Immunology 1996 **87** 559–565

Differential regulation of tissue-specific lymph node high endothelial venule cell adhesion molecules by tumour necrosis factor and transforming growth factor-β_1

Y.-H. CHIN,* M.-W. YE,* J.-P. CAI*† & X.-M. XU* *Departments of *Microbiology and Immunology and †Dermatology and Cutaneous Surgery, University of Miami School of Medicine, Miami, FL, USA*

SUMMARY

Lymphocytes migrate from blood into lymph nodes (LN) of rats specifically at segments of venules lined by high endothelium (HEV). We have previously shown that pretreatment of LN HEV cells with pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), augments their adhesiveness for thoracic duct lymphocytes (TDL). Here we report that a mouse monoclonal antibody, 3C10, recognized tissue-specific endothelial determinants on rat LN HEV cells and blocked their adhesiveness for TDL and EL-4J cells transfected with rat L-selectin. In contrast, 3C10 antibody did not inhibit lymphocyte attachment to Peyer's patch (PP) frozen sections or cultured PP HEV cells. The antibody immunoprecipitated from LN HEV cells two proteins with apparent molecular weights of 90 000 and 50 000. The expression of 3C10 antigen on LN HEV cells was increased by incubation with TNF-α or IFN-γ. Furthermore, pretreatment of cytokine-stimulated LN HEV cells with 3C10 antibody blocked TDL binding in a dose-dependent manner. In contrast, 3C10 antigen expression on LN HEV cells was significantly decreased following incubation of cells with transforming growth factor-β_1 (TGF-β_1). In addition, TGF-β_1 also abrogated the adhesiveness of LN HEV cells stimulated with TNF-α, IFN-γ or both cytokines. Together, these data suggest that endothelial determinants recognized by the 3C10 antibody are tissue-specific ligands for lymphocyte adhesion and cytokines such as TNF-α and TGF-β differentially regulate their expression and function.

INTRODUCTION

Lymphocytes in the bloodstream migrate into lymph nodes (LN) by first adhering to high endothelial cells lining the postcapillary venules (HEV).[1–3] The initial binding is mediated by L-selectin on lymphocytes recognizing complementary ligands (vascular addressins) on LN HEV cells.[4–6] Under shear force conditions, firm adhesion and transendothelial migration of lymphocytes also require the participation of additional ligands such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1).

The establishment of high endothelial cell cultures from rat LN and Peyer's patches (PP) have facilitated investigation into the factors that control the adhesiveness of HEV cells to promote lymphocyte binding.[7–10] There is strong evidence that a network of cytokines differentially regulates the ability of HEV cells to mediate lymphocyte adhesion. Thus, pretreatment of rat LN or PP HEV cells with tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-4 (IL-4) stimulate their adhesiveness for thoracic duct lymphocytes (TDL) in a dose- and time-dependent manner. In contrast, preincubation of PP HEV cells with transforming growth factor-β (TGF-β) leads to a rapid decrease in high endothelial adhesiveness for TDL.[11] Interestingly, TGF-β treatment also abrogated the stimulating effect for TNF-α and IFN-γ on HEV cells. The mechanisms by which these cytokines regulate the adhesiveness of LN HEV cells is unclear at present.

To characterize tissue-specific endothelial determinants on rat LN HEV cells, we developed a mouse monoclonal antibody, designated as 3C10, that selectively inhibited the ability of LN HEV cells, but not PP HEV cells, to bind TDL or rat L-selectin transfectants. We also investigated in this study the differential effects of TNF-α, IFN-γ and TGF-β_1 on the expression and function of 3C10 antigens on LN HEV cells.

MATERIALS AND METHODS

Animals and lymphocytes

Female Sprague–Dawley rats [Cgl: CD(SD)BR] were purchased from Charles River Breeding Laboratories, Wilmington, MA and were used at 8–12 weeks of age. The thoracic ducts of rats were cannulated and lymphocytes were obtained

Received 9 August 1995; revised 5 November 1995; accepted 5 November 1995.

Correspondence: Yee-Hon Chin, Department of Microbiology and Immunology, University of Miami School of Medicine, P.O. Box 016960 (R-138), Miami, FL 33101, USA.

from lymph collected for 3–4 hr beginning at 20 or 40 hr after cannulation.[12] The cells were washed in RPMI medium and resuspended at 5×10^6/ml. Cell viability for all preparations was at least 90%, as measured by trypan blue exclusion.

Cytokines

Murine recombinant TNF-α (rTNF-α), IFN-γ or granulocyte–macrophage colony-stimulating factor (GM-CSF) (3×10^7 U/ml) were purchased from Genzyme, Boston, MA. Porcine platelet-derived TGF-β_1 (R and D Systems, Minneapolis, MN) was reconstituted in 4 mM HCl to 1 μg/ml. The cytokines were stored at −70° and used within 1 month.

