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T Lymphocyte Adhesion to Human Brain Pericytes Is Mediated Via Very Late Antigen-4/Vascular Cell Adhesion Molecule-1 Interactions

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T cell adhesion to the brain microvascular endothelium and subsequent migration into the brain parenchyma is one of the major events in the development of multiple sclerosis (MS). Interactions of the T cell integrin very late antigen-4 (VLA-4) with its receptor, vascular cell adhesion molecule-1 (VCAM-1) have been described to be of crucial importance for the development of MS. We investigated the expression of these adhesion molecules in MS brain tissue by immunohistochemical analysis, and studied their functional involvement in an in vitro T cell adhesion assay. A number of other adhesion molecules were studied for comparison. In cryosections of several MS brains, expression of VCAM-1 was demonstrated not only on the endothelium of vessels surrounding MS plaques, but also in perivascular positions, suggesting expression by pericytes. T cells adhered to both cell types in vitro. Both LFA-1/intercellular adhesion molecule-1 and VLA-4/VCAM-1 interactions were equally involved in the adhesion of T cells to TNF-α-stimulated endothelial cells. However, adhesion of T cells to TNF-α-stimulated pericytes was clearly dominated by VLA-4/VCAM-1 interactions. These results indicate that pericytes, next to endothelial cells, may play an important role in regulating T cell infiltration into the central nervous system. The Journal of Immunology, 1995, 154: 5876–5884.

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Abbreviations used in this paper: MS, multiple sclerosis; VLA-4, very late antigen-4; BBB, blood-brain barrier; EAE, experimental allergic encephalitis; HBP, human brain pericytes; ICAM-1, intercellular adhesion molecule-1; NCS, newborn calf serum; VCAM-1, vascular cell adhesion molecule-1.

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T cell adhesion to cultures of endothelial cells is mediated through the interaction of both the LFA-1 with its receptor, intercellular adhesion molecule-1 (ICAM-1) and through VLA-4/VCAM-1 interactions, whereas in T cell adhesion to pericytes, isolated from human brain microvessels, VLA-4/VCAM-1 was the predominant pathway. These results indicate that pericytes, next to endothelial cells, may play an important role in regulating or guiding T cell infiltration into the brain.

Materials and Methods

Immunohistochemical analysis

Acetone-fixed cryosections (6 μm) from frozen tissue on aminopropyltriethoxysilane-coated glass slides were stained indirectly with the indicated primary mAbs (see Table I) by using peroxidase-labeled rabbit anti-mouse Abs (Dako, Glostrup, Denmark) as secondary Abs, or, alternatively, by using the ABC detection kit (Vector, Burlingame, CA) according to the manufacturer’s description, and the chromogen diaminobenzidine, essentially as described previously (30, 31). Incubation with the polyclonal anti-collagen IV Ab was followed by successive incubations with swine anti-rabbit Abs (Dako) and the peroxidase-anti-peroxidase complex (Dako). Sections were counter-stained with hematoxylin. Tissue samples of white matter from four different MS brains (age 41 to 63 years, postmortem delay: 4.5 to 7.5 h) and Alzheimer brains were obtained from the Netherlands Brain Bank, Amsterdam (Coordinator, Dr. R. Ravid).

Pericytes and endothelial cells

Human brain pericytes (HBP) were isolated and characterized as described previously (33). Brain tissue material used for the isolation procedure was obtained after a rapid autopsy procedure (postmortem delay: between 2 and 6 h) from the following cases: four cases of senile dementia of the Alzheimer type (age 81 to 94); one case of a cerebrovascular accident (age 85); one case of an unidentified metabolic disorder (age 49), and one neuropathologically unaffected case (age 81). Endothelial cells (HUVEC) were isolated from human umbilical cord vein after collagenase treatment essentially as described by Jaffe (32). HBP and HUVEC were subcultured at 37°C and 5% CO2 in Eagle's MEM supplemented with 10% heat-inactivated human serum, 20% newborn calf serum (NCS; Life Technologies, Inc., Paisley, Scotland), 2 mM glutamine (Flow Laboratories, Richmansworth, UK), 150 μg/ml endothelial cell growth factor (isolated from calf brain) (33), 5 U/ml heparin (Organon Teknika, Boxtel, The Netherlands), and 40 μg/ml gentamicin (Schering-Plough, Amstelveen, The Netherlands) in fibronectin-coated tissue culture flasks (Costar, Cambridge, MA) (29, 30). Cells were harvested from culture flasks by using a solution of 0.125% Trypsin (Difco, Detroit, MI) and 0.2% EDTA in PBS. Passages 6 to 12 (HBP) or 3 to 9 (HUVEC) were used, and no effect of passage number was observed in our experiments.

