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Control of lymphocyte migration into brain: selective interactions of lymphocyte subpopulations with brain endothelium

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SUMMARY

We have determined whether particular lymphocyte populations bind preferentially to cerebral endothelium, using adhesion assays and a new method for *in situ* staining of adherent lymphocytes. B cells bind more strongly than T cells, an effect enhanced by lymphocyte activation or endothelial cell stimulation with interferon-gamma (IFN-γ) or tumour necrosis factor-alpha (TNF-α). This is not equated with levels of CD18 expression on the lymphocytes. $CD8^+$ T cells bound more efficiently than $CD4^+$ cells under all conditions. To determine whether there was a population of cells which selectively homes to the brain, we compared adhesion of cervical lymph node cells to brain endothelium, with adhesion of lymphocytes from other nodes. In 50% of the experiments there was significantly enhanced binding of activated cervical lymph cells to cerebral endothelium but not to control (aortic) endothelium. This effect was seen using both normal and IFN-γ-activated endothelium. The explanation for this finding is that cervical lymph nodes frequently, but not invariably, contain higher proportions of $CD8^+$ cells and B cells than other lymph nodes. These data imply that selective adhesion of lymphocytes to brain endothelium is related to the subpopulations involved and this may be reflected in the cell types seen in immunological lesions of the brain, and in the relative proportions of the subpopulations seen in cervical lymph nodes.

INTRODUCTION

Migration of lymphocytes through the brain is normally very limited, although this can be greatly increased in diseases such as viral encephalitis and multiple sclerosis,[1] and in experimental models such as experimental allergic encephalomyelitis (EAE).[2] Observations of lymphocyte cuffing around cerebral microvessels, particularly post-capillary venules, suggest that the main route of migration into the central nervous system (CNS) is across the specialized cerebral endothelium. Following injection of antigen into the brain, antigen-specific B cells are selectively found in cervical lymph nodes in comparison with other nodes.[3] This, and the finding that antigen drains from cerebral ventricles into cervical lymph nodes[4] has lead to the theory that lymphocytes also migrate out of the CNS by this route.

We have previously shown that lymphocytes are much less adhesive for brain microvessel endothelium than extracerebral endothelium, but that adhesion can be increased by stimulation of the endothelial cells over 1–24 hr with low levels of interferon-gamma (IFN-γ) or tumour necrosis factor-alpha (TNF-α).[5] This requires new protein synthesis. Lymphocytes activated by mitogens and T cells activated by their specific antigen also bind more effectively than resting lymphocytes, but this effect is greatest 8–16 hr after activation and disappears as the cells divide.[6]

The aim of this study was to determine whether particular subpopulations of lymphocytes interact with cerebral microvessel endothelium and to see whether there is any evidence for a brain-specific homing population. Experiments were carried out *in vitro*, using different lymphocyte populations and cultured monolayers of rat (LEW) cerebral endothelium or aortic endothelium as a control. These were examined in various intercellular adhesion assays, since binding of lymphocytes to endothelium is thought to be the first critical step which controls migration into CNS.

Our results show that binding of B cells and $CD8^+$ T cells to endothelium is greater than that of $CD4^+$ cells, and this finding explains why cervical lymph node cells (which frequently contain low proportions of $CD4^+$ cells) often bind to brain endothelium more efficiently than cells from other lymph nodes.

Abbreviations: BSA, bovine serum albumin; BSS, balanced salt solution; Con A, concanavalin A; EAE, experimental allergic encephalomyelitis; FCS, foetal calf serum; PBS, phosphate-buffered isotonic saline.

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MATERIALS AND METHODS

Endothelium

Brain microvascular endothelium was isolated from 3-month-old female Lewis rats according to our previously described methods[7] and plated onto 96-well microtitre plates coated with

type 1 collagen (Sigma, Poole, Dorset, U.K.). The cells were grown until they formed confluent monolayers (8–10 days) in HAMS F10 medium (Gibco, Paisley, Renfrewshire, U.K.) containing 20% plasma-derived bovine serum,[8] 40 μg/ml heparin, 75 μg/ml endothelial cell growth supplement (ECGS; Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml vitamin C (growth medium). The cells express von Willebrand factor and angiotensin converting enzyme, and have the tight junctions characteristic of brain endothelium.[9] The cells were used as primary cultures.

