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Lymphocyte adhesion and transendothelial migration in the central nervous system: the role of LFA-1, ICAM-1, VLA-4 and VCAM-1

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SUMMARY

Lymphocyte adhesion to and migration across endothelial cell (EC) monolayers, derived from the rat blood–retinal barrier (BRB), were measured *in vitro*. The binding of concanavalin A (Con A)-activated peripheral lymph node lymphocytes and the migration of $CD4^+$ T-cell lines could be significantly increased by treating the EC with interleukin-1β (IL-1β). To determine the role of various adhesion molecules during the processes of lymphocyte binding and transmonolayer migration (diapedesis), lymphocytes were treated with monoclonal antibody (mAb) specific for CD11a (α_L subunit of leucocyte functional antigen-1; LFA-1), CD18 (β_2 subunit of leucam family) and CD49d (α4 subunit of very late activation antigen-4; VLA-4) and EC with mAb specific for CD54 (intercellular adhesion molecule-1; ICAM-1) and CD106 (vascular cell adhesion molecule-1; VCAM-1). Binding of the highly adhesive but non-migratory Con A-activated lymphocytes was inhibited by mAb to CD11a (reduced to 73% and 65% of control lymphocyte adhesion) and CD18 (42% and 54%) on non-activated and IL-1β-treated EC, respectively, but not by mAb to ICAM-1 or VCAM-1. Diapedesis of the highly migratory T-cell line lymphocytes was also blocked by antibodies to CD11a (reduced to 11% and 10% of control T-cell migration), CD18 (29% and 43%) but in addition was also inhibited by anti-ICAM-1 (17% and 53%) on non-activated and IL-1β treated EC, respectively. Both anti-VLA-4 and anti-VCAM-1 were also effective in producing a smaller reduction in migration, but only on IL-1β activated EC (66% and 58% of control migration, respectively). These studies indicate that lymphocyte adhesion to central nervous system (CNS) vascular EC is largely dependent on LFA-1 but not through its interaction with ICAM-1. In contrast, lymphocyte diapedesis is mostly supported through the LFA-1/ICAM-1 pairing, with a small proportion being mediated by VLA-4/VCAM-1 on IL-1β-activated EC. This latter pathway, however, also appears to be dependent on LFA-1 interacting with the EC.

INTRODUCTION

Recruitment of leucocytes from the circulation is a fundamental property of vascular endothelia and is one of the central elements in the development and resolution of an inflammatory lesion. The underlying mechanisms involved in the capture of leucocytes from the blood and their subsequent migration through the vessel wall has attracted substantial interest and has recently been reviewed by Springer.[1] Most data, however, have been derived from studies on either large vessel endothelia (e.g. human umbilical vein endothelia) or endothelia derived from non-central nervous system (CNS) tissue. These studies do not, therefore, address the unique situation within the CNS where the endothelia form tight junctions of high electrical resistance[2] and which have already been shown to be less adhesive for lymphocytes than other endothelia.[3–5] Although many properties of non-CNS endothelia may be assumed to operate in the CNS, clearly described functional and molecular differences between the endothelial cells (EC) of these vascular beds would indicate that extrapolation of data from one population to another could be misleading. For lymphocytes to extravasate into the brain or retina by crossing the blood–brain (BBB) and blood–retinal barriers (BRB), respectively, they are likely to utilize mechanisms that do not operate elsewhere. Thus, to enter the CNS they must either separate the tight endothelial junctions or migrate through the body of the EC. In recent years this latter means of diapedesis has gained considerable ultrastructural support, as lymphocytes appear to penetrate EC at a parajunctional site. This route of lymphocyte penetration has been proposed in experimental autoimmune encephalomyelitis (EAE) the experimental analogue of multiple sclerosis,[6,7] and in experimental autoimmune uveoretinitis (EAU), the animal model of posterior uveitis.[8]

Investigations into the role of adhesion molecules involved

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Abbreviations: CNS, central nervous system; EC, endothelial cell; ICAM-1/2, intercellular adhesion molecule-1/2; LFA-1, leucocyte functional antigen-1; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late activation antigen-4.

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in lymphocyte migration across non-CNS vasculature from both human[9–12] and non-human sources[13] have shown that leucocyte functional antigen-1 (LFA-1) is of fundamental importance in this process. The ligands to which it binds during the complex process of extravasation and the differential role of such molecules are less clearly understood. Although cytokine-induced increases in lymphocyte adhesion to endothelia closely correlates with an up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression, ICAM-1 only appears to be partly responsible for mediating adhesion.[14,15] Indeed, it is suggested that ICAM-1 plays a negligible role in adhesion[13] but is the major ligand in mediating migration.[16] In addition to LFA-1 there is also some evidence to suggest that under inflammatory conditions the interaction between very late activation antigen-4 (VLA-4) and vascular cell adhesion molecule-1 (VCAM-1) is also important in lymphocyte traffic across the vessel wall.[17,18] In the CNS, adhesion studies also indicate an involvement of LFA-1 and VLA-4,[19–23] but no detailed investigation into the regulation of migration has been reported.

