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Role of LFA-1, ICAM-1, VLA-4 and VCAM-1 in lymphocyte migration across retinal pigment epithelial monolayers *in vitro*

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SUMMARY

The blood–retinal barrier (BRB), which is composed of the retinal pigment epithelium (RPE) and retinal vascular endothelium, normally restricts the traffic of lymphocytes into the retina. During ocular inflammatory conditions such as posterior uveitis there is a large increase in lymphocyte migration across the BRB. The differential role played by the two barrier sites, however, remains unclear. To evaluate the role of the posterior BRB, the migration of a $CD4^+$ antigen-specific T-cell line through rat RPE cell monolayers was investigated *in vitro* using time-lapse videomicroscopy. The adhesion molecules involved in controlling transepithelial migration across normal and interferon-γ (IFN-γ)-activated RPE was assessed with monoclonal antibodies directed against cell adhesion molecules. Lymphocytes were treated with antibodies specific for CD11a (α_L subunit of LFA-1), CD18 (β_2 subunit of the leucam family) and CD49 d (α_4 subunit of very late activation antigen-4, VLA-4), and the RPE with antibodies specific for CD54 (intracellular adhesion molecule-1, ICAM-1) and CD106 (vascular cell adhesion molecule-1, VCAM-1). Migration across unstimulated RPE was inhibited by antibodies to ICAM-1 (48·6 ± 3·5% reduction), leucocyte functional antigen-1 (LFA-1)α (61 ± 5·2%) and LFA-1β (63·2 ± 4·7%), but not by antibodies to VLA-4. VCAM-1 was not expressed on untreated RPE. Following activation of the RPE monolayers for 72 hr with IFN-γ, antibodies to LFA-1α, LFA-1β and ICAM-1 inhibited migration by 49·9 ± 9·4%, 63·6 ± 5·5% and 47·7 ± 4·2% respectively. Antibodies to VLA-4 and VCAM-1 blocked migration by 21·5 ± 8·4% and 32·3 ± 6·2%, respectively, which correlated with the induction of VCAM-1 expression on RPE and increased migration. Under these conditions blocking both VCAM-1 and ICAM-1 reduced migration by 70·9 ± 2·3%, which was greater than the effect of blocking either of these molecules alone. These results demonstrate that the posterior barrier of the BRB utilizes the same principle receptor–ligand pairings in controlling lymphocyte traffic into the retina as the vascular endothelium of the anterior BRB.

INTRODUCTION

Under normal conditions the blood–central nervous system (CNS) barriers are capable of supporting a limited degree of lymphocyte traffic into the neural parenchyma.[1] During inflammatory diseases, however, the degree of lymphocyte migration across these barriers is markedly up-regulated.[2] In general, lymphocyte extravasation is governed by a variety of factors, such as the state of cell activation and the repertoire of adhesion molecules expressed by the local vascular endothelia. Lymphocyte traffic into the retina, however, is controlled not only by the vascular endothelium but also by the retinal pigment epithelium (RPE), which forms a cellular barrier at the back of the eye overlying the permeable choroidal vasculature. Together these two cellular barriers form the blood–retinal barrier (BRB). We have recently shown that the extent of lymphocyte migration across RPE monolayers is dependent on the state of lymphocyte activation[3] and that this is similar to lymphocyte migration across retinal endothelial cells (EC).[4]

Despite an extensive amount of research investigating the role of the adhesion molecules intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in transendothelial migration of leucocytes (recently reviewed by Springer)[5] little is known about whether these molecules are also involved in the trafficking across other cellular barriers. Unlike vascular endothelial cells, the RPE is not involved in capturing immune cells from the circulation and therefore the expression and function of adhesion molecules on RPE cells is likely to differ.

The migration of antigen-specific T-cell line lymphocytes across untreated REC *in vitro* is largely mediated by ICAM-1/leucocyte functional antigen-1 (LFA-1) interactions, with

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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; RPE, retinal pigment epithelium; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late activation antigen-4.

