EA-1, A Novel Adhesion Molecule Involved in the Homing of Progenitor T Lymphocytes to the Thymus

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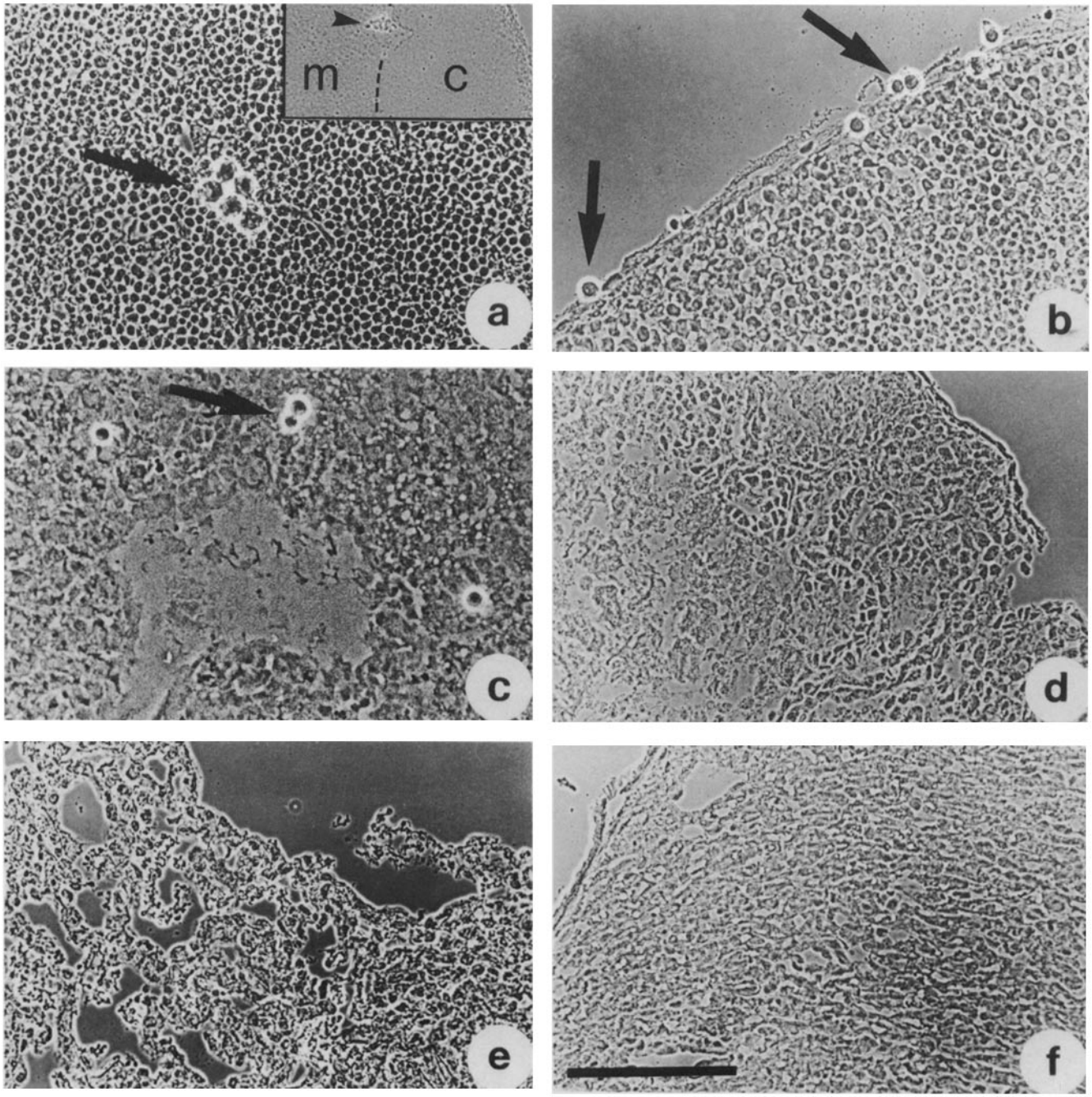
Abstract. The mouse progenitor T lymphocyte (pro-T) cell line FTF1 binds in vitro to thymus blood vessels, the thymic capsule, and liver from newborn mice. A mAb, EA-1, raised against an embryonic mouse endothelial cell line, blocked adhesion. The antibody also interfered with pro-T cell adhesion to a thymus-derived mouse endothelial cell line; it had no effect on the adhesion of mature T lymphocytes and myeloid cells. The antigen recognized by EA-1 is located on the vascular endothelium of various mouse tissues and absent on pro-T cells. EA-1 antibody precipitates molecules with apparent molecular weights of 110,000, 140,000, 160,000, and 200,000. Immunoclearing and binding-inhibition studies with antibodies against known adhesion molecules suggest that the EA-1 antigen is a novel adhesion molecule involved in colonization of the embryonic thymus by T cell progenitors.