T cells

Leukocyte-enriched cell suspensions were obtained by cytophoresis of healthy volunteers as described previously (34). PBMC were isolated by centrifugation on Ficoll (density 1.077 g/ml, Pharmacia, Uppsala, Sweden), 2 ml glutamine (Flow Laboratories, Richmond, UK), 150 μg/ml endothelial cell growth factor (isolated from calf brain) (33), 5 U/ml heparin (Organon Teknika, Boxtel, The Netherlands), and 40 μg/ml gentamicin (Schering-Plough, Amstelveen, The Netherlands) in fibronectin-coated tissue culture flasks (Costar, Cambridge, MA) (29, 30). Cells were harvested from culture flasks by using a solution of 0.125% Trypsin (Difco, Detroit, MI) and 0.2% EDTA in PBS. Passages 6 to 12 (HBP) or 3 to 9 (HUVEC) were used, and no effect of passage number was observed in our experiments.

Flow cytometry analysis

HBP were treated with TNF-α (50 ng/ml) or IFN-γ (200 U/ml) (kind gifts of Boehringer Ingelheim, Ingelheim, Germany) for 48 to 72 h where indicated. The cells were detached from culture flasks by treatment with 0.02% EDTA and subsequent scraping. The cells were washed with PBS containing 1% BSA and 0.1% NaN3 (washing buffer) and incubated in suspension with Abs at 106 cells per incubation. The mAbs used in this study and their origin are listed in Table I. After removing unbound Abs with washing buffer, cells were incubated with FITC-conjugated sheep
anti-mouse F(ab')2 fragments (Cappel). Finally, the cells were fixed in 1% paraformaldehyde and analyzed with a flow cytometer (Coulter Corporation, Hialeah, FL).

**T cell adhesion assay**

HBP and HUVEC were grown to confluence on gelatin-coated 96-well culture plates and were treated with TNF-α (50 ng/ml) for 28 to 48 h as indicated. The cells were fixed with 0.025% glutaraldehyde to prevent cell detachment, washed three times with PBS, and stored at −20°C until usage. T cells were thawed and washed in Eagle’s MEM before incubation at 37°C for another 30 min. After the adhesion period, the concentration of Mn++ was chosen on the basis of a previous study (35). Unstimulated HBP expressed HLA class I, ICAM-1, and VLA-4, as demonstrated by the presence of CD2-positive T cells around blood vessels at the edge of the plaques (Fig. 1a). Of these T cells, the majority expressed CD8 (Fig. 1b) rather than CD4 (not shown). Also, VLA-4-expressing T cells (Fig. 1c) were more abundant than LFA-1-positive T cells (not shown). VCAM-1 expression was found in a number of vessels surrounding the plaques, also extending into nearby grey matter. Careful examination of this vascular staining revealed not only that the endothelium was stained, but also that perivascular staining could be observed, indicating expression by pericytes (Fig. 1d). As expected, the VCAM-1-positive pericytes were enveloped by a basement membrane, visualized by collagen IV staining, as demonstrated by serial section analysis (Fig. 2, a and b). In contrast, in brains of control cases or in unaffected areas of MS brains, VCAM-1 staining was absent. ICAM-1 and, to a lesser extent, LFA-3 were expressed at high levels all over the MS plaques. Vessels within or surrounding the MS plaques were only moderately stained for either Ag (not shown). E-selectin staining was absent in all cases.

**Expression of membrane Ags on HBP**

Before studying the interactions between T cells and HUVEC or HBP, we examined the expression of several adhesion molecules on HBP by flow cytometry analysis (Fig. 3). Flow cytometry analysis of these membrane molecules by HUVEC has been described elsewhere (29, 36, 37). Unstimulated HBP expressed HLA class I, ICAM-1,
FIGURE 2. Immunohistochemical staining of serial MS brain cryosections (white matter). Staining of pericytes for VCAM-1 (a, arrows) and of basement membrane for collagen IV (b). Note the strong staining of the inner basement membrane for collagen IV (b, arrowheads) and weaker staining of the outer basement membrane, enclosing the pericyte cell body (b, arrows). Magnifications: ×330.