Transfections

The full length rat L-selectin cDNA was isolated in our laboratory and directionally cloned into the expression vector pcDNA3 (Invitrogen, San Diego, CA). For transfection, 10 μg of the L-selectin-pcDNA3 vector were electroporated (Bio-Rad electroporator set at 960 uF, 300 V) into 5×10^6 EL4J cells (kindly provided by Dr Tom Malek, University of Miami, FL), a murine T-cell line that does not express L-selectin and lacks the capacity to bind to rat LN HEV. Stable transfectants were selected by G418 (800 μg/ml; Life Technologies, Grand Island, NY) resistance, stained with the A.11.5 antibody and cloned by limiting dilution. Clones expressing high levels of L-selectin were identified by flow cytometry and expanded.

HEV cell culture

Rat LN and PP HEV cells were prepared and characterized as previously described.[9,10] The isolated HEV cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 10% fetal calf serum (FCS), 10 mM HEPES, 1 mM L-glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids supplemented with 5 ng/ml of purified endothelial cell growth factor-α (Collaborative Research, Walham, MA) and 5 U/ml of heparin. The endothelial origin of these cells was confirmed by uptake of acetylated low-density lipoprotein and to incorporate [^{35}S]sodium sulphate. These cells also stained positive with endothelial-specific *Ulex europeus* lectin and the OX43 antibody. The culture cells were routinely used at passages 2 and 3.

Antibodies

The monoclonal antibody 3C10 was prepared by fusion of spleen cells from immunized mice with the P3X63 myeloma cells as detailed elsewhere.[13] Rat LN HEV cells were emulsified in RIBI adjuvant (RIBI Immunochemicals, Hamilton, MT) and groups of five mice were each immunized intraperitoneally (i.p.) with 5×10^6 LN HEV cells. The animals were boosted twice at 2-week intervals with LN HEV cells in RIBI adjuvant. Sera from immunized animals were tested by enzyme-linked immunosorbent assay (ELISA) and immunoperoxidase for reactivity to cultured LN HEV cells and LN frozen sections, respectively. The spleen cells were fused with the murine myeloma cell line P3X63 using standard protocols. In the initial screening, positive clones that reacted with cultured LN HEV cells, but not with the rat epithelial cell line, RT-2, were identified by ELISA. Thereafter, the positive clones were selected by limiting dilution and tested for the ability to detect antigens on HEV by immunoperoxidase staining of LN and PP frozen sections. One clone, designated as 3C10, reacted specifically with HEV of LN but not with HEV of PP and was selected for this study. The isotype of 3C10 monoclonal antibody (mAb) was determined by ELISA to be IgG2a.

The A.11.5 mAb was produced in our laboratory and has previously been shown to block rat lymphocyte binding to LN HEV cells.[10] The antibody immunoprecipitated a 90 000 MW glycoprotein from rat thoracic duct lymphocytes. Molecular cloning of the gene encoding the A.11.5 antigen indicates that the antibody is specific for rat L-selectin.[14] The OX-1 (anti-leucocyte common antigen) mAb and OX-43 (anti-rat endothelial cell) mAb were obtained from Accurate Chemicals (Westbury, NY). The antibodies were purified from ascites by protein-G column chromatography.

The mouse anti-rat VLA-4 antibody TA-2,[15] and the hamster anti-rat L-selectin mAb, HRL-2 and HRL-3,[16] were purchased as purified ascites from Seikagaku America (Rockville, MD). The HRL-3, but not HRL-2, antibody inhibited rat lymphocyte adhesion to peripheral LN frozen sections.[16]

Immunoperoxidase

Five micron-thick frozen sections of rat LN and PP were placed on poly-L-lysine-coated slides and fixed in acetone–formaldehyde as described previously.[17] The tissue sections were sequentially stained with 3C10 mAb (20 μg/ml), affinity-purified biotinylated goat anti-mouse IgG, and the avidin–biotin immunoperoxidase staining system (Dako Immunochemicals, Carpinteria, CA) according to the manufacturer's instructions. Diaminobenzidine in hydrogen peroxide was used as the chromogen and the sections were counterstained with haematoxylin.

Immunoprecipitation and sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

Two million LN HEV cells were surface-labelled with 1 mCi of ^{125}I-labelled sodium iodide by the lactoperoxidase method as previously described.[9] The cells were lysed and membrane proteins extracted in detergent lysis buffer containing 1% Triton-X-100, 0·25% sodium deoxycholate, 0·15 M NaCl, 50 mM Tris, 5 mM EDTA, 1 mM phenylmethylsulphonylchloride, 1 μg/ml leupeptin and 100 U/ml aprotinin as protease inhibitors. Immunoprecipitation was performed as described in detail elsewhere.[14] Immunoprecipitated protein was analysed by SDS–PAGE on 10% gels and analysed by autoradiography; the molecular weight (MW) of the immunoprecipitated protein was identified using ^{14}C-methylated protein markers. For glycosylation analysis, the immunoprecipitated proteins were digested with peptide *N*-glycanase or neuraminidase and *O*-glycanase (Oxford Glycosystems) according to manufacturer's instructions prior to SDS–PAGE.

Immunofluorescence analysis

LN HEV cells (1×10^5) were incubated with 2 μg of purified 3C10 antibody for 60 min at 4°. After three washes with phosphate-buffered saline (PBS) containing 20 mM sodium azide and 1 mg/ml bovine serum albumin (BSA) (Sigma, St Louis, MO), the cells were incubated with a 1/50 dilution of fluorescein isothiocynate (FITC)-labelled F(ab')$_2$ fragments of affinity-purified goat anti-mouse immunoglobulin antibody (Southern Biotechnology, Birmingham, AL) for 60 min at 4°. After three additional washes, the labelled cell samples were

analysed by flow cytometry on a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA).