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VCAM-1/very late activation antigen-4 (VLA-4) interactions playing a role in lymphocyte migration across activated retinal EC.[6] RPE cells also constitutively express ICAM-1,[7,8] which has been shown to adhere neutrophils[7] and lymphocytes.[7,8] While the migration of neutrophils is reported to be entirely ICAM-1 mediated,[7] it has been suggested that lymphocytes also employ alternative adhesion pathways during migration.[7–9] To date, however, VCAM-1 expression has not been described on untreated or cytokine-activated RPE either *in vivo* or *in vitro*.[7]

In this study we have examined the constitutive and interferon-γ (IFN-γ)-induced expression of the adhesion molecules ICAM-1 and VCAM-1 on rat RPE and the role which these molecules play in migration of antigen-specific T-cell line lymphocytes across RPE monolayers *in vitro*.

MATERIALS AND METHODS

Reagents

Purified recombinant interleukin-2 (IL-2) and dispase were obtained from Boehringer Mannheim (Lewes, 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 and 0·5 mM mercaptoethanol (all Sigma, Poole, UK). RPE cells were grown in Hams F-10 supplemented with 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES and 7·5% sodium bicarbonate. Sodium azide-free monoclonal antibodies (mAb) specific for rat CD11a (α_L subunit of LFA-1; WT1)[10], CD18 (β_2 subunit of leucam family; WT3)[10], CD54 (ICAM-1; 1A29)[11], CD4 (W3/25), CD5 (pan T cell; OX19), CD8 (OX8), CD25 (IL-2 receptor; OX39) and CD45RC (OX22) were obtained from Serotec (Oxford, UK). Anti-rat CD49 d (α_4 subunit of VLA-4; TA-2)[12] was obtained from AMS Biotechnology Ltd (Oxford, UK), and anti-rat CD106 (VCAM-1; 5F10) was a generous gift from Dr R. Lobb, (Biogen, Cambridge, MA). An irrelevant control antibody to human C3b inactivator (OX21) was a generous gift of Dr M. Puklavec (MRC, Oxford University, UK).

RPE cultures

Specific pathogen-free PVG rats (6–8 days old; bred in house) were used to isolate the RPE cells according to a previously described method.[13] Briefly, eyes were enucleated and the intact globes digested with 2% dispase for 30 min. Following this incubation the cornea, lens and vitreous were removed and discarded. The retina was then carefully isolated and incubated in culture medium for a further 15 min, after which sheets of RPE cells could be separated from the neuroretina. The sheet of RPE cells was trypsinized to obtain a single suspension of cells, which were then plated onto 24- or 96-well plates. RPE cells were grown to confluence in 5% CO_2 at 37°, with the culture medium being replaced every 2–3 days.

RPE cultures were used for the migration assay either as untreated cells or activated with IFN-γ (100 U/ml) for 24 or 72 hr. For enzyme-linked immunosorbent assay (ELISA), RPE cells were activated with IFN-γ (100 U/ml) for up to 5 days.

Lymphocytes

A terminally differentiated IL-2-dependent $CD4^+$ T-cell line from Lewis rats specific for the retinal antigen, retinal soluble antigen (SAg), was used (a generous gift from Dr V. Calder, Institute of Ophthalmology) for the migration assays. The cell line was established according to a previously described method.[14,15] Briefly, Lewis rats were immunized with bovine SAg and after the onset of experimental autoimmune uveoretinitis (EAU) the popliteal lymph nodes were harvested for lymphocytes. The cells were maintained by weekly stimulations with SAg (10 μg/ml) using 50-fold irradiated autologous thymocytes as antigen-presenting cells. Three to 4 days after antigen stimulation, dead feeder cells were removed by density centrifugation over metrizoate–Ficoll and viable cells were grown at 2×10^5/ml in the presence of purified recombinant IL-2 (20 U/ml). Lymphocytes from the third to the sixth passage were used, with no functional differences observed between passages. In proliferation assays this SAg T-cell line responds to antigen in the presence of professional antigen-presenting cells (thymocytes), giving an eightfold proliferative response with 10 μg/ml SAg.[16] The same T-cell line has also been shown previously to be highly migratory across both RPE[3] and retinal EC[4] monolayers.

The T-cell line phenotype was assessed by flow cytometry, as described below.