COLONIZATION of the mouse thymus by T lymphocyte progenitors from fetal liver occurs apparently in a wave starting at day 10 of embryonic development (Moore and Owen, 1967; Salaün et al., 1986; Jotereau et al., 1987; Savagner et al., 1988; Palacios and Samaridis, 1991). In postnatal life, T cell progenitors (pro-T cells)[1] from the bone marrow continually migrate into the thymus, albeit in smaller number, and there they develop into the various thymocyte populations (Scollay et al., 1986; Kyewski, 1987; Adkins et al., 1987; Savagner et al., 1988; Deugnier et al., 1989; Palacios et al., 1990). pro-T cells, the earliest differentiation stage in the T-lymphocyte lineages (Palacios and Pelkonen, 1988), are transported to the thymus via the blood circulation (Savagner et al., 1986; Dunon et al., 1990; Palacios et al., 1990). A critical first step in the process of thymus colonization is the adhesion of pro-T cells to the perithymic vascular endothelium.

Adhesion of mature hemopoietic cells to endothelium has been extensively studied (Harlan, 1985; Rosen, 1989; Stoolman, 1989; Osborn,, 1990; Springer, 1990*a*; Albelda and Buck, 1990). Several classes of adhesion molecules present on endothelium participate in this process. ELAM-1 and PADGEM/GMP-140 constitute the so called selectin family or LECAM (Bevilacqua et al., 1989; Johnston et al., 1989). ICAM-1, ICAM-2, PECAM-1/EndoCAM, and VCAM belong to the group designated as cell adhesion molecules (CAM) and more widely to the Ig structure superfamily (Simmons et al., 1988; Horley et al., 1989; Staunton et al., 1989; Albelda et al., 1990; Newman et al., 1990; Elices et al., 1990). Most of these molecules can be induced to be expressed on certain endothelia by tissue inflammation in vivo or by cytokine treatment in vitro (Osborn, 1990). Finally the integrins, consisting of an α and a β subunit, are widely distributed on different tissues and participate in both, cell–cell and cell–substrate interaction (Albelda and Buck, 1990). All these molecules seem to be involved in homing of various hemopoietic cell lineages to a variety of tissues. A more restricted tissue or organ-specific homing has been found in the lymphoid system (Streeter et al., 1988*a*; Springer, 1990*b*). Thus, lymph nodes and Peyer's patches contain specialized blood vessels with a characteristic morphology, the high endothelial venules (HEV), to which mature lymphocytes bind. mAbs, directed against HEV antigens, block lymphocyte adhesion on HEV in the mucosa and others in peripheral lymph nodes (Streeter et al., 1988*b*; Nakache et al., 1989). Because such endothelial adhesion molecules are expressed in specific organs and because they seem to determine where circulating mature lymphocytes will exit the bloodstream, they are referred to as addressins (Berg et al., 1989).

Little is known about the interaction of lymphocyte progenitors with endothelium during development. Moreover, little is known about the existence of adhesion molecules that participate in the homing of lymphocyte progenitors to particular tissues or organs (Tavassoli and Hardy, 1990). We generated a mAb, EA-1, that reacts with a protein expressed on mouse endothelial cells. It inhibits the binding of pro-T cells to thymic endothelium, but it has no effect on adhesion of mature T lymphocytes or myeloid cells to tissues. Thus we provide evidence for restricted binding of T-cell progenitors to thymic endothelium, and we have identified on the surface of endothelial cells, a novel protein involved in that process.

1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; DPBS, Dulbecco's PB; HEV, high endothelial venules; IMDM, Iscoves modified MEM; pro-T cells, T cell progenitors.