In vitro *lymphocyte adhesion assay*

The lymphocyte–frozen tissue section adherence assay was performed as described.[12] Briefly, 0·2 ml of rat TDL at the indicated concentrations were overlaid onto frozen sections of rat LN or PP and the slides were rotated at 80 r.p.m. for 30 min at 7°. At the end of the incubation period, the non-adherent cells were removed, the slides refixed in glutaraldehyde, and stained with methyl green-thionin. Data were expressed as the mean number of positive HEV (more than two lymphocytes bound per venule) ± SEM of eight sections.

The procedures used to assay for lymphocyte binding to cultured HEV cells have been described in detail recently.[9] To assay the effects of cytokines on endothelial adhesiveness, confluent monolayers of LN HEV cells in 48-well plates were incubated at 37° with the cytokines for the indicated period of time. The HEV cells were washed extensively with DMEM medium and overlaid with $2{\cdot}5 \times 10^6$ ^{51}Cr-labelled TDL in assay medium, and incubated for 1 hr at 37° in 5% CO_2. The non-adherent cells were then removed by washing with assay medium, the adherent cells lysed in 1% nonident P-40 (NP-40), and the radioactivity bound obtained by counting in a LKB gamma counter. The percentage of lymphocytes adhering to the HEV monolayer was calculated as: percentage bound = (c.p.m. in 0·5 ml lysate divided by c.p.m. in 0·5 ml of original lymphocyte suspension) × 100. Statistical analysis was performed by the Student's *t*-test.

Effect of mAb on lymphocyte–HEV adhesion

For blocking rat TDL or L-selectin-transfected EL4J cell adhesion to LN and PP frozen sections, each tissue section was pretreated with the indicated concentrations of 3C10 or the control OX43 mAb for 30 min at 4°. The slides were then washed three times in PBS and overlaid with lymphocytes for the frozen section adhesion assay. To test the effect of 3C10 mAb on the lymphocyte-binding properties of cultured HEV cells, the HEV cells were incubated with 3C10 or OX43 mAb for 30 min at 37°, washed once, and overlaid with ^{51}Cr-labelled TDL. In some experiments, TDL were incubated with 20 μg of A.11.5, HRL-3, anti-rat VLA-4, or the control OX1 antibody for 30 min at 37° and assayed for binding to unstimulated and cytokine-stimulated LN HEV cells.

RESULTS

Tissue distribution of 3C10 endothelial determinants

The tissue distribution of the 3C10 antigen was evaluated by immunoperoxidase staining for reactivity to rat lymphoid and stromal tissues. Among the tissues tested, the 3C10 mAb stained specifically antigens on cervical and mesenteric LN frozen sections. As shown in Fig. 1, the pattern of staining in the LN was restricted to postcapillary high endothelial venules. The affinity-purified goat anti-mouse IgG secondary antibody was pre-absorbed with rat immunoglobulin and did not stain LN frozen sections (data not shown). In addition, there was no staining of PP, spleen, thymus, liver, lung, or normal skin (data not shown).

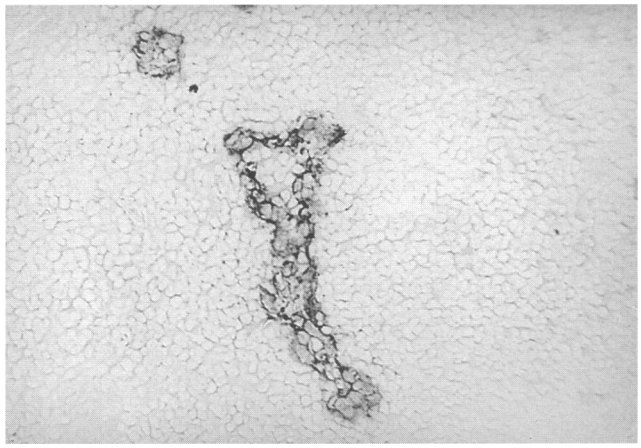

Figure 1. Immunoperoxidase staining of rat LN by the 3C10 mAb. The immunoperoxidase staining was performed on frozen LN sections as described in the Materials and Methods. Note the specific staining to discrete areas of HEV and lack of staining to capillaries and large blood vessels. Original modification × 400.

Immunoprecipitation and SDS–PAGE analysis of 3C10 antigens

Immunoprecipitation and SDS–PAGE experiments were performed to analyse the antigens on LN HEV cells detected by the 3C10 antibody. As shown in Fig. 2a, the 3C10 antibody immunoprecipitated from lysates of peripheral and mesenteric LN HEV cells two polypeptides with apparent MW of 87 000 and 50 000. Treatment of the immunoprecipitates with peptide *N*-glycanase did not alter the sizes of the proteins, whereas incubation with neuraminidase and *O*-glycanase reduced the size of the 50 000 band to 37 000 (Fig. 2b).