In this study we have utilized antibody-blocking techniques to investigate the role of the adhesion molecules LFA-1, VLA-4, ICAM-1 and VCAM-1 in both the adhesive and migratory stages of lymphocyte transmigration across retinal EC monolayers. These results are compared with previous studies on endothelia from other vascular beds.

MATERIALS AND METHODS

Reagents

Purified recombinant interleukin-2 (IL-2) and collagenase/dispase was obtained from Boehringer Mannheim (Lewes, UK). Hank's balanced salt solution (HBSS) and plastic culture dishes were from Gibco (Paisley, UK). Lymphocytes were cultured in RPMI-1640 containing 10% fetal calf serum (FCS), 1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all Gibco) and 5×10^{-5} M 2-mercaptoethanol (Sigma, Poole, UK). EC were grown in Hams F-10 medium supplemented with 7·5 μg/ml endothelial cell growth supplement, 80 μg/ml heparin, 2 mM glutamine, 0·5 μg/ml vitamin C (all Sigma) and 100 U/ml penicillin/streptomycin, and 17·5% plasma-derived serum (Advanced Protein Products, Brockmoor, UK). Concanavalin A (Con A; type V), Percoll and collagen (type 1) were all from Sigma. Sodium azide-free monoclonal antibody (mAb) specific for rat CD11a (α_L subunit of LFA-1; WT1),[24] CD18 (β_2 subunit of leucam family; WT3)[24] and CD54 (ICAM-1; 1A29)[25] were obtained from Serotec (Oxford, UK). Anti-rat CD49d (α4 subunit of VLA-4; TA-2)[26] was purchased from AMS Biotechnology (UK) Ltd (Oxford, UK) and anti-rat CD106 (VCAM-1; 5F10)[27] was a generous gift from Dr R. Lobb (Biogen, Cambridge, MA). An identical isotype to an irrelevant antigen (human C3b inactivator; OX21) was a generous gift of Dr M. Puklavec (Oxford University, Oxford, UK). The anti-rat EC antibody RECA-1 was a generous gift of Dr A. M. Duijvestijn (University of Limburg, the Netherlands).

Endothelial cells

Specific pathogen-free female Lewis rats (50–120 g; Charles River Ltd, Margate, UK) were used throughout. Retinal microvessels were isolated and primary cultures of EC were grown to confluency according to a previously described method.[28] Briefly, retinas were removed from the eyes of young female Lewis rats and digested in collagenase/dispase for 1 hr. Microvessel fragments were isolated from contaminating single cells by density centrifugation, further enzymatic digestion and finally by separation on a 50% preformed Percoll gradient. The microvascular fragments were finally suspended in culture medium[28] and plated out onto collagen-coated (type 1) plastic culture plates. The EC were allowed to grow to near confluence over a 2–3 week period in 5% CO_2 at 37°, with the culture medium being replaced every 2–3 days. Primary cultures that have been fully characterized previously[28] were used throughout.

Retinal EC monolayers were used either as untreated cells or activated with IL-1β (5 U/ml) for 24 hr prior to the adhesion and migration assays. This dose and duration has been shown previously to produce maximal adhesion.[5]

Lymphocytes

Two separate populations of lymphocytes were used in these studies. Firstly, a mixed population of lymphocytes isolated from peripheral lymph nodes (PLN) of female Lewis rats[5] was used on the day of separation or following mitogen activation with Con A (5 μg/ml) for 18 hr. These cells have been previously shown to be inducible for adhesion to retinal endothelia[5] but not for migration through an EC monolayer.[29]

The second population of lymphocytes to be used comprised two terminally differentiated IL-2-dependent $CD4^+$ T-cell lines specifically recognizing retinal soluble antigen (SAg). The cell lines were between three and six passages and no functional differences were observed at each passage. These cells were established and maintained by weekly restimulations with SAg (10 μg/ml) using 50-fold irradiated autologous thymocytes as feeder cells,[30] and have been shown to be $CD4^+$, $CD45Rc^{low}$ and $CD25^+$.[31] Three days after antigen restimulation, dead feeder cells were removed by density centrifugation over metrizoate–Ficoll.[30] Cells were grown at 2×10^5/ml in the presence of purified recombinant IL-2 and restimulated with antigen prior to use. These T-cell line cells are highly migratory,[29] with maximal migration occurring between 3 and 5 days post antigen stimulation (G. Pryce, D. Male, I. Campbell & J. Greenwood, unpublished data).