VCAM-1, and the TGF-β-binding molecule endoglin (CD105). Treatment of HBP with TNF-α resulted in an increased expression of HLA class I, ICAM-1, and VCAM-1, but had a slightly negative effect on endoglin expression. Similar treatment with IFN-γ induced HLA class II expression by HBP, and increased HLA class I and ICAM-1 expression, whereas VCAM-1 and endoglin expression remained unaffected. LFA-3, CD44, and the high m.w.-melanoma-associated Ag (a staining marker for HBP) were constitutively expressed by HBP, and remained unaffected by cytokine treatment (not shown). Furthermore, HBP did not express ICAM-2, E-selectin, or B7/BB1, and expression of these molecules was not induced by exposure to cytokines (not shown).

Adhesion of T cells to confluent cultures of HBP and HUVEC

TNF-α-stimulated HBP expressed high levels of various adhesion molecules that are known to mediate interactions between T cells and endothelial cells. We compared the capacity of T cells to adhere to HBP or HUVEC. A small number of T cells (3 to 4%) adhered to unstimulated HBP or HUVEC (Fig. 4). Stimulation of HBP or HUVEC with TNF-α resulted in a threefold increase of T cell adhesion. Activation of T cells had a more profound effect on adhesion, however. Treatment with the phorbol ester PMA resulted in a three- to fourfold increase of T cell adhesion, and applying both PMA and TNF-α resulted in an 8- to 10-fold increase in T cell adhesion. T cell adhesion to either cell type was strongly augmented when T cells were treated with the divalent cation Mn²⁺ which, upon binding, results in the activation of T cell integrins (35, 38). Of the Mn²⁺-stimulated T cells, 25% or 35% adhered to unstimulated HUVEC or HBP, respectively (eightfold increase). Moreover, 60% of these cells adhered to TNF-α-stimulated HUVEC or HBP (20-fold increase; see Fig. 4).

The effect of Mn²⁺ treatment seemed to be time dependent

T cell adhesion to TNF-α-treated or untreated HBP or HUVEC increased sharply between 0 and 15 min, reached maximal values between 15 and 45 min, and slowly declined again (not shown). On the basis of these results, additional experiments were performed with T cells treated with Mn²⁺ for 15 to 20 min.

VLA-4/VCAM-1 interactions in the adhesion of T cells to HBP

The most striking difference in expression of adhesion molecules between HBP and HUVEC was the basal expression of VCAM-1 on the former cell type. This prompted us to investigate whether T cell adhesion to HUVEC or HBP could be related to specific interactions between adhesion molecules on either cell type. Adhesion of Mn²⁺-stimulated T cells to TNF-α-treated HBP or HUVEC could be partially inhibited by mAbs against the α- or β-chain of LFA-1 and the α-chain of VLA-4, whereas an anti-VLA-4β mAb only had a minor effect (Fig. 5A). Moreover, the contribution of LFA-1 in T cell
adhesion to HUVEC seemed to be more important than in T cell adhesion to HBP. Also, the contribution of the LFA-1 receptor ICAM-1 seemed to be higher in T cell adhesion to HUVEC than to HBP (Fig. 5B), whereas VCAM-1 seemed to play an equal role in T cell adhesion to either cell type. Neither ICAM-2 nor LFA-3 seemed to be involved in T cell adhesion to HUVEC or HBP. Conversely, by using mAbs directed against the counterstructure of LFA-3, i.e., CD2, a small but insignificant inhibitory effect was observed in T cell adhesion to HUVEC (Fig. 5A) only.

The use of combinations of mAbs directed against couples of adhesion molecules revealed that VLA-4/VCAM-1 interactions formed the major route for T cell adhesion to HBP, whereas LFA-1/ICAM-1 and VLA-4/VCAM-1 interactions contributed equally to the adhesion of T cells to HUVEC (Fig. 5C). CD2/LFA-3 interactions were marginally involved in T cell adhesion to HUVEC, but not to HBP. When combinations of anti-ICAM-1 and/or anti-LFA-1-α and -β mAbs were applied in the inhibition assays, an unexpected effect on the adhesion of T cells to HBP or HUVEC was observed, compared with anti-LFA-1-α mAbs alone (Fig. 5, A and C). The inhibitory effect was reduced from 36% to 8% (HBP) and from 58% to 45% (HUVEC), which remains unexplained.

