-1 monolayers. Second, using

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Effect of anti-ICAM-1 2D5 and 3D6 mAbs on PMN migration across CHO monolayers expressing ICAM-1 or PECAM-1. The experiment was performed in the presence of complete plasma; in some cases, 2D5 or 3D6 mAbs were added to PMN and CHO cells in the presence of complete plasma. After a 10-min incubation period, mAbs added to CHO monolayers were washed out and PMN incubated 5 min with 2D5 or 3D6 were added to CHO cells without removing the mAbs. Transmigration assay was conducted as described in Materials and Methods. Data represent mean ± SD of three separate experiments.

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** Effect of anti-ICAM-1 2D5 or 3D6 mAbs on PMN transmigration across CHO monolayers expressing ICAM-1. The experiment was performed in the presence of complete plasma either in the absence of 2D5 or with 2D5 incubated on PMN and CHO cells in combination or alone. 3D6 mAb was incubated on CHO cells alone. In each condition, mAbs were removed from monolayers or PMN by extensive washes. Percentage obtained with WT CHO was subtracted for all of the experiments. Transmigration assay was performed as described in Materials and Methods. Data shown represent mean ± SD of three separate experiments.
anti-lg polyclonal Ab followed by an immunofluorescence experiment, we have demonstrated that fg was able to interact with WT ICAM-1 and mutated ICAM-1 (data not shown).

We have investigated whether this inhibition in transmigration resulted from a defect in a signaling event which in the WT ICAM-1 could be induced by interaction of ICAM-1 cytoplasmic domain with an intracellular protein. It has been shown that ICAM-1 can associate via its cytoplasmic domain with a-actinin, an actin-binding cytoskeletal protein (24). The small GTP-binding protein Rho is a key mediator of actin cytoskeletal remodeling induced by intracellular signals (25). The observation that ICAM-1 can associate with components of the actin cytoskeleton suggested that Rho protein could be involved in regulating adhesion and transmigration mediated by this receptor. Furthermore, endothelial ICAM-1 has been implicated recently in lymphocyte migration across brain EC via a Rho-dependent pathway (23). Since deletion of the ICAM-1 cytoplasmic sequence dramatically inhibited PMN transmigration, the potential role of Rho was assessed in our PMN transmigration assay. CHO cell monolayers were treated with the bacterial toxin C3 transferase, which is capable of entering the cell surface, and the ability to form cohesive and confluent monolayers. In these recombinant cell lines, we obtained expression levels similar to those found in EC, with an ICAM-1 expression level comparable to TNF-α-stimulated HUVEC. As revealed by immunofluorescence experiments, ICAM-1 is localized uniformly at the cell surface, whereas PECAM-1 is concentrated at the cell-cell contacts of these recombinant cells; these findings are in agreement with ICAM-1 and PECAM-1 localization on EC and on recombinant cells described in other studies (31–34).

The results obtained with our model of CHO cells expressing ICAM-1 alone indicate that in addition to its well-known function in leukocyte adhesion, ICAM-1 plays a direct role in the migration of these cells across the junction. This enhanced transmigration mediated by ICAM-1 could be the end result of the increase in adhesion, with other molecules expressed at the cell junctions being responsible for the transmigration. Our results indicate that this is not the case. First, if the above hypothesis was correct, CHO ICAM-1 would have an increase in leukocyte adhesion, with no modification of the transmigration level, while CHO ICAM-1/PECAM-1 would be expected to show an even higher increase in transmigration. However, transmigration across CHO ICAM-1/PECAM-1 or ICAM-1 monolayers was very similar, suggesting that in our system, ICAM-1 participates extensively in PMN transmigration, independently of PECAM-1. This is supported by the fact that CD31-deficient mice exhibit leukocyte transendothelial migration to the same degree as WT mice (35), thus suggesting the existence of a CD31-independent pathway to sustain leukocyte migration. Second, results obtained with cells expressing ICAM-1Δcyt clearly demonstrate that adhesion can be dissociated from transmigration. Cells transfected with this mutant protein are able to support adhesion, to a level similar to the one obtained with WT ICAM-1, but can no longer support PMN transmigration.

One surprising result was that there was no additive effect of ICAM-1 and PECAM-1 in our model of recombinant CHO cells.
One possibility is that ICAM-1 is already stimulating transmigration to its maximum and that the addition of PECAM-1 produces no further effect. This is supported by the fact that in our model, ICAM-1-mediated transmigration reached the same level as PMN transmigration across CHO PECAM-1 monolayers.