Flow cytometry

A single-cell suspension of untreated or IL-1β-treated (24 hr with 5 U/ml IL-1) retinal EC was obtained by washing confluent monolayers three times with Mg^{2+}/Ca^{2+}-free HBSS containing 0·02% EDTA, followed by enzymatic dissociation with collagenase/dispase (0·1%) for 1 hr. The single cells prepared by this procedure exhibited >80% viability by trypan blue staining. To confirm the purity of the retinal EC they were stained with the anti-rat EC antibody RECA-1.[32] In addition the level of expression of ICAM-1 and VCAM-1 on both resting and IL-1β-activated EC was assessed. The cells were resuspended in phosphate-buffered saline (PBS) and 5×10^4 cells/vial were incubated for 1 hr with the antibodies 1A29 (anti-CD54), 5F10 (anti-CD106) or RECA-1. The cells were then incubated for a further 1 hr with fluorescence isothiocyanate–rat anti-mouse immunoglobulin (FITC-RAMIg) in the presence of 20% normal rat serum. After washing twice, cells were resuspended in PBS medium

and used for analysis by flow cytometry (FACScan; Becton Dickinson, Oxford, UK). Unstained cells were used to set the parameters, and cells stained with the FITC–RAMIg alone were used to set background control.

Expression of the adhesion molecules LFA-1 and VLA-4 on Con A-activated lymphocytes and T-cell line lymphocytes was also determined. Briefly, lymphocytes were washed and stained with the antibodies WT1 (anti-CD11a), WT3 (anti-CD18) and TA-2 (anti-CD49d) as described above.

Adhesion assay

The adhesion assay was carried out as previously reported.[4,5] Lymphocytes were washed twice in HBSS, centrifuged and the cell pellet labelled with 3 μCi $^{51}Cr/10^6$ cells for 90 min at 37°. After washing the cells twice with HBSS they were resuspended in RPMI-1640 medium containing 10% FCS. Endothelial monolayers grown on 96-well plates were prepared by removing the culture medium and washing the cells four times with HBSS. Lymphocytes at a concentration of 2×10^5 in 200 μl were then added to each well and incubated at 37° for 1·5 hr. For each assay, γ-emissions in quadruplicate blank wells were determined to provide a value for the total amount of radioactivity added per well, and to allow for calculating the specific activity of the cells. After the incubation period non-adherent cells were washed off with four separate washes from the four poles of the well with 37° HBSS, as previously described.[3] Adherent lymphocytes were lysed with 2% SDS, the lysate removed and γ-emissions counted, the number of counts per well being a measure of the number of adherent lymphocytes.

The results of the controls are expressed as the percentage of lymphocytes adhering to the EC monolayer as a proportion of the total number of lymphocytes added. All other experiments are expressed as a percentage of the control results. Results were obtained from a minimum of 12 separate wells. The results are expressed as the means and standard error of means (SEM) and significant differences between groups determined by Student's *t*-test.

Migration assay

Lymphocyte migration was assayed using time-lapse videomicroscopy, as reported previously.[29] Briefly, Con A-activated PLN cells or antigen-specific T cells (2×10^5 cells/ml/well) suspended in culture medium were added to retinal EC monolayers grown in 24-well plates. Five to 10 min prior to the 4 hr time-point of each coculture, a field of 200 μm^2 was randomly selected by inverse phase contrast microscopy and recorded for 10–15 min. After each series of recordings the tapes were replayed at 160× normal speed and analysed by enumerating the number of cells within the field that had migrated through the monolayer and were underneath between the culture dish and the underside of the endothelia. The migrated lymphocytes could be readily distinguished from those remaining on the surface by their distinctive morphology and refractive appearance.[29] The data were then expressed as the percentage of lymphocytes within the field that had migrated underneath the monolayer, and these were then expressed as a percentage of the controls run at the same time. Controls were run in parallel in each assay to overcome any possible minor variations in the migratory ability of the T-cell lines.

All values are given as means ± SEM and differences between groups were assessed using Students *t*-test.

Antibody blocking

Antibodies to rat CD54 (ICAM-1), rat CD106 (VCAM-1), rat CD49d (VLA-4), rat CD11a (LFA-1), rat CD18 (leucam) and to an irrelevant antigen (human C3b inactivator; OX21) were used. Treatment of lymphocytes was carried out by resuspending the cells in 100 μl PBS, to which was added the antibody. The cells were then kept at 4° for 1 hr, after which the cells were resuspended in the presence of the antibody in culture medium and added to the EC monolayer. EC were treated by washing the monolayer followed by removal of the medium and addition of the antibody. The culture plate was then kept at 4° for 1 hr prior to adding the lymphocytes in the presence of the antibody. For each assay untreated cells were used to give control values of adhesion and migration. The level of adhesion/migration following antibody treatment was then expressed as a percentage of the control levels of adhesion/migration.