Aortic endothelium was isolated by a method based on that described by McGuire & Orkin.[10] Briefly rat aorta was removed by dissection, cut into small pieces (2–5 mm^2) and placed endothelium side down on collagen-coated plates in growth medium. After 3 days the explants were removed from the plate and outgrowing cells were expanded and passaged by trypsinization. At confluence these cells developed the cobblestone appearance characteristic of large vessel endothelium. They expressed von Willebrand factor, and were able to grow indefinitely in MEM d-valine, a capacity lacking in fibroblasts and smooth muscle cells.[11] Unlike cerebral endothelium, aortic endothelium does grow indefinitely in tissue culture but in these experiments was always used between passage 4 and 10. Passage 3 is the earliest stage at which sufficient cells are available from an aortic explant to do useful experiments.

The cells were pulsed with the stated doses of recombinant rat IFN-γ or human TNF-α in growth medium, lacking ECGS, for 24 hr before use in adhesion assays. Rat IFN-γ was a generous gift of Dr P. H. Van der Meide of the Primate Research Centre, Rijswijk, The Netherlands. TNF-α was kindly supplied by Dr G. R. Adolf, Genentech, Vienna, Austria.

Lymphocytes

Lymphocytes were obtained from peripheral lymph nodes of 8–14-week-old female Lewis rats, on the day before the adhesion assays. Cells were isolated from either cervical lymph nodes or mesenteric lymph nodes or mixed other lymph nodes (inguinal, popliteal, brachial and subaxillary nodes). Pooled peripheral lymph node cells were prepared from a mixture of all these lymph nodes. These were teased apart with forceps, into BSS containing 10% foetal calf serum (FCS), and the cell suspension was passed through sterile gauze to remove clumps. The cells were washed three times in BSS before culturing overnight in RPMI-1640 containing 10% FCS and antibiotics. Activated cells were treated for 16 hr before assay with 5 μg/ml Con A (Sigma). We have shown elsewhere that these conditions induce a maximal increase in adhesion molecules, which falls as the cells move into M phase.[5]

Populations enriched for T or B cells were prepared using nylon wool columns.[12] The purity of the T-cell populations by this method was $96{\cdot}1\% \pm 2{\cdot}5\%$ ($CD2^+$) and of the B cells $82\% \pm 3\%$ ($CD45R^+$ (=CD45R.ABC)).

Adhesion assay

Lymphocytes were washed twice in BSS and then labelled with ^{51}Cr in BSS (3 μCi/10^6 cells) for 90 min, then the cells were spun down and washed twice more with BSS. They were resuspended in BSS containing 10% FCS at a density of 2×10^7/ml at 37°. Endothelial cell monolayers were prepared by removing culture medium and washing the cells *in situ* four times with BSS. Aliquots of 0·15 ml of lymphocytes were applied to the wells, and the plates were incubated at 37° in 5% CO_2 for 2 hr. Non-adherent lymphocytes were aspirated off the monolayers, which were then washed four times with prewarmed (37°C) BSS. Best results were obtained when washing was carried out from each of the four quadrants of the microwells. The adherent cells were then lysed with 2% SDS and the lysate and washings from each well were counted on a gamma counter.