Effects of 3C10 mAb on lymphocyte–HEV binding

Table 1 shows the effect of antibody pretreatment on TDL binding to LN and PP frozen sections. The 3C10 mAb tested at 10 or 20 μg/ml significantly ($P < 0{\cdot}001$) inhibited TDL binding to HEV of LN. In contrast, the antibody treatment had no effect on lymphocyte binding to HEV of PP at concentrations

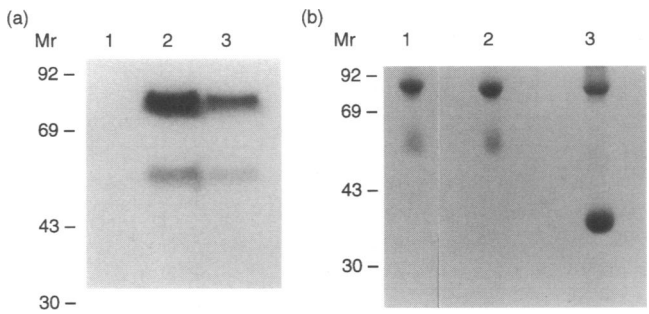

Figure 2. (a) Autoradiograph of SDS–PAGE analysis of 3C10 immunoprecipitated ^{125}I-labelled protein from Peyer's patch (lane 1), peripheral LN (lane 2) and mesenteric LN (lane 3) HEV cells. (b) 3C10 immunoprecipitated radioiodinated protein from peripheral LN (lane 1), digested with *N*-glycanase (lane 2), or with neuraminidase and *O*-glycanase (lane 3). The relative MW were established with methylated ^{14}C-protein standards.

Table 1. 3C10 antibody blocks the endothelial adhesiveness of HEV of LN but not HEV of PP for TDL

Source of tissue section	Section pretreatment*	No. positive HEV† per section
LN	3C10 (20 μg/ml)	4 ± 1‡
	OX43 (20 μg/ml)	41 ± 6
LN	3C10 (10 μg/ml)	8 ± 3‡
	OX43 (10 μg/ml)	44 ± 5
LN	3C10 (1 μg/ml)	46 ± 10
	OX43 (1 μg/ml)	54 ± 7
PP	3C10 (40 μg/ml)	13 ± 3
	OX43 (40 μg/ml)	14 ± 3
PP	3C10 (20 μg/ml)	20 ± 4
	OX43 (20 μg/ml)	18 ± 5

* Each tissue section was pretreated with 3C10 antibody for 30 min at 7°; the sections were then rinsed once, were overlaid with untreated TDL and the HEV binding assays were performed as described in the Materials and Methods.

† Mean ± SEM of four sections.

‡ Significantly reduced ($P < 0.001$).

as high as 40 μg/ml. The antibody did not react with lymphocyte surface determinants, because no inhibition occurred when TDL were pretreated with 3C10 mAb, washed and overlaid onto untreated LN sections (data not shown). Comparable results have been obtained in five different experiments.

These findings suggest that the endothelial determinants recognized by 3C10 mAb mediate the binding of lymphocytes to LN HEV but not to PP HEV. To investigate further this possibility, we analysed the ability of 3C10 mAb to block the adhesiveness of LN tissue sections for rat L-selectin-transfected EL4J cells. As shown in Table 2, rat L-selectin transfectants, but not untransfected cells or cells transfected with the expression vector alone, adhered selectively to LN HEV. At the functional level, the binding of L-selectin transfectants to

Table 2. 3C10 antibody blocks the endothelial adhesiveness of LN HEV to bind L-selectin-transfected EL4J cells

Lymphocyte*	Tissue section pretreatment†	No. positive HEV per section‡
EL4J	None	1 ± 1
EL4J-pcDNA3	None	1 ± 1
EL4J-L-selectin	None	38 ± 7
EL4J-L-selectin	3C10	5 ± 2§

* EL4J cells (5×10^6) were overlaid onto LN frozen sections and the HEV binding assays were performed as described.

† Each LN section was untreated or treated with 20 μg of 3C10 antibody for 30 min at 7°; the sections were then rinsed once and overlaid with EL-4J cells for the HEV binding assay.

‡ Mean ± SEM of four sections.

§ Significantly reduced ($P < 0.005$).

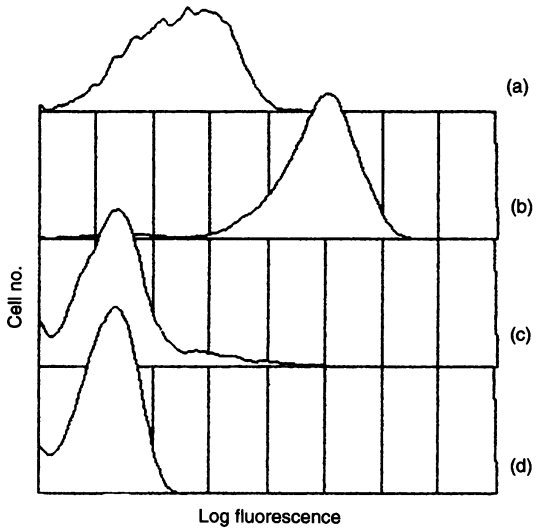

Figure 3. Expression of 3C10 antigens on TNF-stimulated LN HEV cells. The LN HEV cells were unstimulated (panel a), or stimulated with TNF (panel b) alone for 8 hr, or stimulated with TNF for 8 hr and then incubated in TGF-β for an additional 8 hr (panel c). The cells were then stained with 3C10 mAb followed by FITC-conjugated goat anti-mouse IgG as described in the Materials and Methods. For each experiment, a total of 5×10^3 cells was analysed on a FACScan flow cytometer. In panel d, unstimulated or cytokine-stimulated LN HEV cells were stained with isotype-matched mouse immunoglobulin and FITC-conjugated goat anti-mouse IgG and represented control fluorescence. The given profiles are representative of four separate experiments.