ELISA

The time–course of ICAM-1 and VCAM-1 expression on RPE monolayers following IFN-γ activation was determined using an ELISA. RPE monolayers grown on 96-well plates were treated with 100 U/ml IFN-γ for 1–5 days, after which the cells were fixed for 15 min with 0·1% glutaraldehyde in PBS before washing and blocking for 20 min with 0·05 M Tris–HCl (pH 7·4). The cells were then incubated for 1 hr with the primary antibodies 1A29 (anti-CD54/ICAM-1) and 5F10 (anti-CD106/VCAM-1). After washing three times with 0·3% bovine serum albumin in phosphate-buffered saline (BSA–PBS), cells were incubated for 1 hr with biotinylated sheep anti-mouse IgG (1 : 10 000 dilution) followed by 1 hr with streptavidin peroxidase (1 : 5000). Each well was washed three times with 0·3% BSA–PBS between each step.

The plates were developed using 100 μg/ml tetramethylbenzidine in 97 mM sodium acetate/3 mM citric acid containing 0·5 μl/ml H_2O_2, for approximately 15 min. The reaction was stopped with 1 M H_2SO_4 and the optical density measured at 450 nm using an ELISA plate reader (Titertek Multiskan, Flow Laboratories, Ayrshire, UK).

Flow cytometry

To evaluate the percentage expression of ICAM-1 and VCAM-1 on normal and IFN-γ-activated (100 U/ml for 24 hr) RPE monolayers, flow cytometry was employed. Single cell suspensions were obtained by washing the cells in Mg^{2+}/Ca^{2+}-free Hanks' balanced salt solution, followed by enzymatic dissociation with collagenase/dispase (0·1%) for 1 hr. The cells were resuspended in PBS at 5×10^4 cells/vial and incubated for 1 hr with the antibodies 1A29 (anti-ICAM-1) or 5F10 (anti-VCAM-1). Following this incubation, the cells were then incubated for a further 45 min with fluorescein isothiocyanate–rat anti-mouse immunoglobulin (FITC-RAMIG) in the presence of 20% normal rat serum. After washing the cells twice, they were resuspended in PBS and the percentage of positive cells determined using flow cytometry (FACScan; Becton Dickinson, Oxford, UK). Unstained cells were used to set the parameters and cells stained with FITC–RAMIG alone were used to set the background control.

Expression of the adhesion molecules LFA-1 and VLA-4 on 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.

For T-cell phenotyping, antibodies recognizing rat CD4 (W3/25), CD8 (OX8), CD5 (pan T cell; OX19), CD25 (IL-2 receptor; OX39) and CD45RC (OX22) were used.

Migration assay

Lymphocyte migration was evaluated using time-lapse videomicroscopy, as previously reported.[3,4] Lymphocytes (2×10^5 cells/ml/well) were suspended in culture medium and added to RPE monolayers grown in 24-well plates. Five to 10 min prior to the 4-hr time-point of each co-culture, a field of 200 μm^2 was randomly selected by inverse phase contrast microscopy and recorded for 10 min. After each series of recordings the tape was replayed at 160× normal speed and analysed by calculating the number of cells above and below the monolayer. The T cells that had migrated below the RPE monolayer could be easily distinguished by their phase contrast appearance and attenuated morphology. The data were expressed as the percentage of lymphocytes within the field that had migrated underneath the monolayer. Controls (untreated cells) were run in parallel with the antibody-treated cells to overcome any possible minor variations in the migratory ability of the T-cell lines on different days. All values are given as means ± SEM and differences between groups were assessed using Student's *t*-test.

Electron microscopy

Monolayers of RPE were grown in 24-well plates until confluent. Antigen-specific T cells were then added and the co-culture left for 4 hr at 37°, after which they were fixed with a mixture of 1% paraformaldehyde and 3% glutaraldehyde in 0·1 M sodium cacodylate buffer (pH 7·4). After washing, the cells were osmicated (1% osmium tetroxide) and dehydrated through ascending concentrations of ethanol. The cells were then embedded in araldite and sections cut and counterstained with lead citrate and uranyl acetate prior to viewing on a Jeol 1010 transmission electron microscope (TEM).

Antibody blocking

The sodium azide-free blocking antibodies directed against rat CD11a (LFA-1),[10] rat CD18 (leucam family),[10] rat CD49d (VLA-4),[12] rat CD54 (ICAM-1),[11] rat CD106 (VCAM-1) and to an irrelevant antigen (human C3b inactivator; 20 μl of hybridoma supernatant) were used to investigate the role of adhesion molecules in transepithelial T-cell migration. Lymphocytes were pretreated with the antibodies at a saturating concentration to either CD11a (20 μl of hybridoma supernatant), CD18 (20 μl of hybridoma supernatant) or CD49d (10 μg/ml) by resuspending 10^6 T cells in 100 μl PBS containing the antibody. The cells were then kept at 4° for 1 hr, after which the cells were resuspended in culture medium and added to untreated or IFN-γ-treated (100 U/ml for 24 hr) RPE monolayers in the presence of the antibody. RPE cells were treated with saturating concentrations of antibodies directed against either CD54 (ICAM-1; 10 μg/ml) or CD106 (VCAM-1; 10 μg/ml) or both for 1 hr at 4° prior to adding the lymphocytes.