By using a combination of all our mAbs against T cell adhesion molecules, the adhesion to HBP or HUVEC could be blocked for 70% or 80%, respectively. Inhibition was 63% and 84%, respectively, when a mixture of anti-HBP or anti-HUVEC mAbs was applied. Combining these mAb mixtures resulted in a 85% inhibition of the adhesion to either cell type (Fig. 5D).

Similar to the adhesion of T cells to TNF-α-treated HBP or HUVEC, we observed a tendency toward the involvement of VLA-4/VCAM-1 interactions in Mn++-stimulated T cell adhesion to unstimulated HBP, but these experiments did not result in significant differences in adhesion values, because of the low numbers of T cells that adhered to unstimulated HBP or HUVEC (results not shown).

### Discussion

Extravasation of T cells into the brain parenchyma requires interaction of T cell adhesion molecules with their counterstructures expressed by the cells constituting the brain microvasculature. Endothelial cells are the main constituents of the BBB, but pericytes may play a complementary role in its maintenance (11-14). After initial close interaction with the endothelial cells, T cells have to migrate along pericytes for subsequent migration into the brain parenchyma. Although the interactions between T cells and perivascular cells are hardly studied, they may be crucial to the process of T cell infiltration into the central nervous system. Therefore, we analyzed the interactions of T cells with pericytes and compared these with endothelial cells. In a previous report we described the isolation and characterization of pericytes from human cerebral microvessels and showed that these cells expressed high levels of the adhesion molecule ICAM-1 and, to a lesser extent, VCAM-1 (30). In this report we show that the expression of either adhesion molecule is sensitive to cytokine treatment, in a way similar to that described previously for HUVEC (39-41).

In an in vitro adhesion assay, we studied the interactions of T cells with HUVEC and HBP to identify the adhesion molecules essential for these interactions. Because we did not succeed in growing sufficient numbers of endothelial cells from human cerebral microvessels, we chose to study HUVEC for the interaction with T cells. This choice seems to be justified by the observation that HUVEC and cerebral endothelial cells respond similarly to cytokine treatment with regard to the expression of adhesion molecules (41) and that these latter cells in vitro rapidly lose their specific properties. T cell adhesion to either HUVEC or HBP was augmented after treatment with Mn++, PMA, or TNF-α, which is in line with previous reports (35, 38, 40, 42, 43). It has been suggested that by this in vitro treatment T cell integrins assume the activated conformation required for their in vivo high affinity binding to their counterstructures. The adhesion of T cells to HUVEC was mediated by both the LFA-1/ICAM-1 and VLA-4/VCAM-1 pathways,
FIGURE 5. The effect of inhibitory mAbs in Mn^{+ +}-stimulated T cell adhesion to TNF-α-treated HBP (black bars) or HUVEC (shaded bars). A: The effect of anti-T cell mAbs. Control mAbs (WT1, WT31, not shown) slightly inhibited T cell adhesion to HBP (9%) or inhibited adhesion to HUVEC (4%). B: The inhibitory effect of anti-HBP/HUVEC mAbs. Control mAbs were PN-E2 and 2A7. C: The inhibitory effect of combinations of anti-T-cell and anti-HBP/HUVEC mAbs. D: The inhibitory effect of a combination of anti-T cell mAbs (directed against LFA-1, VLA-4, and CD2), a combination of anti-HUVEC/HBP mAbs (directed against ICAM-1, ICAM-2, LFA-3, and VCAM-1), and a mixture of all these mAbs. The number of experiments is indicated beside each bar. The mean ± SD of these measurements is shown. Statistical analysis was performed as described in the legend of Figure 4. In D, all p values were <0.001.

whereas, in contrast, adhesion of T cells to HBP was dominated by the VLA-4/VCAM-1 route.