RESULTS

Morphology

The retinal EC grew to form uniform monolayers of spindle-shaped cells, as described previously.[28] With the addition of IL-1β it was noted that concentrations of 10 U/ml and above caused a rapid (< 2 hr) separation of many of the junctions between the cells. As a result of this finding all adhesion and migration assays were carried out at concentrations of 5 U/ml of IL-1β, where this effect was not observed.

Flow cytometry

The purity of the dissociated primary cultures of retinal EC was confirmed using indirect immunofluorescence staining with the RECA-1 antibody. With flow cytometry it was found that the majority of dissociated EC (95%) were positive for the RECA-1 antigen (Fig. 1). The expression of the surface adhesion molecule ICAM-1 in primary cultures of retinal EC was also evaluated. In an untreated population of retinal EC, 60% were found to be positive for ICAM-1 but negative for VCAM-1. Following activation of the EC monolayer with 5 U/ml of IL-1β for 24 hr, the level of expression of ICAM-1 was significantly increased both in the percentage of positive cells (93%) and in the intensity of fluorescence, corresponding to an increase in the number of surface molecules expressed (Fig. 1). After 24 hr, of IL-1β activation there was also a significant induction of VCAM-1 expression, with 21% of the cells expressing this molecule.

The expression of CD11a, CD18 and CD49d on Con A-activated lymphocytes and SAg-specific T-cell lines are shown in Fig. 2. The majority of both Con A-activated lymphocytes and SAg-specific T cells expressed CD11a (98% and 100%, respectively), CD18 (97% and 100%, respectively) and CD49d (both 99%). Although the percentage of both Con A and SAg T-cell line lymphocytes expressing these molecules was of the order of 100%, the latter exhibited a 10-fold greater intensity of fluorescence staining for both CD11a and CD18.

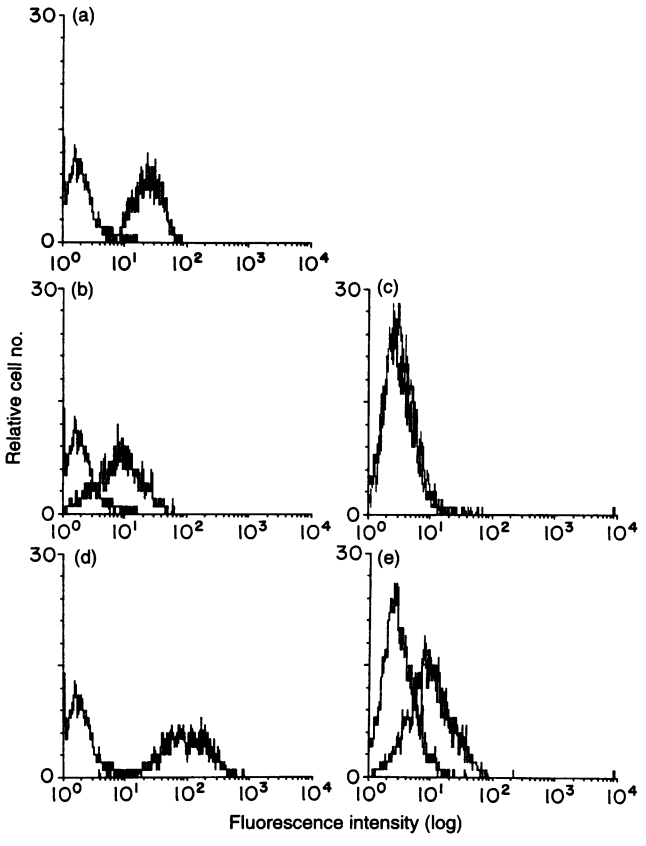

Figure 1. Representative plots from flow cytometric analysis of retinal EC surface expression of (a) RECA-1 antigen on non-activated EC; (b) ICAM-1 on non-activated EC; (c) VCAM-1 on non-activated EC (control and VCAM-1 histogram overlap); (d) ICAM-1 on IL-1β-activated EC; (e) VCAM-1 on IL-1β-activated EC. The controls (left-hand histograms) were set using FITC-labelled RAMIg.