The data are expressed as the percentage inhibition compared to controls, which were run at the same time, and values are given as means ± SEM. Differences between groups were assessed using Student's *t*-test.

RESULTS

Morphology and electron microscopy

After approximately 2 weeks from seeding, the RPE formed uniform monolayers of pigmented hexagonal cells. TEM revealed the presence of melanosomes and stunted microvilli. SAg-specific T cells could be seen adhering to the surface of the monolayer, penetrating the monolayer and lying underneath (Fig. 1). These findings clearly demonstrated the ability of the T cells to migrate through the RPE monolayer.

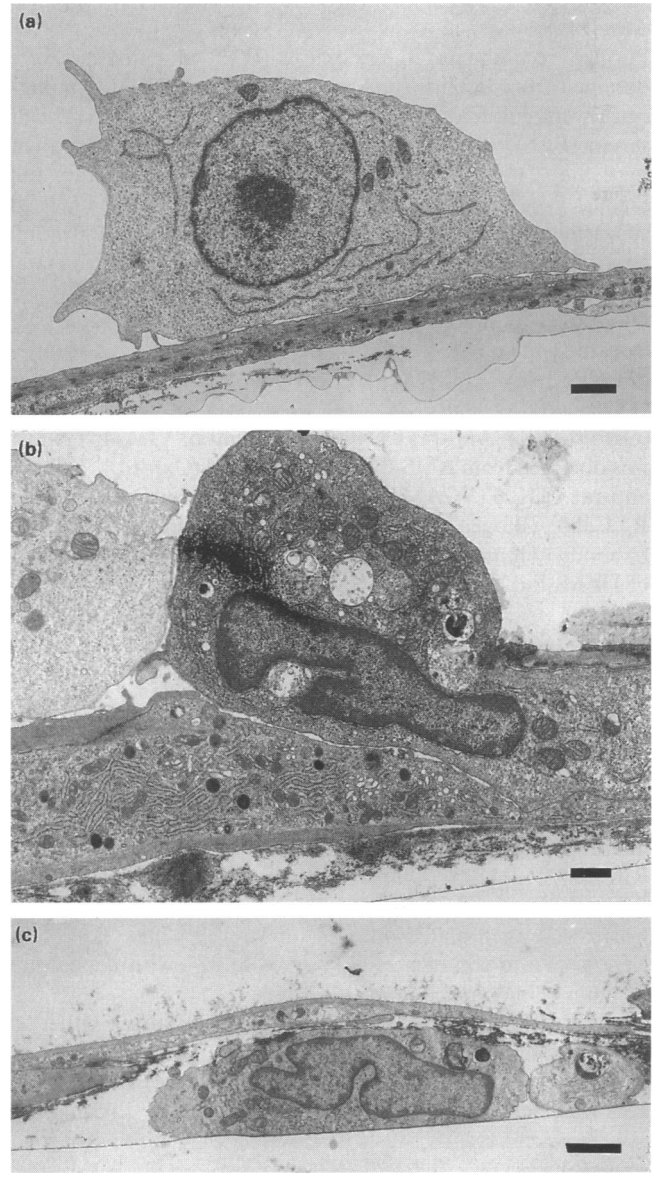

Figure 1. TEM of SAg-specific T-cell co-culture with RPE cell monolayers. (a) Lymphocyte adhering to RPE cell (bar = 1 μm). (b) Lymphocyte migrating through RPE monolayer (bar = 1 μm). (c) Lymphocyte lying underneath RPE monolayer (bar = 2 μm).