A possible role for the T cell integrin α4β7 (44, 45) in the interaction with VCAM-1 could not be excluded, however, as anti-VLA-4β (β1) mAbs only had a partial inhibiting effect compared with the effect of anti-VLA-4α and anti-VCAM-1 mAbs. It is unlikely that the absence of inhibition by 4B4 is caused by the Mn^{+ +} treatment, as it has been described that the interaction of β1 integrins, expressed by melanoma cells, with extracellular matrix components is completely inhibited by this mAb both in resting (46) and Mn^{+ +}-treated cells (E. H. J. Danen, personal communication). Furthermore, it is clear from our findings that the anti-LFA-1 and anti-VLA-4α mAbs also inhibit the interaction of Mn^{+ +}-activated integrins with their respective ligands. The adhesion of T cells to HBP or HUVEC could be maximally blocked only for 80 to 85% by using mixtures of mAbs in the inhibition assays. These results suggest that the α4β7 integrin, or other presently unknown adhesion molecules, may play a role as well in the interaction of T cells with HUVEC and HBP.
It has been described that the adhesion of T lymphocytes to HUVEC is dependent on the activation state of LFA-1 (42). T cell adhesion via VLA-4/VCAM-1 would only take place when LFA-1 is not available, or not in the activated conformation, to mediate adhesion. Here we show, however, that two different cell types (HBP and HUVEC), with similar expression levels of ICAM-1 and VCAM-1 after TNF-α treatment, interact differentially with T cells. These results suggest that T cell adhesion is not only regulated at the level of T cell integrins, but that HUVEC and HBP may also possess mechanisms to regulate the functionality of their adhesion molecules.

Our results confirm previous reports that both the LFA-1/ICAM-1 and VLA-4/VCAM-1 pathways are involved in T cell binding to HUVEC (25, 37, 42, 47). The migration of T cells through the endothelial cell layer, however, is mainly dependent on the LFA-1/ICAM-1 pathway (47). An alternative mechanism seems to operate once T cells have escaped from the blood flow and passed the endothelial cell layer. Our results suggest that the VLA-4/VCAM-1 pathway then predominantly mediates further migration of T cells along pericytes into the brain parenchyma.

A number of studies have presented evidence for the involvement of adhesion molecules during T cell infiltration into the brain, resulting in the development of MS. Although ICAM-1 expression is up-regulated in MS microvessels (48, 49), treatment of EAE with anti-ICAM-1 Abs has been reported to yield controversial results (50–52). These studies are compatible with our findings that the LFA-1/ICAM-1 pathway only partially mediated the binding of T cells to HUVEC. Thus, in EAE animals treated with anti-LFA-1 or anti-ICAM-1 Abs, T cells could still extravasate by using the VLA-4/VCAM-1 pathway.

Indeed, unambiguous evidence has been provided for an important role of this latter pathway in T cell infiltration into the MS or EAE brain. VCAM-1 is up-regulated in the vasculature of mice affected by EAE (7) and in MS microvessels (8, 49), and the expression of its receptor VLA-4 by T cells has been described to be essential for T cell invasion into the brain (9, 10). Furthermore, EAE development could be prevented by anti-VLA-4a Abs (6). Our data are compatible with this inhibitory effect, indicating that under these conditions T cells are still able to pass the endothelial cell barrier, but that subsequent migration of the T cells through the pericyte layer is blocked by the anti-α4 Abs. The possible involvement of VLA-4/VCAM-1 interactions in MS development was supported by our immunohistochemical demonstration of VCAM-1 in endothelial cells and in perivascular positions, possibly pericytes, at the edges of MS plaques. VCAM-1 expression could not be demonstrated in all plaques, but it indicates that VCAM-1 can be up-regulated under certain conditions in MS. For example, it is well known that VCAM-1 expression can be up-regulated by cytokines like TNF-α and IL-1 (36, 37), which may be produced especially during the active phases of MS plaque development (2). Furthermore, perivascular T cells expressed VLA-4 rather than LFA-1, emphasizing the relative importance of VLA-4-mediated interactions in the extravasation processes.

In summary, the results described in this study indicate that lymphocytes leaving the circulation to migrate into the brain may be actively guided to their goal or, alternatively, be retained in a perivascular position by interacting differentially with endothelial cells and pericytes. In accordance with earlier studies, an important role for VCAM-1 in the interaction of T cells with the brain vasculature was demonstrated (6, 7), but in addition we showed that these VCAM-1/VLA-4 interactions are exclusive at the level of the pericyte. A more detailed knowledge of these control mechanisms may be important in developing therapeutic strategies aimed at inhibition of T cell infiltration in MS brain tissue.

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