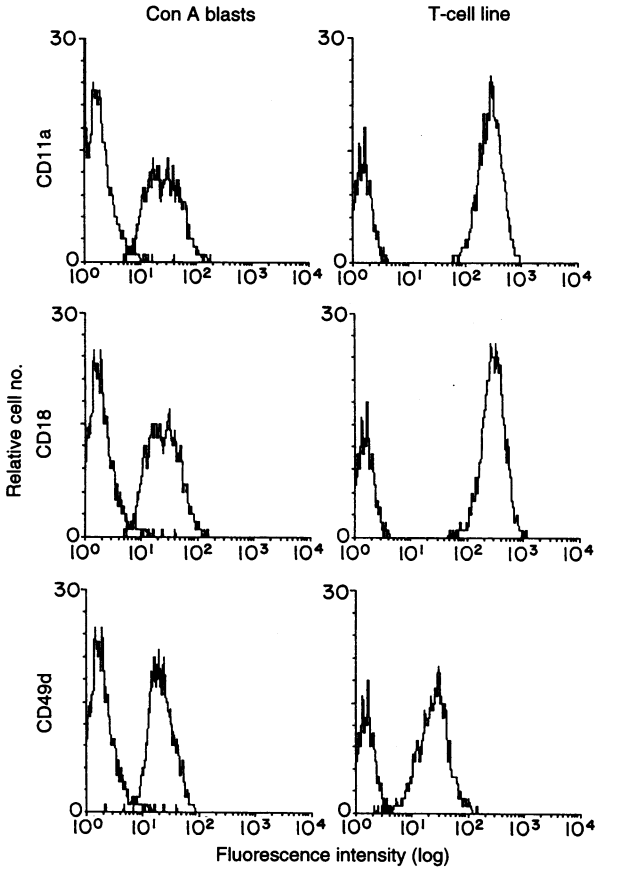

Figure 2. Representative plots from flow cytometric analysis of the surface expression of CD11a, CD18 and CD49d on both Con A blasts and T-cell line lymphocytes. The controls (left-hand histograms) were set using FITC-labelled RAMIg.

Adhesion of Con A-activated PLN cells

The percentage of Con A-activated PLN lymphocytes that adhered to IL-1β-activated retinal EC monolayers was significantly greater than to resting EC monolayers (Fig. 3; $P < 0{\cdot}0005$).

The level of adhesion, expressed as a percentage of control lymphocyte adhesion, following treatment with antibodies to various cell surface molecules is given in Fig. 4. Irrespective of whether the EC monolayer was treated with IL-1β or not, anti-CD54, anti-CD106 and anti-C3b inactivator failed to inhibit Con A blast adhesion. In comparison, antibodies to CD11a, CD18 and CD49d all caused a significant reduction in Con A-activated lymphocyte adhesion to retinal EC monolayers. Using a combination of both anti-CD11a and CD49d antibodies no further reduction in adhesion was observed. With each individual antibody treatment there was no difference in response between IL-1β activated and untreated EC monolayers.

When untreated EC monolayers were used the degree of inhibition of adhesion with anti-CD18 was significantly greater than with anti-CD11a ($P < 0{\cdot}005$). With IL-1β-treated EC, however, no difference in the ability to block adhesion was recorded.

Adhesion of T-cell lines

The percentage of T-cell line lymphocytes that adhered to IL-1β-activated retinal EC monolayers was significantly greater than to non-activated EC (Fig. 3; $P < 0{\cdot}005$). Furthermore, these antigen-specific cells appeared to adhere to untreated and IL-1β-treated retinal EC to a greater extent than did the Con A-activated lymphocytes ($P < 0{\cdot}0001$ in both cases).

The level of adhesion, relative to controls, following treatment with antibodies is given in Fig. 5. The anti-C3b inactivator antibody did not bring about any reduction in T-cell adhesion to either the resting or IL-1β-treated EC monolayer. With the anti-CD106 antibody there was also no effect on T-cell adhesion to resting EC, but a significant reduction in apparent binding following IL-1β activation. Antibodies to CD54, CD11a, CD18 and CD49d all brought about a significant reduction in the apparent adhesion of T cell line lymphocytes to retinal EC monolayers. Combinations of both anti-CD11a and anti-CD49d did not further decrease the extent of adhesion. As with the Con A-activated lymphocytes, each antibody treatment gave no significant difference in response between IL-1β-activated and untreated endothelia.

Migration of T-cell lines

Migration of the SAg-specific T-cell lines across IL-1β-activated

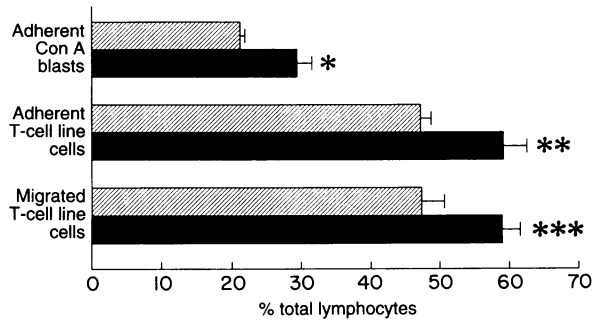

Figure 3. The adhesion of Con A-activated PLN cells and the adhesion and migration of an antigen-specific T-cell line on untreated (hatched bars) and IL-1β-treated (solid bars) retinal EC. Values are expressed as the percentage of cells that have adhered to or migrated across a monolayer of retinal EC (mean ± SEM of a minimum of six separate wells). * $P < 0.0005$, ** $P < 0.005$ and *** $P < 0.01$ compared to untreated EC.

retinal EC monolayers was significantly greater than through untreated monolayers (Fig. 3; $P < 0.01$). Addition of the control antibody OX21 to the endothelia failed to alter the level of migration on either IL-1β-activated or untreated EC monolayers.