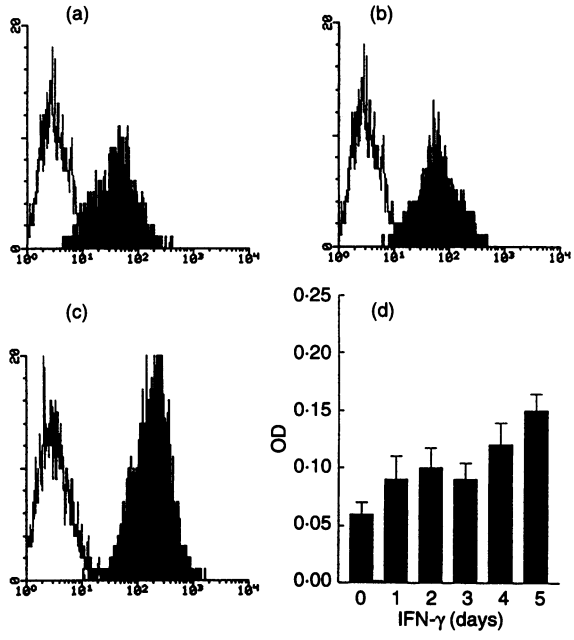

Figure 2. The expression of ICAM-1 on untreated and IFN-γ-treated (100 U/ml) RPE cells. Representative plots from flow cytometric analysis of ICAM-1 expression on (a) untreated RPE, (b) RPE activated with IFN-γ for 24 hr, and (c) RPE activated with IFN-γ for 72 hr. The controls (open histograms) were set using FITC-labelled RAMIG. Horizontal axis represents log of fluorescence and vertical axis relative cell number. (d) Time–course of ICAM-1 expression over 5 days activation with IFN-γ, as determined by ELISA (mean ± SEM).

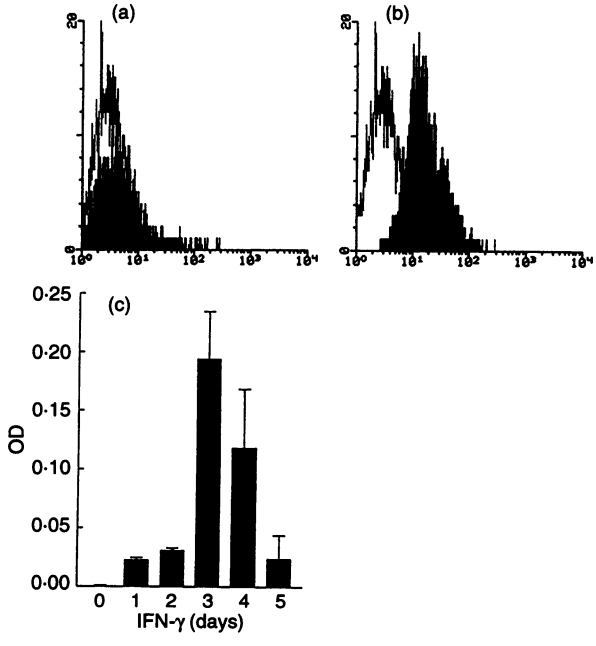

Figure 3. The expression of VCAM-1 on untreated and IFN-γ-treated (100 U/ml) RPE cells. Representative plots from flow cytometric analysis of VCAM-1 expression on (a) untreated RPE and (b) RPE activated with IFN-γ for 72 hr. The controls (open histograms) were set using FITC-labelled RAMIG. Horizontal axis represents log of fluorescence and vertical axis relative cell number. (c) Time–course of VCAM-1 expression over 5 days activation with IFN-γ, as determined by ELISA (mean ± SEM).

Flow cytometry and ELISA

Results from both flow cytometry and ELISA demonstrated that ICAM-1 was constitutively expressed on RPE cells and that the level of expression could be induced over 5 days following activation with IFN-γ (Fig. 2). VCAM-1, on the other hand, was not constitutively expressed but reached maximal expression 3 days after activation (Fig. 3), and started to decline thereafter. From flow cytometric analysis, the majority of cells were positive for ICAM-1, with an increase in the intensity of expression occurring at 24 and 72 hr following activation with IFN-γ (Fig. 2). VCAM-1 was not expressed on untreated RPE but was induced by IFN-γ, with the level of expression peaking at 25·0 ± 4·2% by 72 hr activation (Fig. 3).

The SAg-specific T-cell line cells were characterized using flow cytometry and found to be $CD4^+$, $CD8^-$, $CD5^+$, $CD25^+$ and $CD45Rc^{low}$. These cells were also found to express constitutively the adhesion molecules CD11a, CD18 and CD49 d (Fig. 4).