In situ *staining*

To determine the surface phenotype of lymphocytes adherent to the monolayers, we developed a new assay, in which lymphocytes binding to the monolayers were fixed and stained *in situ*. For this technique, the adhesion assay was carried out exactly as indicated above, except that lymphocyte labelling with ^{51}Cr was omitted. After removing unbound cells by washing, the adherent cells were rinsed once with phosphate-buffered saline (PBS) and fixed for 10 min in a fresh solution of 4% paraformaldehyde in PBS. The plates were blocked with 200 μl per well of 0·05 M Tris-HCl, pH 7·5, for 20 min, and then washed three times in PBS containing 0·1% Tween-20 (washing buffer). The lymphocytes were then stained for cell surface antigens using monoclonal antibodies W3/25 (CD4), OX8 (CD8), OX33 (B-cell restricted form of CD45) and OX34 (CD2), all from Seralab, Crawley Down, Sussex, UK. The antibodies were applied at 1/5 dilutions of tissue culture supernatants in PBS containing 10 mg/ml BSA (Miles; reagent grade) and 0·1% Tween 20 (antibody diluent), for 90 min. The plates were washed three times, and the second layer, 1/500 biotinylated anti-mouse Ig (Amersham, Amersham, Bucks, U.K.), added in antibody diluent for 1 hr. The plates were washed again three times and the third layer, 1/500 streptavidin biotinylated horseradish peroxidase (Amersham), applied for 1 hr. The plates were again washed three times, rinsed in PBS and developed using 200 μl of 30 μg/ml diaminobenzidine in PBS containing 1 μl/ml 30% H_2O_2. Development was stopped after 20 min by washing with PBS. Cells were scored visually (see Fig. 2 for example). Each determination was carried out in four or five identical wells and the results are derived from the mean proportion of positively stained cells present on the monolayers, counting 100–250 cells. The variation between wells (standard deviation) was always $< 2\%$ of the mean value for $CD4^+$ cells and $< 5\%$ of the mean value for $CD8^+$ cells.

Immunofluorescence

Lymphocytes were washed twice in PBS containing 0·2% BSA and 0·2% sodium azide, and stained with 1/4 dilutions of anti-CD2, -CD4, -CD8, and -CD45R (as above) in this buffer. They were then developed with 1/300 biotinylated anti-mouse immunoglobulin and 1/300 fluoresceinated streptavidin (Amersham). These cells were scored visually, positive or negative. For analysis of CD18 expression, cells were stained with OX42 (kindly supplied by Dr D. Mason, MRC Cellular Immunology Unit, Oxford, U.K.). The staining was completed as above, and the cells resuspended in PBS for analysis on a Beckton-Dickinson 'FACS' analyser. Results are expressed as numbers of cells against fluorescence, detected using logarithmic detector amplification.

Replication and analysis of data

All results are expressed as the percentage of applied lymphocytes which adhere to the monolayers. The data points are the

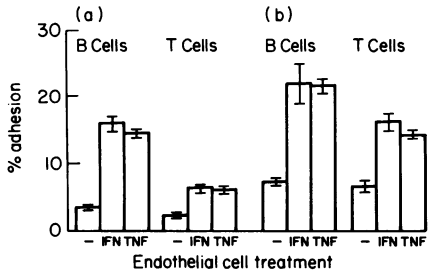

Figure 1. B and T cell-enriched populations were applied to brain endothelial monolayers, untreated (−) or activated 24 hr previously with 50 U/ml of IFN-γ (IFN) or 25 ng/ml TNF-α (TNF), in an adhesion assay. The lymphocytes were either untreated (a) or activated for 16 hr with Con A (b).

mean and SEM of five or six identical determinations performed on the same plate. Each experiment was carried out three times with concordant results, except for the binding of separate lymph node populations, which was performed six times. Correlation between proportions of cell types within a lymph node and the adhesion of those cells to brain endothelium was determined by Fischer's rank correlation test. FACS analysis of CD18-positive cells was carried out twice with identical results. Other immunofluorescence assays were done on three to six separate occasions, and results expressed as mean and standard deviation.

RESULTS

Lymphocytes prepared from pooled peripheral lymph nodes were enriched for T cells and B cells using nylon-wool columns. Half of each population was cultured for 16 hr with 5 μg/ml Con A. They were then labelled with ^{51}Cr and applied to normal brain endothelium, or endothelium activated with IFN-γ or TNF-α for 24 hr, and adhesion to the monolayers was measured (Fig. 1). The results showed that normal B cells bind slightly more strongly to the endothelium than normal T cells, and that binding of both cell types is enhanced by cytokines. Endothelial activation has a greater effect on B-cell adhesion than T-cell adhesion. Activation of lymphocytes enhances adhesion for both cell types, and again activated B cells bind more strongly than activated T cells.