The addition of anti-CD54, anti-CD11a and anti-CD18 antibodies to the migration assay led to a significant decrease in transmonolayer trafficking (Fig. 6; all $P < 0.0001$). There was no difference in the percentage reduction between activated and untreated EC following treatment with either anti-CD11a or anti-CD18 antibodies. With the anti-CD54 antibody, however, a much reduced level of inhibition was observed with the IL-1β-activated EC compared to resting EC monolayers ($P < 0.0001$).

When T cells were treated with either the anti-CD49d or anti-CD106 antibody it was found that only with the IL-1β-activated EC was there a significant reduction in lymphocyte migration (Fig. 6; $P < 0.0001$).

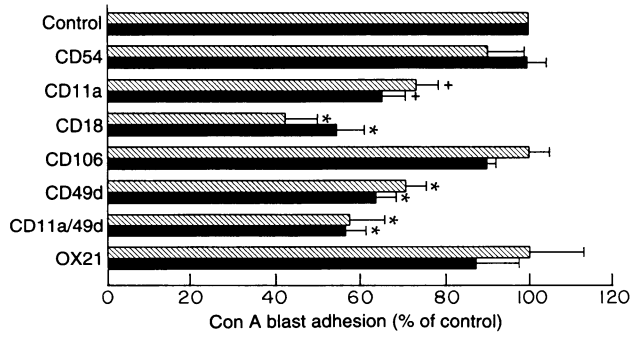

Figure 4. The effect of treating cells with antibodies to CD54, CD11a, CD18, CD106, CD49d and an irrelevant antibody (OX21) on Con A blast adhesion to untreated (hatched bars) and IL-1β-treated (solid bars) retinal EC. Antibodies to CD11a, CD18 and CD49d caused a reduction in the apparent adhesion to both untreated and IL-1β-activated EC (* $P < 0.0001$ and ** $P < 0.005$). The antibodies directed against CD54, CD106 and an irrelevant antigen did not cause any inhibition. Values are expressed as a percentage of adhesion in the absence of antibody (mean ± SEM of a minimum of 12 separate wells).

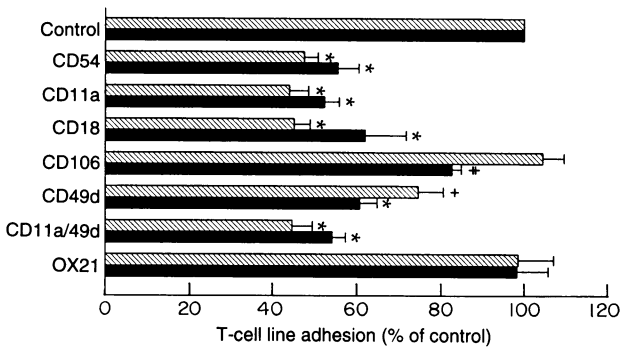

Figure 5. The effect of treating cells with antibodies to CD54, CD11a, CD18, CD106, CD49d and an irrelevant antibody (OX21) on antigen-specific T-cell line lymphocyte adhesion to untreated (hatched bars) and IL-1β-treated (solid bars) retinal EC. Antibodies to CD54, CD11a, CD18 and CD49d bring about a reduction in the apparent adhesion to both untreated and IL-1β-activated retinal EC (* $P < 0.0001$ and ** $P < 0.005$). The antibody to CD106 caused a small reduction in apparent adhesion on IL-1β-activated EC (*** $P < 0.05$), while the antibody directed against an irrelevant antigen did not cause any inhibition. Values are expressed as a percentage of adhesion in the absence of antibody (mean ± SEM of a minimum of 12 separate wells).

DISCUSSION

For lymphocytes to enter the CNS they must first traverse the vascular wall. This process involves a number of stages, which have been well characterized in non-CNS vascular studies,[1] although the vasculature of the CNS, being structurally and functionally different from that of other organs, may differ in its control of lymphocyte migration. Molecules expressed on the surface of both EC and lymphocytes are believed to direct the traffic of the latter into the inflamed CNS, a process which is