T-cell line migration across RPE monolayers

The percentage of antigen-specific T-cell line lymphocytes that

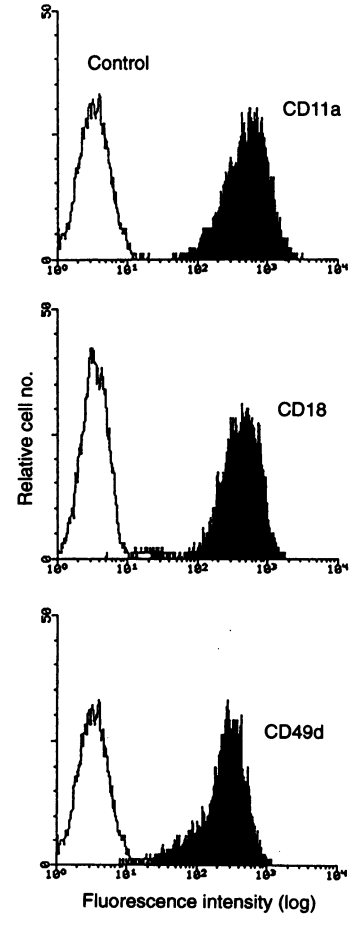

Figure 4. Representative plots from flow cytometric analysis of the surface expression of CD11a, CD18 and CD49 d on SAg T-cell line lymphocytes. The controls (open histograms) were set using FITC-labelled RAMIG.

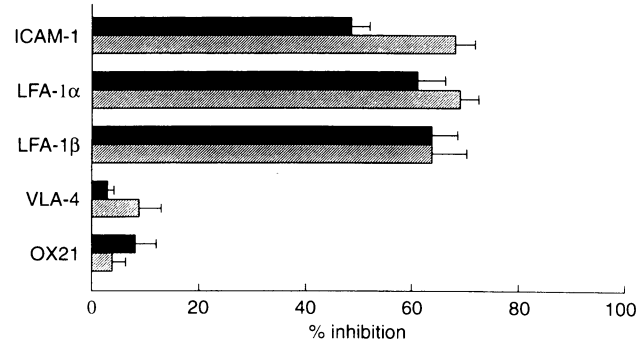

Figure 5. The effect of treating cells with antibodies to CD54 (ICAM-1), CD11a (LFA-1α), CD18 (LFA-1β), CD49d (VLA-4) and an irrelevant antibody (OX21) on SAg-specific T-cell line lymphocyte migration through RPE monolayers. Antibodies to CD54, CD11a and CD18 inhibited lymphocyte migration through untreated RPE (solid bars) and RPE monolayers activated for 24 hr with IFN-γ (hatched bars). The anti-CD49d and irrelevant antibody (OX21) had no effect on lymphocyte migration. Values are expressed as the percentage inhibition compared with controls (mean ± SEM of a minimum of six separate wells).

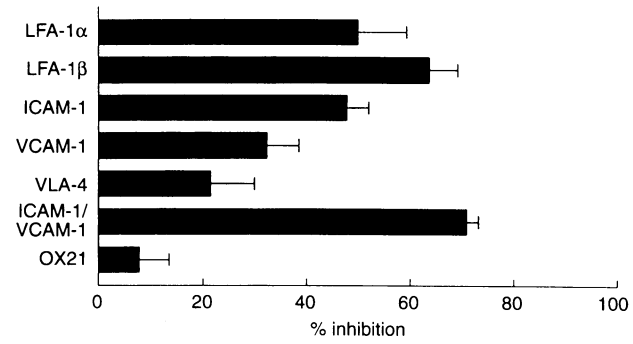

Figure 6. The effect of treating cells with antibodies to CD54 (ICAM-1), CD11a (LFA-1α), CD18 (LFA-1β), CD106 (VCAM-1), CD49d (VLA-4) and an irrelevant antibody (OX21) on SAg-specific T-cell line lymphocyte migration through RPE monolayers activated for 72 hr with IFN-γ. Antibodies to CD54, CD11a, CD18, CD54 and CD106 all inhibited lymphocyte migration. The irrelevant antibody (OX21) had no effect on lymphocyte migration. A combination of both anti-CD54 and anti-CD49d antibodies brought about a greater inhibition than with either antibody alone. Values are expressed as the percentage inhibition compared with controls (mean ± SEM of a minimum of six separate wells).