In the next series of experiments we analysed the relative binding strength of $CD4^+$ and $CD8^+$ T cells. In the first experiments we applied whole populations of lymph node cells to the endothelium and analysed relative binding efficiency by a new method which allows staining of adherent cells *in situ*. Examples of this method are shown in Fig. 2. The advantage of this method is that whole populations of lymphocytes can be analysed for their binding efficiency, without preliminary purification steps, involving either adhesion or panning—we were concerned that such preliminary steps might skew the cell populations with respect to their adhesion properties.

We measured the percentage of each phenotype in the population applied to the monolayers by immunofluorescence and the percentage bound by *in situ* staining on the monolayers. Results are expressed as relative binding: binding ratio (CD4) = %$CD4^+$ cells bound/%$CD4^+$ cells applied; binding ratio (CD8) = %$CD8^+$ cells bound/%$CD8^+$ cells applied.

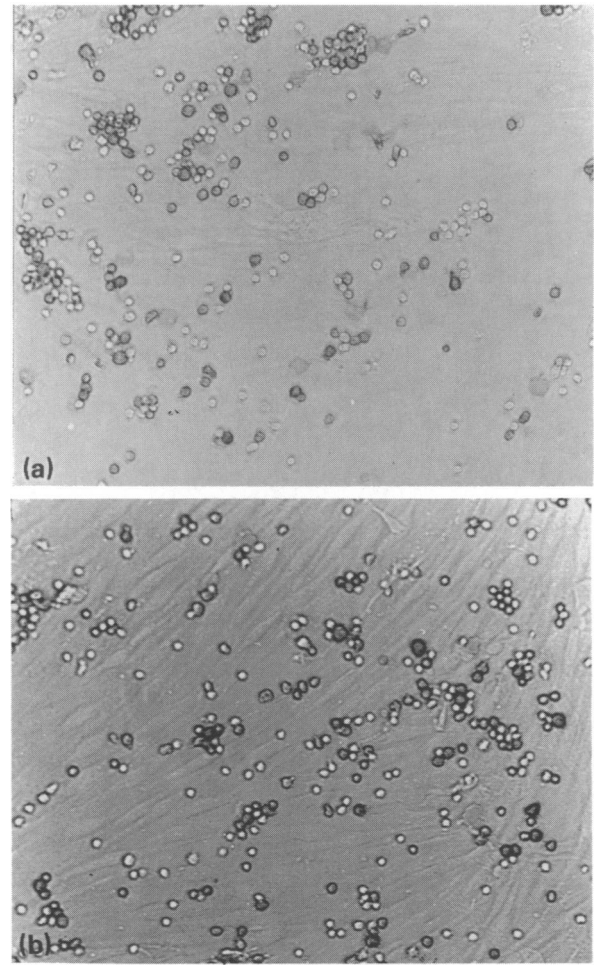

Figure 2. Examples of *in situ* staining of adherent Con A-activated cells from a mixed lymph node population. Antibody to CD4 is shown in (a) and anti-CD8 in (b).

In every case, using either activated or control lymphocytes and activated or control endothelium, we found that $CD8^+$ cells bound more strongly than $CD4^+$ cells (Table 1). We also carried out the same experiment using nylon-wool enriched T cells (Table 1c), which confirmed the result with whole lymph node populations.

We then determined whether the differences between the binding of the lymphocyte subpopulations or the differences between activated and control lymphocytes could be attributed to their surface levels of LFA-1, detected by anti-CD18, since it has been proposed that lymphocytes bind to ICAM-1 and ICAM-2 on endothelium using LFA-1. Contrary to this hypothesis, normal T cells expressed higher surface levels of CD18 than normal or activated B cells or activated T cells (Fig. 3).

We wanted to know whether there is a particular population of lymphocytes which migrates through CNS. It would be anticipated that such cells would accumulate in cervical lymph nodes. Therefore we compared the adhesion of lymphocytes from different lymph nodes to brain endothelium (Fig. 4). In three of six experiments we noted a significantly enhanced binding of activated (but not control) cervical lymph node cells to brain endothelium, but in no case did these cells bind more

Table 1.