LN HEV was significantly ($P < 0.01$) inhibited when the tissue sections were pretreated with 20 μg of 3C10 antibody. Importantly, binding to untreated LN HEV was also blocked by pretreatment of the L-selectin-transfectants with either A.11.5 or the HRL-3 antibody.

We investigated the effect of 3C10 mAb treatment on the adhesiveness of cultured LN or PP HEV cells for TDL at 37°. Treatment of cultured LN HEV cells with 3 μg/ml of 3C10 IgG blocked the adhesion of TDL, with maximal inhibition observed at 5 μg/ml of antibody. Furthermore, the 3C10 antibody also inhibited binding of L-selectin-transfected EL4J cells to LN HEV cells. The antibody, however, did not interfere with the capacity of PP HEV cells to bind to TDL (data not shown).

Effect of 3C10 antibody on adhesiveness of cytokine-stimulated LN HEV cells

The next series of experiments were designed to investigate the expression and function of the 3C10 endothelial determinants on cytokine-stimulated LN HEV cells. Flow cytometry analysis indicated the 3C10 antigen was expressed on cultured LN HEV cells (Fig. 3, panel a). Furthermore, incubation of HEV cells with 20 U/ml of recombinant murine TNF-α for 8 hr significantly increased the level of expression (Fig. 3, panel b). Similarly, incubation of LN HEV cells with 20 U/ml of IFN-γ also stimulated 3C10 antigen expression (data not shown).

To investigate the functional roles of 3C10 determinants to promote endothelial adhesiveness for lymphocytes, cultured LN HEV cells were incubated with TNF-α for 8 hr, washed and

Table 3. Effect of 3C10 mAb on the adhesiveness of TNF-α and IFN-γ stimulated LN HEV cells

	LN HEV cells incubated with			
mAb treatment*	Medium	TNF-α†	IFN-γ†	TNF-α + IFN-γ†
None	14·5 ± 3·2‡	34·2 ± 4·9	37·5 ± 3·8	43·3 ± 3·7
OX43	11·3 ± 2·2	36·4 ± 2·8	34·2 ± 4·5	47·1 ± 4·2
3C10	3·6 ± 1·1§	7·2 ± 1·4§	4·9 ± 1·2§	8·6 ± 2·1§

* LN HEV cells incubated in culture medium or cytokines were pretreated with 20 μg/ml of OX43 or 3C10 mAb, or medium alone for 30 min at 37°. The cells were then washed once and assayed for TDL adhesion.

† LN HEV cells were incubated with 20 U/ml of recombinant murine TNF-α, IFN-γ, or a combination of TNF-α and IFN-γ for 8 hr at 37°. The cytokine-treated cells were washed three times in culture medium and treated with mAb or medium and then assayed for lymphocyte binding.

‡ Values equal mean percentage of radioactivity bound ± SD of quadruplicate experiments.

§ $P < 0.005$ as compared with TDL binding to LN HEV cells treated with medium alone or control OX43 antibody.

treated with either 3C10 or OX43 mAb for 60 min and then tested for lymphocyte binding. The results in Table 3 showed that treatment of TNF-α-stimulated LN HEV cells with 20 μg/ml of 3C10 antibody significantly ($P < 0.005$) decreased the adhesiveness for lymphocytes as compared to LN HEV cells treated with medium or with the control OX-43 mAb alone (7% versus 34%). In addition, 3C10 antibody blocked the adhesiveness of HEV cells stimulated with 20 U/ml of IFN-γ, or a combination of TNF-α and IFN-γ (Table 3).

Effect of TGF-β on cytokine-stimulated LN HEV cells

In the next series of experiments, we investigated the effect of TGF-β treatment on the lymphocyte-binding properties of unstimulated and cytokine-stimulated LN HEV cells. As shown in Fig. 4, TGF-β decreased the adhesiveness of LN HEV cells in a dose-dependent manner; TGF-β at concentration as little as 0·5 ng/ml decreased TDL binding by > 65%. The inhibitory effects were most pronounced when LN HEV cells were incubated for 8–48 hr in the continued presence of 0·5 ng/ml TGF-β (data not shown).

The inhibitory effects of TGF-β could be extended to TNF-α and IFN-γ-stimulated LN HEV cells. When LN HEV cells were stimulated with optimal doses of TNF-α or IFN-γ and then incubated with 0·5 ng/ml of TGF-β, flow cytometry analysis indicated that TGF-β significantly decreased 3C10 antigen expression on LN HEV cells stimulated with TNF-α (Fig. 3, panel c) or IFN-γ (data not shown). TGF-β treatment also significantly abrogated the HEV adhesiveness stimulated by optimal doses of TNF-α or IFN-γ, or both cytokines (Fig. 5).