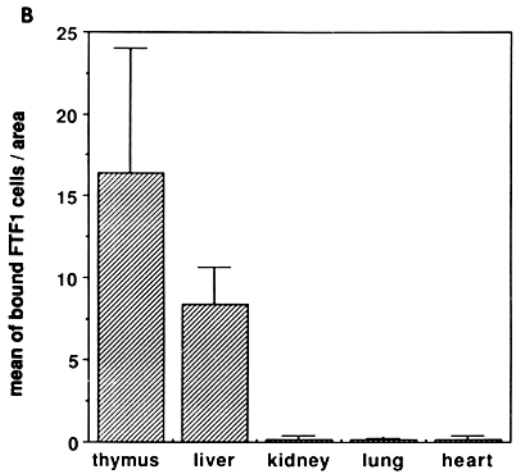


Figure 1. Binding of FTF1 pro-T cells to frozen sections of tissue from newborn mice. (*A*) Photograph of frozen sections from the binding assay. FTF1 appear as large round cells with a shiny surface easily distinguishable from the frozen sections (see *arrows*). (*a*) Cortico-medullary junction of thymus; FTF1 cells bound to a blood vessel. (*b*) Cortical region and capsule of the thymus; FTF1 cells bound to the capsule. (*c*) Liver; (*d*) kidney; (*e*) lung; (*f*) heart. The inset in *a* shows medulla and cortex of a newborn thymus. The arrowhead points to FTF1 cells bound to a blood vessel. (*B*) Quantitation of bound cells per microscope field. Mean values represent five independent assays with a total of 20 counted microscope fields per assay. Bar, 100 μm.

Table I.

	pro-T FTF1	B lymphoma L10A62	Pre-B lymphoma 18.81	Early pre-B lymphoma 40E1	Carcinoma KLN205
A. Binding capacity of cell lines on tissue sections					
Thymus	++	−	−	−	+
Liver	+	+	−	+	+
Kidney	−	+	−	+	++
Lung	−	−	−	+	++
Heart	−	−	−	−	++
B. Inhibition of cell binding by EA-1 antibody					
Thymus	+++	−	−	−	+
Liver	++	−	−	−	+++
Kidney	−	−	−	−	++
Lung	−	−	−	+++	++
Heart	−	−	−	−	+

In *A* the symbols are as follows: −, <3 cells/field; +, 3–15 cells/field; ++, 16–40 cells/field. In *B* the symbols are as follows: −, 0%; +, 1–30%; ++, 31–60%; +++, 61–100%.

Materials and Methods

Cells

The development, characterization, and functional potential of the pro-T cell clone FTF1 are described elsewhere (Pelkonen et al., 1987; Palacios et al., 1989). They were grown in culture medium in the presence of Interleukin 2 as described (Palacios et al., 1989). For further binding studies we used the pre-B cell lymphoma 18-81 (Alt et al., 1981), early pre-B cell lymphoma 40E1 (Alt et al., 1981), the mature B cell line L10A62 (Kim et al., 1979) and the carcinoma cell line KLN 205 (American Type Culture Collection, Rockville, MD). Endothelial cells, eEnd.2 (derived from mouse embryo) and tEnd.1 (derived from young mouse thymus), obtained from E. Wagner, Institute for Molecular Pathology (Vienna), are both endothelioma cell lines transformed by the polyoma middle T oncogene and cultured as described (Williams et al., 1989).

FITC Coupling to pro-T Cells

FITC (Sigma Chemical Co., St. Louis, MO) was prepared as a 25.7 mM stock solution in DMSO (Fluka AG, Buchs, Switzerland) and processed as described (Imhof et al., 1990). Briefly, 100 μl stock solution was added to a 1 ml cell suspension containing between 0.5 and 4 × 10^7 cells in DME (Gibco BRL, Paisley, UK) and incubated at 4°C for 15 min in the dark. Labeled cells were then centrifuged through a layer of 5 ml 10% BSA for 15 min at 460 *g* at room temperature. Cells were washed with DME, and cell viability was assessed by the trypan blue dye exclusion test.

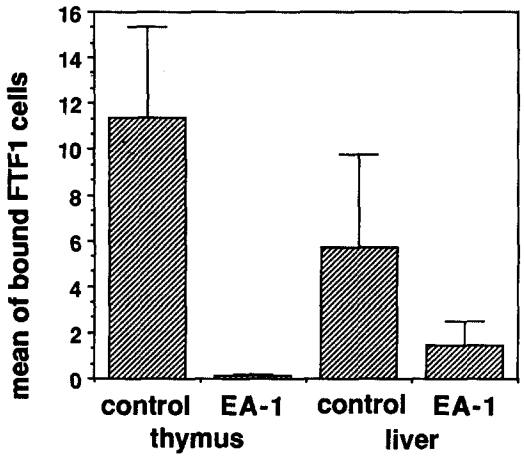


Figure 2. Inhibition of pro-T cell binding on frozen sections of newborn thymus and liver by EA-1 antibody. FTF1 cells were mixed with SP 2/0 supernatant (*control*) or with EA-1 hybridoma supernatant (*EA-1*) and used in the binding assay. Control rat antibodies of the same isotype as EA-1 gave identical results as the SP 2/0 control (not shown).