Marker	Exp.	Resting lymphocytes		Con A lymphocytes	
		Control	IFN-γ	Control	IFN-γ endothelium
CD2	a	1·42	1·00	1·25	1·04
	b	1·04	1·10	1·03	1·02
	c	0·94	0·76	1·05	1·04
CD4	a	0·88	0·59	1·03	0·69
	b	0·68	0·56	0·87	0·75
	c	0·62	0·33	0·56	0·51
CD8	a	1·22	1·65	1·69	1·46
	b	1·53	1·84	2·13	2·17
	c	1·56	3·30	2·35	3·16

Binding ratios of $CD2^+$, $CD4^+$ and $CD8^+$ T cells to brain endothelium in three separate experiments (a, b, c). In (a) and (b) whole lymph node populations were used (72% T cells) and in (c) nylon-wool purified T cells were applied (95% T cells). Lymphocytes were either untreated (resting) or Con A stimulated for 16 hr before assay (Con A). Endothelium was either untreated (control) or stimulated for 24 hr before assay with 50 U/ml IFN-γ (IFN-γ).

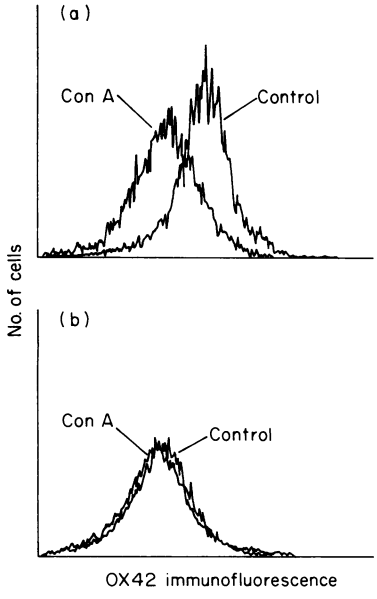

Figure 3. FACS analysis of enriched T cells (a) and B cells (b) stained with OX42. Normal T cells showed highest expression.

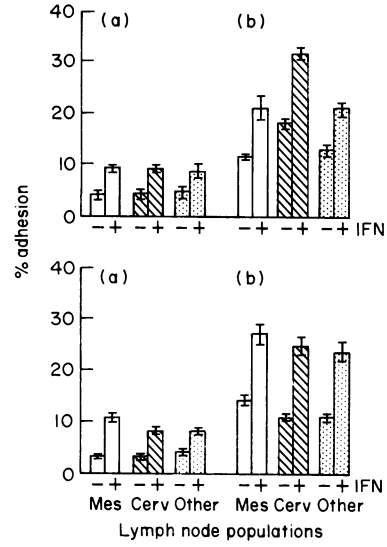

Figure 4. Two examples of how lymphocytes from different groups of lymph nodes (PLN cells) bind to brain endothelium. (a) Shows binding of normal lymphocytes, and (b) shows Con A-activated cells. Binding is shown for either normal (−) or IFN-γ-activated endothelium (+). The upper experiment is typical of those where binding of activated lymphocytes to cervical lymph node cells (Cerv) was greater than that of mesenteric (Mes) or other nodes. The lower experiment is typical of those experiments which showed no such differential.

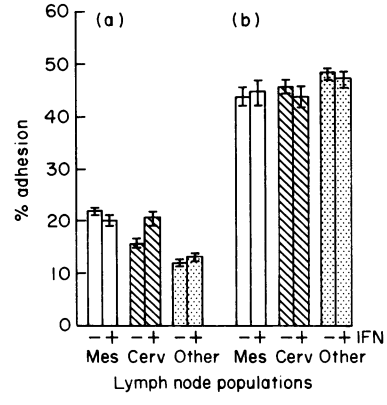

Figure 5. Binding of lymphocytes from peripheral lymph nodes (PLN cells) to aortic endothelium. Normal lymphocytes are shown in (a) and activated cells in (b). Adhesion was measured to normal (−) or IFN-γ-activated endothelium (+).

strongly to aortic endothelium than did other lymphocytes (Fig. 5). This demonstrated that the preferential binding of cervical lymph node cells is not due to greater non-specific adhesiveness of these cells. However, the findings with cerebral endothelium were not always consistent (Fig. 4, lower panel). Complete data for six experiments using activated lymphocytes is shown in Table 2.