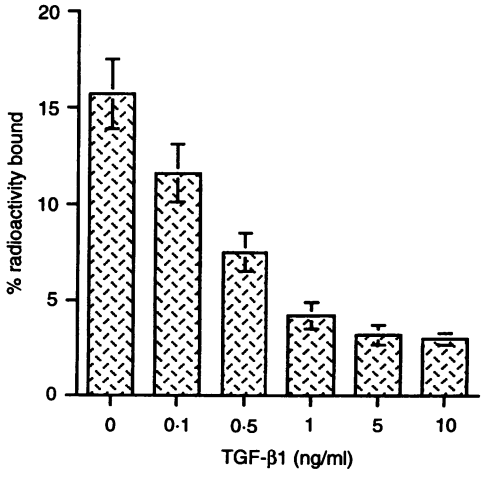

Figure 4. Modulation of LN HEV cell adhesiveness for rat TDL by TGF-β. Cultured LN HEV cells were incubated with increasing doses of TGF-β for 8 hr, washed, and ^{51}Cr-labelled TDL were then added to assay for lymphocyte binding. The results are expressed as mean percentage radioactivity bound ± SD of triplicates and are representative of three separate experiments.

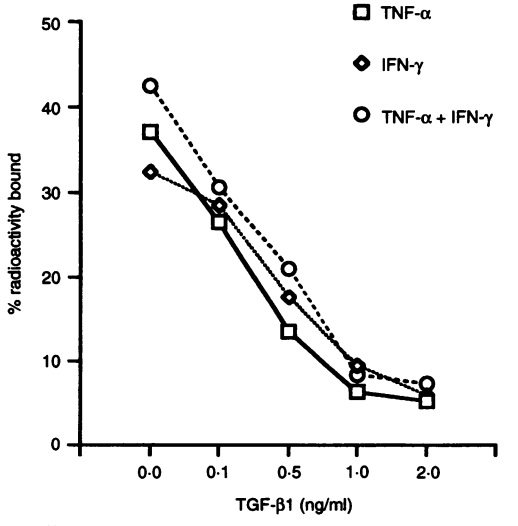

Figure 5. Effects of TGF-β on cytokine-stimulated LN HEV cell adhesiveness. The LN HEV cells were pretreated with 20 unit of TNF, or IFN-γ, or with both cytokines for 8 hr. Increasing doses of TGF-β or culture medium alone were then added and the cells were incubated for an additional 8 hr before assaying for lymphocyte binding. Each point represented the mean ± SEM of quadruplicate determinations.

DISCUSSION

The present study demonstrates that endothelial determinants detected by 3C10 mAb are involved in the binding of LN HEV cells to lymphocytes *in vitro*. This conclusion is supported by the observation that 3C10 antibody blocked the ability of HEV of LN, but not HEV of PP to bind rat lymphocytes. In addition, the finding that pretreatment of LN frozen sections with 3C10 antibody blocked binding of rat L-selectin-transfected EL4J cells provides strong evidence that the antibody recognizes tissue specific endothelial determinants on HEV cells.

We have demonstrated previously cytokines such as TNF-α or IFN-γ stimulate the adhesiveness of LN HEV cells for lymphocytes. Treatment of TDL with either A.11.5 or HRL-3 antibody significantly reduced adhesion to TNF-α or IFN-γ-stimulated LN HEV cells, suggesting that lymphocyte binding was mediated by tissue-specific adhesion molecules in this model. We now show that the adhesion-promoting effect of cytokines such as TNF-α and IFN-γ is associated with an increase in 3C10 antigen expression on LN HEV cells. Importantly, the 3C10 mAb also selectively inhibited the ability of cytokine-stimulated LN HEV cells to support TDL binding. If similar changes in 3C10-determinant expression are induced *in situ* on HEV cells by locally released cytokines during antigenic stimulation, they would provide a mechanism to facilitate the recruitment of lymphocytes from blood into LN across the endothelial layer.

Over the past several years, it has been postulated that selectins interact with multiple ligands on endothelial cells[18–20] as well as extravascular ligands in non-lymphoid tissues.[21,22] The L-selectin lectin domain displays affinity for glycoconjugates, particularly the sialylated Lewis X and Lewis A antigens.[23] However, high-affinity interactions may require the epidermal growth factor and the complement-binding protein-like domains.[24,25] Recently, two glycoproteins of 50 000 and 90 000 have been shown to bind murine recombinant L-selectin–IgG chimeric protein.[26] Cloning of the 50 000 molecule, designated as GlyCAM-1, indicates a secreted protein with extensive *O*-linked glycosylation.[27] In contrast, the 90 000 glycoprotein has a protein core identical to the sialomucin CD34,[28] a marker for haematopoietic stem cells. Both GlyCAM-1 and CD34 require extensive post-translational sialylation and sulphation of the core polypeptide for binding to L-selectin.[29] GlyCAM-1 is also antigenically related to the peripheral lymph node vascular addressin.[30] In other studies, recombinant rat L-selectin-mouse IgG fusion proteins precipitated multiple sulphated-glycoproteins from LN lysates with apparent MW of 55 000, 65 000, 120 000, 190 000 and $>250\,000$.[31] In our present study, the 3C10 mAb immunoprecipitated from LN HEV cells two proteins with relative MW of 87 000 and 50 000. The 50 000 protein appears to be extensively *O*-linked glycosylated and sulphated. Experiments are now in progress to investigate whether soluble rat L-selectin exhibits specificity for each of these protein and to analyse the relationship of 3C10 antigens to GlyCAM-1 and CD34.