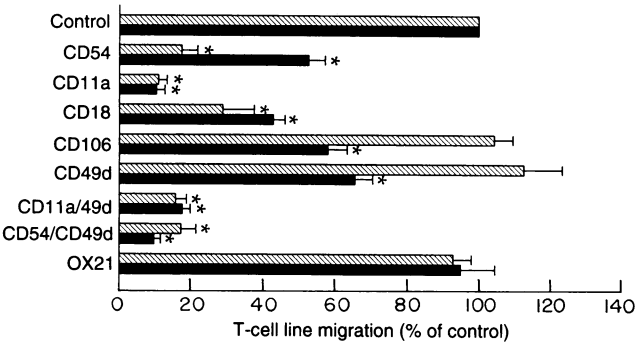

Figure 6. The effect of treating cells with antibodies to CD54, CD11a, CD18, CD106, CD49d and an irrelevant antibody (OX21) on antigen-specific T-cell line lymphocyte migration through untreated (hatched bars) and IL-1β-treated (solid bars) retinal EC monolayers. Antibodies to CD54, CD11a and CD18 inhibited lymphocyte migration (* $P < 0.0001$). With anti-CD54 the effect was more pronounced on untreated than on IL-1β-activated EC. The anti-CD49d and anti-CD106 antibodies had no effect on untreated EC but brought about a significant reduction in migration on IL-1β-activated EC. A combination of both anti-CD54 and anti-CD49d antibodies brought about maximal inhibition on the IL-1β-activated EC. The irrelevant antibody had no effect on migration. Values are expressed as a percentage of adhesion in the absence of antibody (mean ± SEM of a minimum of six separate wells).

regulated through both quantitative and qualitative changes in these molecules. In EAE, for example, the adhesion molecule ICAM-1 is strongly expressed on the vasculature[33,34] and is therefore thought to play an important role in recruiting cells from the circulation. The differential involvement of these molecules during the separate and complex processes of lymphocyte extravasation, however, remains to be fully resolved.

Throughout the numerous studies investigating the role of adhesion molecules during lymphocyte extravasation, few have considered their function at the vascular barriers of the CNS. In those studies in which this has been addressed it is clear that lymphocyte binding to EC of the BBB *in vitro* is less than to non-CNS endothelia[3,4] and that this process can be blocked by antibodies directed against the LFA-1 molecule expressed on the lymphocyte.[21,23] We have previously shown that retinal EC, which are structurally and functionally identical to brain endothelial cells, are equally poor at binding lymphocytes.[5] In this study we have extended this work to investigate the differential role played by adhesion molecules not only in adhesion, but also in facilitating the subsequent process of lymphocyte diapedesis.

As we have reported previously,[5] treatment of retinal endothelia with IL-1β significantly increased the degree of lymphocyte binding. However, unlike interferon-γ (IFN-γ), which increases adhesion[3–5] but not migration[29,35] to CNS-derived endothelia, IL-1β also increases the level of trans-monolayer migration, but whether this has any biological significance is not clear. It was also of interest to note that concentrations of 10 U IL-1β/ml and above caused separation of the EC junctions, a process that may have some relevance to BBB and BRB breakdown *in vivo* where it has been reported to cause barrier disruption.[36,37]

We have previously shown that Con A activation of PLN cells dramatically up-regulates their adhesion to retinal EC but does not affect their inability to migrate through a retinal endothelial monolayer. In contrast, T-cell lines exhibit a high degree of transmonolayer migration.[29] These two populations of lymphocytes have therefore enabled us to investigate the surface molecules involved during the separate processes of adhesion and migration.

The inability of the anti-ICAM-1 antibody to block Con A-activated lymphocyte adhesion confirms previous studies using brain endothelia[21] and would suggest that ICAM-1 is not involved in the adhesion of activated lymphocytes to CNS-derived EC. Its counter-receptor LFA-1, however, plays a significant role in lymphocyte binding. Antibodies directed against either CD11a or CD18 caused a marked reduction in adhesion, being similar to that previously reported with rat brain endothelium.[21] There are several possible explanations for the discrepancy between the anti-ICAM-1 and anti-LFA-1 results. Firstly, the antibody directed against ICAM-1 may bind to a non-functional site on the molecule although it does bind to a site which blocks migration (see below). Secondly, it is possible that LFA-1 is adhering to another ligand on the EC, such as ICAM-2. This, however, is inconsistent with the temporal increase in adhesion following cytokine activation of the EC, which correlates closely with an increased expression of ICAM-1.[21,38,39] The ligand ICAM-2 is not thought to be inducible on CNS EC,[38] although it is possible that cytokines are able to alter its surface distribution or conformation and hence its avidity. Finally, it is feasible that a further ligand is present on EC that is able to bind to LFA-1 and which is cytokine inducible, although this remains to be demonstrated. As the anti-LFA-1 antibody failed to block completely the adhesion of Con A blasts to the retinal EC monolayers, this may also suggest that other receptor pairings may also be involved in this process.