All of the above experiments were done in the presence of autologous plasma to overcome the problem of neutrophil viability in the absence of protein. As we have previously shown that fg-ICAM-1 interactions enhance leukocyte transmigration across resting HUVEC monolayers (12), we have performed experiments to address the question of the importance of fg-ICAM-1 interactions using our recombinant model. Replacement of normal plasma with fg-depleted plasma led to a decrease of the observed transmigration to control level, demonstrating the involvement of fg-ICAM-1 interaction in neutrophil transmigration. Addition of purified fg to fg-depleted plasma counteracted the inhibition due to the depletion of fg. These data clearly demonstrate the fundamental implication of fg in ICAM-1-mediated neutrophil transmigration. In contrast, fg depletion has no effect on PMN transmigration across WT CHO or CHO PECAM-1 monolayers.

To further confirm the implication of fg-ICAM-1 interaction in PMN transmigration, we used anti-ICAM-1 mAbs. 2D5, which interacts with domain 1 and inhibits fg-ICAM-1 recognition (12), had no effect on PECAM-1-induced transmigration but completely suppressed PMN migration across CHO ICAM-1. Interestingly, this 2D5 inhibitory effect was observed when CHO monolayers were preincubated with the mAb, but not when PMN alone were preincubated. These results indicate that the enhanced PMN transmigration is directly mediated by ICAM-1 expressed on CHO cells. Using anti-Mac-1 Abs, we conclude that on PMN, fg interacts with the β₂ integrin Mac-1, as previously demonstrated by Altieri et al. (9).

The previous data are consistent with the hypothesis of an intracellular signal in the cell monolayer. The mechanism by which fg-ICAM-1 interaction leads to the increase of transmigrated neutrophils remains to be determined. Several mechanisms can explain this marked increase. Interaction between endothelial ICAM-1 and a ligand expressed on leukocytes or a soluble ligand, like fg, could induce an intracellular signaling cascade leading to the opening of intercellular junctions associated with leukocyte transmigration. Indeed, Hicks et al. (36) have shown that fg, via its binding to ICAM-1, initiates signaling pathways leading to the synthesis of vasoactive mediators. ICAM-1 has also been implicated in signal transduction mechanism involving tyrosine phosphorylation of cytoskeleton-associated proteins (22, 37), resulting in enhanced lymphocyte migration across brain EC monolayers (23). To test the hypothesis of an intracellular signaling pathway, we investigated the implication of ICAM-1 cytoplasmic domain. Interestingly, in the presence or absence of fg, ICAM-1 Δcyt protein supports PMN adhesion, whereas ICAM-1-mediated PMN transmigration is totally abolished.

This result is consistent with a role for ICAM-1 in intracellular signaling events which may lead to facilitated PMN infiltration into tissues. Since the Rho signaling pathway was previously implicated in lymphocyte migration across brain EC (23), we investigated its implication in our model of PMN transmigration by pretreatment of CHO monolayers with C3 transferase, a specific inhibitor of Rho proteins. PMN adhesion is not affected by C3, whereas ICAM-1-mediated transmigration is totally abolished. These results demonstrate that ICAM-1 is implicated in a signaling...
cascade involving Rho molecules expressed in CHO cells. As previously reported (38), Rho itself may be activated through cell surface signals propagated through the actin cytoskeleton. A possible mechanism is that ICAM-1-fg interactions induce strong adhesion of PMN on cell monolayers which led to intracellular signals involving ICAM-1 cytoplasmic domain and Rho proteins. One hypothesis to explain the observed effect of fg-ICAM-1 interaction on PMN transmigration is that the subsequent activation of Rho might induce some modifications of junctional proteins which may facilitate PMN infiltration.

Another likely possibility is that neutrophils may migrate more easily on the surface of EC using fg-ICAM-1 interactions, leading to an increase in the number of leukocytes reaching the intercellular junctions. This hypothesis is supported by the demonstration that VCAM-1 was able to increase monocyte transendothelial chemotaxis (4) by facilitating lateral migration of monocytes on EC. We are currently investigating this possibility.

Taken together, these results demonstrate the implication of ICAM-1 in neutrophil transmigration via its interaction with fg. It is the first time that the role of ICAM-1 by itself is directly demonstrated in the process of neutrophil transmigration, without the interfering presence of other adhesion molecules expressed by EC. In addition, we demonstrate that this effect is mediated through the ICAM-1 cytoplasmic domain and the activation of Rho.

Further studies are now in progress to elucidate the mechanisms underlying this phenomenon. In addition, this recombinant CHO model will be useful to precisely determine which amino acids in the ICAM-1 sequence are important in fg-dependent transmigration.

Acknowledgments

We thank Nathalie Bertacchi for technical assistance.

References