It has been postulated the lymphocyte emigration from the vasculature is a multiple step process and involves different receptors and ligands.[32] There is strong evidence that L-selectin is important for the initial attachment, as represented by tethering and rolling of leucocytes along the luminal surface of the vasculature. In contrast, tight adhesion and transendothelial migration are thought to be mediated by integrins (reviewed in ref. 33). Although we hypothesize that 3C10 determinants are involved in the initial attachment of lymphocytes to HEV, it is possible these molecules may participate in subsequent adhesive steps. Additional studies are required to investigate the role of 3C10 antigens in the migration of lymphocytes across lymph node high endothelium *in vivo*.

In earlier studies, TGF-β has been shown to decrease the ability of TNF-α- and IL-1β-stimulated human umbilical vein and microvascular endothelial cells to bind neutrophils and T cells, suggesting an active role for this cytokine in decreasing inflammatory cell recruitment.[34–36] Interestingly, TGF-β selectively decreases E-selectin cell surface and mRNA expression on TNF-stimulated human umbilical vein endothelial cells, but does not interfere with ICAM-1 and VCAM-1 expression.[37] Our finding that TGF-β appears to down-regulate preferentially the 3C10-dependent adhesive pathway provides further support for the idea that perivascular TGF-β_1 may limit the magnitude of lymphocyte migration under physiological conditions by regulating the ability of HEV cells to mediate lymphocyte adhesion.

ACKNOWLEDGEMENTS

We wish to thank James Phillips for expert flow cytometric analysis and Michelle Perez for secretarial assistance. This work was supported by NIH Grants AI-23426 and AI-26761 and multidisciplinary projects grant # 9403002 from the American Heart Association, Florida affiliate. J.P.C. was the recipient of a Glaxo Research Fellowship awarded by the Dermatology Foundation.

REFERENCES

1. Gowans J.L. & Knight E.J. (1964) The route of recirculation of lymphocytes in the rat. *Proc R Soc Lond (Biol)* **159,** 257.
2. Marchesi V.T. & Gowans J.L. (1964) The migration of lymphocytes through the endothelium of venules in lymph nodes: an electron microscopic study. *Proc R Soc Lond (Biol)* **159,** 283.
3. Sugimura M., Furuhata K., Kudo N., Takahata K. & Mifune Y. (1968) Fine structure of post-capillary venules in mouse lymph nodes. *J J Vet Res* **12,** 38.
4. Siegelman M., van de Rijn M. & Weissman I. (1989) Mouse lymph node homing receptor cDNA clone encodes a glycoprotein revealing tandem interaction repeats. *Science* **243,** 1165.
5. Lasky L., Yednock T., Singer M. *et al.* (1989) Cloning of a lymphocyte homing receptor reveals a lectin domain. *Cell* **56,** 1045.
6. Tedder T., Issacs C., Ernst T., Demetri G., Adler D. & Disteche C. (1989) Isolation and chromosomal localization of cDNAs encoding a novel human lymphocyte cell surface molecule, LAM-1. *J Exp Med* **170,** 123.
7. Ager A. & Mistry S. (1988) Interaction between lymphocytes and cultured high endothelial cells: an *in vitro* model of lymphocyte migration across high endothelial venule high endothelium. *Eur J Immunol* **18,** 1265.
8. Ise Y., Yamaguchi K., Sato K. *et al.* (1988) Molecular mechanisms underlying lymphocyte recirculation. I. Functional, phenotypical and morphological characterization of high endothelial cultures *in vitro*. *Eur J Immunol* **18,** 1235.
9. Chin Y., Cai J. & Johnson K. (1990) Lymphocyte adhesion to cultured Peyer's patch high endothelial venule cells is mediated by organ-specific homing receptors and can be regulated by cytokines. *J Immunol* **145,** 3669.
10. Chin Y.H., Cai J. & Xu X. (1991) Tissue-specific homing receptor

mediates lymphocyte adhesion to cytokine-stimulated rat lymph node high endothelial cells. *Immunology* **74,** 478.