In view of our previous findings, we considered the possibility that the preferential binding of cervical lymph node cells to brain endothelium, could be explained by a skewed distribution of lymphocyte subpopulations within these nodes. Table 3 shows that there is no systematic difference between the numbers of $CD2^+$, $CD4^+$ and $CD8^+$ T cells or B cells in the different lymph nodes. However, when individual experiments were analysed separately, there was seen to be a negative correlation between the numbers of $CD4^+$ cells in any particular lymph node and the overall level of adhesion to endothelium (Fig. 6). The results were significant using activated lymphocytes ($Rho = -0{\cdot}7$, $P < 0{\cdot}05$) but only marginal with normal lymphocytes ($Rho = -0{\cdot}533$, $0{\cdot}05 < P < 0{\cdot}1$). There were corresponding

Table 2.

	Normal endothelium		IFN- endothelium	
	Control LNC	Cervical LNC	Control LNC	Cervical LNC
1	11·74±0·76	18·22±2·57***	21·74±5·18	31·86±2·76***
2	13·32±2·83	15·59±1·55*	22·47±3·57	28·01±4·19***
3	14·75±2·37	11·23±1·32††	27·64±4·87	25·40±1·04
4	13·75±1·59	11·99±6·8	30·34±4·51	23·86±3·71†
5	15·69±1·74	16·00±4·93	41·54±2·97	35·94±4·81†
6	10·01±5·11	9·09±3·03	13·45±2·59	19·52±9·02***

The binding of activated lymph node cells (LNC), derived from control (non-cervical) and cervical lymph nodes, was measured on normal and IFN-γ-activated brain endothelium. Results of six different experiments are shown. Data are expressed as mean percentage adhesion and standard deviation, from six identical determinations in each experiment. The one-tailed *t*-test was used to examine the hypothesis that the binding of cervical cells is no greater than that of control cells (***$P<0.001$: *$P<0.05$) Experiments 1, 2 and 6 show significantly greater binding by cervical lymph node cells. Re-analysis of the data, to determine whether the populations of cervical cells differed in any way (higher or lower) from control LNC, showed that in three cases the binding of these cells was actually significantly lower (†$P<0.05$: ††$P<0.01$).

Table 3.

	CD2	CD4	CD8	CD45R.ABC
Lymph nodes				
Cervical	63·1±9·2	51·8±6·9	19·6±1·6	28·9±8·4
Mesenteric	66·6±5·2	51·0±7·1	18·1±4·3	20·2±9·9
Other	74·9±7·0	63·0±6·3	19·7±7·8	22·4±6·5

Percentages of lymphocytes expressing surface markers CD2, CD4, CD8 and CD45R.ABC (B-cell restricted) in preparations derived from different groups of lymph nodes. Data are mean and standard deviation of individual preparations from five different rats.

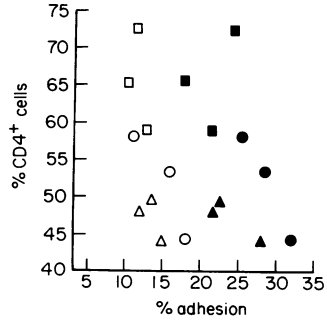

Figure 6. Correlation between percentage of Con A-activated CD4+ cells present in a lymph node preparation and the adhesion level (percentage adhesion) of those cells to brain endothelium. Open symbols = adhesion to untreated endothelium. Closed symbols = adhesion to IFN-γ-activated endothelium. Triangles = mesenteric lymph node cells. Circles = cervical lymph node cells. Squares = mixed other lymph node cells.

weak positive correlations between the numbers of B cells or CD8+ cells and adhesion. This result shows that the cervical lymph nodes sometimes contain a CNS preferential-homing population, but this can be simply explained in terms of the percentages of B cells and CD8+ cells present, and it is not necessary to propose a brain-specific homing mechanism.