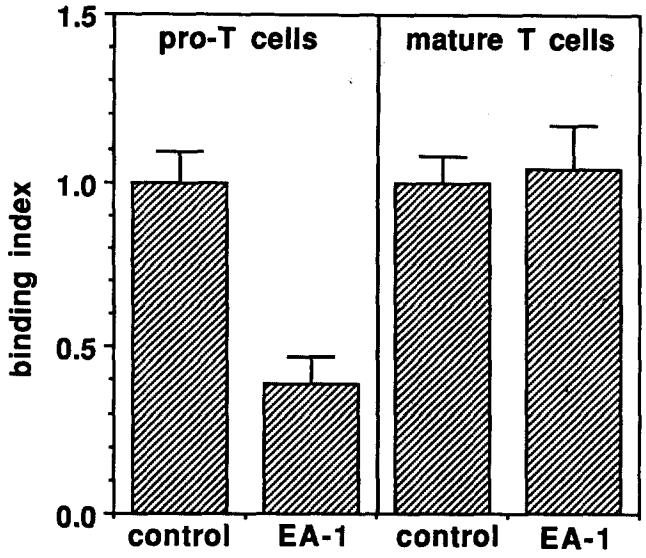


Figure 3. Binding of pro-T cells and peripheral mature T lymphocytes on thymus-derived endothelial cell line, tEnd.1. FTF1 and T cells were first FITC labeled and then used for binding on endothelial cells plated in microtiter plates. Bound cells were counted by measuring fluorescence on a Fluoroskan II microtiter reader. Pro-T cell binding was inhibited by antibody EA-1; binding of mature peripheral T cells was not. A mean of 5.5 × 10^3 ± 0.5 × 10^3 FTF1 cells and 3.3 × 10^3 ± 0.25 × 10^3 peripheral T cells (both taken as binding index 1) bound to a monolayer containing 3 × 10^4 t.End.1 cells per well. Three independent experiments were taken with 10 wells per value.

Cell Adhesion Assays

On Frozen Sections. In situ binding was performed on freshly prepared frozen sections from neonatal mouse tissue (Woodruff et al., 1987). Organs were embedded and frozen in Tissue-Tek, O.C.T. Compound (Miles Laboratories Inc., Elkhart, IN), sectioned to 5 μm thickness, and mounted onto glass slides. The sections were outlined by a PapPen (SCI Science Services, Munich, Germany) and immediately placed into Dulbecco's PBS (DPBS)