11. CHIN Y.H., CAI J.P. & XU X. (1992) Transforming growth factor beta 1 and IL-4 regulate the adhesiveness of Peyer's patch high endothelial venule cells for lymphocytes. *J Immunol* **148,** 1106.
12. STAMPER H.B. & WOODRUFF J.J. (1976) Lymphocyte homing into lymph nodes: *in vitro* demonstration of the selective affinity of recirculating lymphocytes for high endothelial venules. *J Exp Med* **144,** 828.
13. CHIN Y., RASMUSSEN R., WOODRUFF J.J. & EASTON T.G. (1986) Monoclonal anti-$HEBF_{PP}$ antibody with specificity for lymphocyte surface molecules mediating adhesion to Peyer's patch high endothelium of the rat. *J Immunol* **136,** 2556.
14. SACKSTEIN R., MENG L., XU X. & CHIN Y.H. (1995) Evidence of post-transcriptional regulation of L-selectin gene expression in rat lymphoid cell. *Immunology* **85,** 269.
15. ISSEKUTZ T. & WYKRETOWICZ A. (1991) Effects of a new monoclonal antibody, TA-2, that inhibits lymphocyte adherence to cytokine stimulated endothelium in the rat. *J Immunol* **147,** 109.
16. TAMATANI T., KATAMURA F., KRIDA K. *et al.* (1993) Characterization of rat LECAM-1 (L-selectin) by the use of monoclonal antibodies and evidence for the presence of soluble LECAM-1 in rat sera. *Eur J Immunol* **23,** 2181.
17. STAMPER H.B. & WOODRUFF J.J. (1977) An *in vitro* model of lymphocyte homing. I. Characterization of the interaction between thoracic duct lymphocytes and specialized high endothelial venules of lymph nodes. *J Immunol* **119,** 772.
18. PICKER L.J., WARNOCK R., BURNS A., DOERSCHUK C., BERG E.L. & BUTCHER E.C. (1991) The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. *Cell* **66,** 921.
19. SIEGELMAN M., CHENG I., WEISSMAN I. & WAKELAND E. (1990) The mouse lymph node homing receptor is identical with the lymphocyte surface marker Ly-22: role of the EGF domain in endothelial binding. *Cell* **61,** 611.
20. JUTILA M., WATTS G., WALCHECK B. & KANSAS G.S. (1992) Characterization of a functionally important and evolutionary well-conserved epitope mapped to the short consensus repeats of E-selectin and L-selectin. *J Exp Med* **175,** 1565.
21. PICKER L.J., KISHIMOTO T., SMITH C., WARNOCK R. & BUTCHER E.C. (1991) ELAM-1 is an adhesion molecule for skin-homing T cells. *Nature* **349,** 796.
22. HUANG K., GEOFFREY J.S., SINGER M. & ROSEN S.D. (1991) A lymphocyte homing receptor (L-selectin) mediates the *in vitro* attachment of lymphocytes to myelinated tracts of the central nervous system. *J Clin Invest* **88,** 178.
23. FOXALL C., WATSON S., DOWBENKO D. *et al.* (1992) The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl $Lewis^x$ oligosaccharide. *J Cell Biol* **117,** 895.
24. KANSAS G., SPERTINI O., STOOLMAN L. & TEDDER T.F. (1991) Molecular mapping of functional domains of the leukocyte receptor for endothelium, LAM-1. *J Cell Biol* **114,** 351.
25. WATSON S., IMAI Y., FENNIE C. *et al.* (1991) The complement binding-like domains of the murine homing receptor facilitate lectin activity. *J Cell Biol* **115,** 235.
26. BERG E., ROBINSON M., WARNOCK R. & BUTCHER E.C. (1991) The human peripheral lymph node vascular addressin is a ligand for LECAM-1, the peripheral lymph node homing receptor. *J Cell Biol* **114,** 343.
27. IMAI Y., SINGER M.S., FENNE C., LASKY L. & ROSEN S.D. (1991) Identification of a carbohydrate-based endothelial cell ligand for a lymphocyte homing receptor. *J Cell Biol* **113,** 1213.
28. LASKY L., SINGER M.S., DOWBENKO D. *et al.* (1992) An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell* **69,** 927.
29. BAUMHUETER S., SINGER M., HENZEL W. *et al.* (1993) Binding of L-selectin to the vascular sialomucin, CD34. *Science* **262,** 436.
30. IMAI Y., LASKY L.A. & ROSEN S.D. (1993) Sulfation requirement for GlyCAM-1, an endothelial ligand for L-selectin. *Nature* **361,** 555.
31. TAMATANI T., KUIDA K., WATANABE T., KOIKE S. & MIYASAKA M. (1993) Molecular mechanisms underlying lymphocyte recirculation. III. Characterization of the LECAM-1 (L-selectin)-dependent adhesion pathway in rats. *J Immunol* **150,** 1735.
32. BUTCHER E.C. (1991) Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell* **67,** 1033.
33. SPRINGER T.A. (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76,** 301.
34. GAMBLE J. & VADAS M. (1988) Endothelial adhesiveness for blood neutrophils is inhibited by transforming growth factor-beta. *Science* **242,** 97.
35. GAMBLE J.R. & VADAS M. (1991) Endothelial cell adhesiveness for human T lymphocytes is inhibited by TGF-β. *J Immunol* **146,** 1149.
36. CAI J., FALANGA V. & CHIN Y.H. (1991) Transforming growth factor-beta 1 regulated the adhesion interaction between microvascular endothelial cells and lymphocytes and monocytes. *J Invest Dermatol* **97,** 169.
37. GAMBLE J.R., KHEW-GOODALL Y. & VADAS M. (1993) Transforming growth factor-beta inhibits E-selectin expression on human and endothelial cells. *J Immunol* **150,** 4494.