migrated through the untreated RPE monolayer over a 4-hr assay was 36·4 ± 2·3% (mean ± SEM). Following treatment of the RPE monolayer with the anti-ICAM-1 antibody, migration was reduced from control levels by 48·6 ± 3·5%. This level of inhibition was not significantly different from that achieved by blocking the ICAM-1 counter-receptor, LFA-1, as antibodies directed against LFA-1α (CD11a) and LFA-1β (CD18) on the lymphocyte brought about a reduction in migration of 61 ± 5·2% and 63·2 ± 4·7%, respectively. Blocking the VLA-4 receptor with antibody or treating the lymphocytes with the control antibody (OX21) had no effect on migration (Fig. 5).

The amount of lymphocyte migration that could be blocked by the anti-ICAM-1 antibody following activation of the RPE with IFN-γ for 24 hr was greater than that achieved with untreated RPE (68·2 ± 3·7%; $P < 0.01$, cf. untreated RPE). Blocking LFA-1α and LFA-1β on lymphocytes also reduced migration by a similar amount, being 69·1 ± 3·5% and 63·8 ± 6·5%, respectively. As with untreated RPE, antibodies to VLA-4 did not cause a significant reduction in lymphocyte migration across RPE activated with IFN-γ for 24 hr (Fig. 5).

Activation of the RPE with IFN-γ for 72 hr significantly increased the percentage of T cells migrating through the monolayer, to 47·6 ± 3·5% ($P < 0.01$, cf. untreated RPE) over the 4-hr assay. Conversely, the percentage of migration that was inhibited by blocking ICAM-1 was significantly less than with RPE monolayers activated for 24 hr with IFN-γ ($P < 0.05$). A similar degree of inhibition to anti-ICAM-1 was also achieved with the antibody to LFA-1α (49·9 ± 9·4%). The level of inhibition brought about by the anti-CD18 antibody, however, was greater (63·6 ± 5·5%) but was not significantly different. With RPE monolayers activated with IFN-γ for 72 hr, antibodies to both VLA-4 and VCAM-1 also reduced migration from control values, by 32·3 ± 6·2% and 21·6 ± 8·4%, respectively. By blocking both adhesion pathways with antibodies to ICAM-1 and VCAM-1, the percentage inhibition was significantly greater than with either antibody alone (70·9 ± 2·3%, $P < 0.01$) (Fig. 6).

DISCUSSION

The relative importance of the two barrier sites that constitute the BRB in controlling lymphocyte migration into the retina is not clearly understood. By investigating the migration of ocular antigen-specific T cells across RPE monolayers *in vitro*, we have endeavoured to define the molecules that assist in facilitating lymphocyte traffic across the posterior BRB. By using antigen-specific T cells, which we have previously shown to be highly migratory through both RPE[3] and retinal endothelia,[4] we have been able to investigate the adhesion molecules involved in this process. It is also clear from these and other studies[1] that it is antigen activation that induces the migratory phenotype and that the organ-specificity of the antigen is irrelevant in determining lymphocyte migration. Furthermore, a T-cell line was chosen as resting or mitogen-activated peripheral lymph node lymphocytes do not exhibit sufficient levels of migration.[3,4]

We have demonstrated that under these experimental conditions lymphocyte migration was mediated in part by the LFA-1/ICAM-1 receptor–ligand interaction. With unstimulated RPE the reduction in migration following antibody blockade of these molecules was less than that previously observed with retinal vascular endothelial cells.[6] Moreover, the migration of T cells across airway epithelium has been found to be almost entirely ICAM-1/LFA-1 dependent and did not involve other interactions such as with extracellular matrix components.[17] These data suggest that, in contrast to retinal EC and airway epithelia, receptor–ligand interactions other than LFA-1/ICAM-1 and VLA-4/VCAM-1 are of significant importance in controlling migration across unstimulated RPE. What remains unclear, however, is at which stage of lymphocyte/RPE interaction these adhesion molecules are involved in, as inhibition of migration under these conditions could be a result of blocking either the preceding adhesive stage or to the process of diapedesis itself.