DISCUSSION

Patterns of selective lymphocyte migration into secondary lymphoid tissues have been well documented and involve interaction of circulating cells with high endothelial venules in lymph nodes, Peyer's patches and other sites. Small resting lymphocytes tend to migrate into these tissues using homing receptors such as MEL-14, VLA-4 and CD44.[13] In contrast, large activated lymphocytes tend to migrate into non-lymphoid tissues, particularly at sites of inflammation.[14] Selective migration appears to be mediated by a combination of locally expressed receptors on the endothelium interacting with ligands preferentially expressed on particular lymphocyte populations. Endothelial receptors may be site-specific, such as the vascular addressins, or may be induced by cytokines (e.g. ICAM-1).[15] Likewise, lymphocyte adhesion to endothelium depends both on cell lineage and its state of activation. We have previously shown that lymphocyte adhesion to cerebral endothelium depends both on endothelial activation by cytokines and the stage of the lymphocyte in its cell cycle.[6] In this study we wanted to examine the possibility that there is selective migration of particular lymphocyte subsets across cerebral endothelium.

Observations of multiple sclerosis plaques have shown there to be a surprising preponderance of CD8+ T cells, ranging from a CD8/CD4 ratio of 1:1 to 50:1 in different studies.[16-18] In EAE the importance of the CD4+ myelin-specific T cells in initiation of the lesions is undisputed,[19] but other mononuclear cells are recruited shortly afterwards[20] and in the latest stages CD8+ T cells may predominate.[21] These findings have lead to the suggestion that whereas CD4+ cells are essential for initiation of acute disease, CD8+ cells come to predominate as the CNS lesions progress. Our findings that CD8+ cells bind to IFN-γ-activated cerebral endothelium more efficiently than CD4+ cells indicates a mechanism by which this shift in subpopulations could occur.

Several other studies have examined selective lymphocyte adhesion to non-lymphoid endothelium. These include the observation of enhanced binding of CD4+,29+ cells to large vessel endothelium[22] and of increased natural killer (NK) cell binding to microvascular (foreskin) endothelium *in vitro*.[23] The differences between these various studies is probably best explained by the heterogeneity of the endothelia.[24]

It is thought that migration of cells into non-lymphoid tissues is controlled by different sets of adhesion molecules to those which control migration into lymphoid tissues. Moreover, there is evidence for at least three groups of interacting molecules;[25] some are induced by cytokines, while others mediate basal levels of adhesion. We considered the possibility that LFA-1 on the lymphocytes, interacting with ICAM-1 on the endothelium, is involved in mediating binding of lymphocytes to cytokine-activated endothelium.[26] ICAM-1 is induced by IFN-γ on brain endothelium (our unpublished observations) and has been identified immunohistochemically at inflammatory sites on brain endothelium in some species. The time-

course of ICAM-1 induction corresponds with the rate of change of lymphocyte adhesion to brain endothelium, following cytokine activation.[7] We have also shown that anti-CD18 can partly block the enhanced binding of normal lymphocytes to IFN-γ-activated endothelium.[27] For these reasons, we considered ICAM-1/LFA-1 interaction to be a candidate for controlling lymphocyte movement into CNS when immune reactions are underway. The observation that CD18 levels do not correspond with adhesiveness of different subsets suggests that this ligand is not the only one controlling adhesion. However, we cannot exclude the possibility that the LFA-1 exists in different affinity states on the populations, as occurs following T-cell activation.[28] We have noted that removal of either Ca^{2+} or Mg^{2+} from adhering cells partly blocks adhesion, and this also indicates that the interaction cannot be mediated by LFA-1 alone (Mg^{2+}-dependent). The finding accords with that of Pitzalis and colleagues who found that preferential adhesion of subsets of $CD4^+$ cells was also unrelated to LFA-1 expression,[22] although another study had suggested that ICAM-1 adherent cells are skewed towards CD8 positivity.[29]

In total our work shows that different populations of lymphocytes have differential binding to cerebral endothelium, which is often reflected in the cervical lymph node populations. Although LFA-1/ICAM-1 may contribute to these lymphocyte/endothelial interactions, it is not the only adhesion system.

ACKNOWLEDGMENT

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