The partial inhibition of Con A-activated lymphocyte adhesion to non-activated retinal EC with the anti-α4 (VLA-4) antibody was unexpected as the EC ligand for VLA-4 is VCAM-1 and is not expressed on resting EC. A more likely explanation for the apparent reduction of adhesion is that the antibody can cause lymphocyte activation via VLA-4 and induce homotypic aggregation. The formation of small aggregates of lymphocytes which we observed in our studies would then make them more likely to be removed by the washing stage of the assay, thus producing an apparent reduction in binding. The failure of the anti-VCAM-1 mAb to significantly inhibit Con A-activated lymphocyte adhesion would suggest that this pairing is not a major component in adhesion. A recent report with bovine brain EC, however, suggests that anti-VLA-4 antibody can bring about almost total inhibition of adhesion,[23] which may reflect differences in the blocking ability of the anti-VCAM-1 antibody.

With T-cell line cells the adhesion assay must also be interpreted with care. The adhesion of Con A blasts can be readily determined as only a very small proportion of the cells migrate. The T-cell line lymphocytes, however, are highly migratory and the adhesion assay does not differentiate between adherent and transmigrated cells. During the 1·5-hr adhesion assay a significant proportion of these T cells will migrate through the monolayer. Indeed, the seemingly higher level of T-cell line adhesion over that of the Con A blasts (26% greater with resting EC and 30% with IL-1β-activated EC) correlates fairly closely with the number of T-cell line cells predicted to migrate through the monolayer during the adhesion assay (18% and 22%, respectively). Thus, in view of the fact that Con A blast adhesion is not blocked by anti-ICAM-1 the apparent blocking of T-cell line adhesion is likely to be due to inhibition of the subsequent process of migration. Similarly, the reduction in apparent adhesion on IL-1β-activated EC with anti-VCAM-1 mAb would also be consistent with an inhibition of migration and not adhesion.

The migration of T-cell lines through both untreated and IL-1β-activated retinal EC monolayers could be significantly reduced by antibodies directed against ICAM-1, CD11a and CD18. Blocking the CD11a chain of LFA-1 brought about the greatest inhibition, with a reduction to approximately 10% of controls in both non-activated and IL-1β activated EC. In contrast, the antibody to the β-chain was less effective at inhibiting migration. On non-activated EC both anti-ICAM-1 and anti-CD11a brought about a similar level of reduction in migration. Following IL-1β activation, however, although migration was still markedly inhibited it remained significantly greater than with untreated EC. This suggests that an additional ligand is being induced on the EC that is capable of sustaining a small level of migration. This is supported by the finding that antibodies directed against VLA-4 and VCAM-1 had no effect on resting EC but reduced migration on IL-1β-activated EC. The reduction in migration following the blocking of VLA-4 and VACM-1 equates with the greater

level of migration found when IL-1β-activated EC are used in the presence of anti-ICAM-1 compared to non-activated EC. This strongly suggests that although the receptor pairing of LFA-1/ICAM-1 is of fundamental importance in diapedesis, at inflammatory sites within the CNS where the EC are likely to be activated and VCAM-1 induced, migration may also be supported by the VLA-4/VCAM-1 pairing. This interpretation is further supported by the observation that on IL-1β activated endothelia migration can be reduced to less than 10% if antibodies to both to both VLA-4 and ICAM-1 are employed together. This result may partly explain the findings of Yednock *et al.*[40] where administration of an anti-VLA-4 antibody attenuated the clinical and pathological symptoms of EAE.

If the VLA-4/VCAM-1 pathway is responsible for around 30% of migration through activated EC, then one would also expect that the anti-CD11a mAb would be less effective at inhibiting migration through these cells. The observation that anti-CD11a equally inhibits migration through both activated and non-activated EC implies that migration mediated by VLA-4/VCAM-1 also involves some interaction between LFA-1 and the activated EC. Thus, blocking CD11a, which inhibits both the adhesive and migratory pathways, also appears to interfere with the VLA-4/VCAM-1-mediated migration.

In human studies where human umbilical vein endothelial cells (HUVEC) have been used, LFA-1 has also been strongly implicated in lymphocyte migration.[9] Oppenheimer-Marks *et al.*[16] have demonstrated that irrespective of the state of EC or T-cell activation, transmonolayer migration is mediated largely by the LFA-1/ICAM-1 pairing, even when ICAM-1 was not involved in adhesion. They further suggest that, in contrast to our studies, VCAM-1 does not play any part in diapedesis. This finding may reflect a genuine difference in the utilization of adhesion molecules during lymphocyte migration across CNS and non-CNS vascular beds.

The reason that Con A-activated lymphocytes are not as migratory as the T-cell line is not clear as a high proportion of both these lymphocyte populations express LFA-1 and VLA-4. The T-cell line lymphocytes, however, exhibit a greater intensity of expression of LFA-1, but whether this increased level of expression is sufficient to explain the differences in the degree of migration remains unresolved. A likely explanation for these differences is the functional state of the adhesion molecules as mitogen activation may not provide all the necessary signals for full activation of the migratory phenotype. Current studies in our laboratory would support this view.

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