In a previous study in which leucocyte adhesion to human RPE cells was investigated, the level of lymphocyte adhesion

following antibody blockade of ICAM-1 could be reduced by 30%,[7] indicating the presence of alternative adhesive interactions. In contrast, lymphocyte adhesion to human RPE has also been reported to be reduced by approximately 90% with anti-ICAM-1 antibodies,[8] which equates with the reduction in neutrophil adhesion following antibody blockade.[7] These assays, however, do not differentiate between lymphocyte adhesion and migration, which can lead to difficulties in interpretation. Furthermore, the relevance of lymphocyte adhesion to RPE *in vitro* to the mechanisms operating *in vivo* is questionable as RPE do not interface with circulating lymphocytes and thus are not involved in their capture from the blood. What these studies do demonstrate is the existence of adhesive forces between the two cell types, which may provide attachment points for lymphocyte migration through the RPE barrier that is situated within the ocular tissue.

As predicted, anti-VLA-4 antibody did not inhibit lymphocyte migration as its ligand, VCAM-1, was not constitutively expressed on untreated RPE. Unlike retinal EC,[6] activation of the RPE with IFN-γ for 24 hr did not induce VCAM-1 expression and, consequently, anti-VLA-4 antibody did not alter the level of lymphocyte migration. After 72 hr post-IFN-γ activation, however, VCAM-1 was expressed and antibodies to either VLA-4 or VCAM-1 caused a significant reduction in transepithelial migration. This demonstrates for the first time that the VLA-4/VCAM-1 pathway is capable of supporting lymphocyte migration across the posterior BRB. By blocking both ICAM-1 and VCAM-1 so that both receptor–ligand pathways were inhibited, the degree of inhibition of lymphocyte migration was greater than when either antibody was used alone. Therefore, during inflammatory conditions of the retina where the RPE will be activated and VCAM-1 expressed, the migration of lymphocytes across the posterior BRB could be facilitated by both the LFA-1/ICAM-1 and VLA-4/VCAM-1 interactions.

Despite some differences, these results were generally similar to those recently reported for lymphocyte migration through retinal endothelial monolayers.[6] The migration of lymphocytes across normal retinal endothelium was predominantly LFA-1/ICAM-1-dependent, but following cytokine activation of the endothelium for 24 hr migration also occurred via the VLA-4/VCAM-1 pathway. The temporal difference in VCAM-1 induction at the two barrier sites may provide an explanation for the observation that leucocyte migration into the retina in EAU occurs initially via the retinal vascular endothelium,[18] with migration across the posterior barrier occurring later in the disease. This would suggest that the VLA-4/VCAM-1 interaction is of greater importance in recruiting lymphocytes into the neuroretina than the LFA-1/ICAM-1 pathway. This is supported by *in vivo* data where antibodies to either LFA-1 or VLA-4 have been administered to animals with the experimental model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). Following anti-VLA-4 treatment there was a significant attenuation of the disease,[19] whereas antibodies to LFA-1 caused no reduction in the severity of the disease.[20,21] Contrary to these findings, however, is the successful prevention of EAU following the administration of anti-ICAM-1 antibody.[22] Whether this reflects a genuine difference between the retina and the rest of the CNS or is a function of the experimental protocols employed remains to be resolved.

The failure to bring about complete inhibition of migration with any of these antibodies is unlikely to be owing to submaximal inhibition, as saturating concentrations of antibody were used. Furthermore, in our previous study using retinal endothelia[6] where an identical protocol was used, migration was inhibited by approximately 90%. In addition, as the T-cell line used is homogeneous in its expression of both LFA-1 and VLA-4, it is unlikely that the antibodies to these epitopes are preferentially blocking a subpopulation. This strongly suggests, therefore, that transepithelial lymphocyte migration can also be mediated by other receptor–ligand interactions. As trafficking across the RPE *in vivo* is actually migration within a solid tissue, it is possible that extracellular matrix components are also involved. For example, elements of the extracellular matrix, such as fibronectin and laminin, can interact with VLA integrins on lymphocytes.[23–25] RPE cells *in vitro* have been shown to produce fibronectin constitutively,[26] and although migration was not blocked by anti-VLA-4 it is possible that the monoclonal antibody used does not block the fibronectin binding site or that other VLA integrins are involved. This may also explain the high level of migration of the memory T-cell line lymphocytes used, as memory lymphocytes express three- to fourfold more VLA-4, VLA-5 and VLA-6 than do naive cells, and bind more efficiently through them to fibronectin and laminin.[25]

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