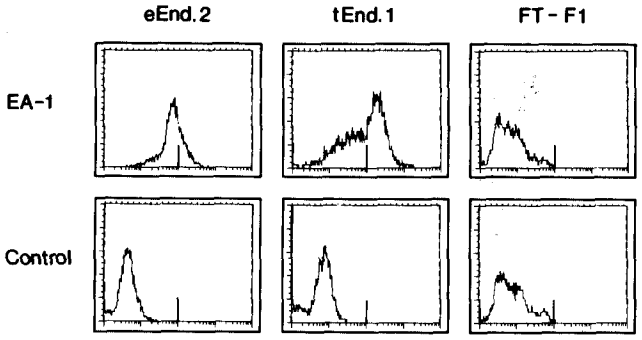
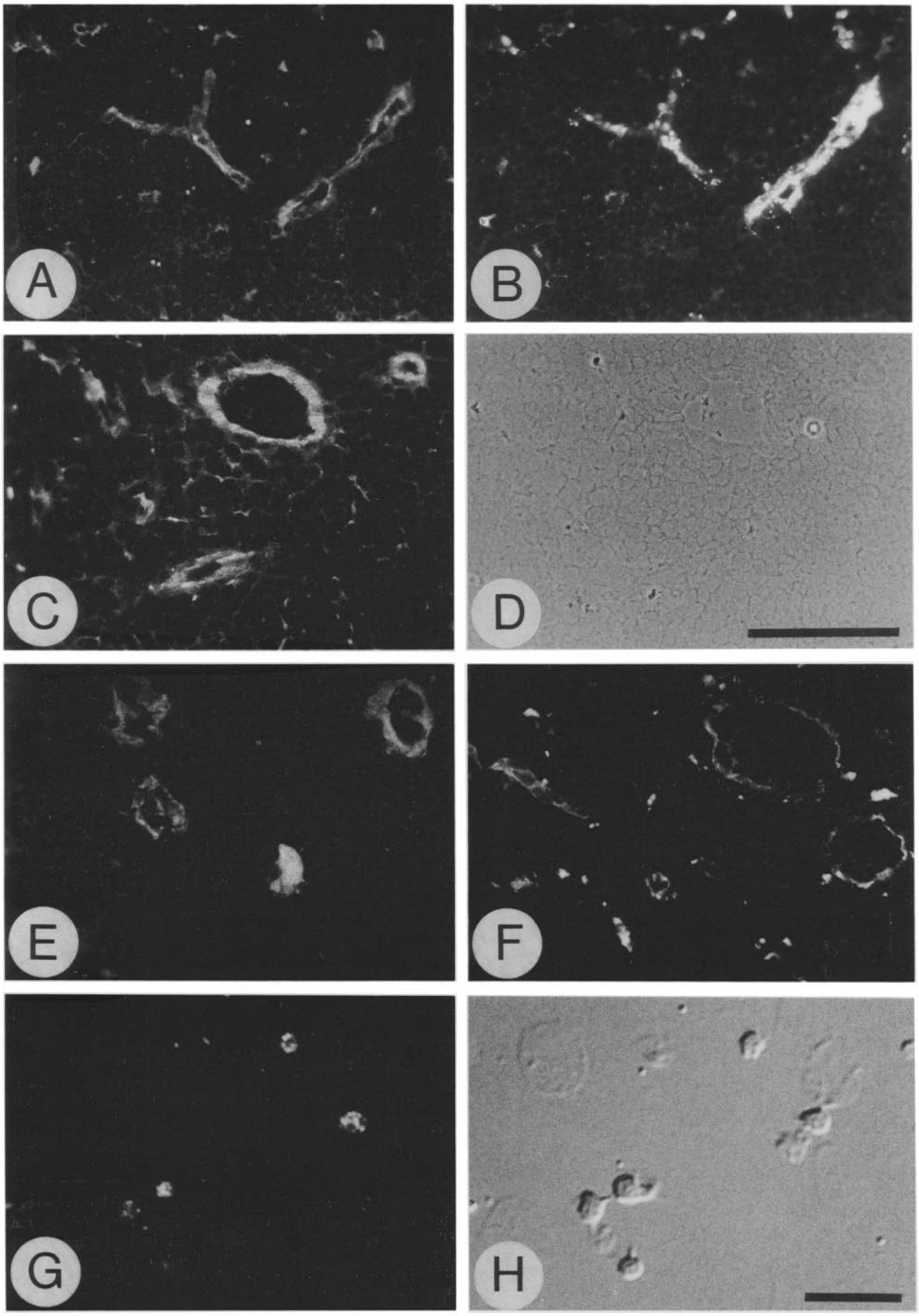


Figure 4. Analysis of surface staining by antibody EA-1 on two endothelial cell lines (*eEnd.2* and *tEnd.1*) and on the pro-T cell line (*FTF1*). Adherent cells were rapidly detached by trypsin and stained in suspension; the fluorescence detected on a FACScan. Cell number is plotted on the linear scale, fluorescence is plotted on a logarithmic scale.


A
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containing 1% BSA for at least 10 min. Slides were dried around the PapPen marked area and the tissue section then loaded with 10^5 pro-T, lymphoma or carcinoma cells in 200 μl DPBS. Binding was allowed to occur for 40 min at 8°C on a mini-shaker (A. Kühner AG, Basel, Switzerland) at 50 rpm. Slides were then placed vertically into DPBS containing 0.5% glutaraldehyde and 2% formaldehyde, where nonbound cells were allowed to fall off. After 20 min of fixation at room temperature, pro-T cells bound to the tissue section under study were counted using a light microscope (Axiophot; Zeiss, Oberkochen, Germany).

On Cultured Cells. Endothelial cells were seeded into 96-well (no. 3596; Costar Data Packaging, Cambridge, MA) microtiter plates (3×10^4 cells/well) and cultured for 24 hr. After removing the medium, FITC-labeled pro-T cells, peripheral T cells, or polymorphonuclear leukocytes were added (10^5 cells/200 μl/well) and incubated at 20°C for 2 h. Unbound cells were removed by washing three times with DPBS containing 1% BSA; the plates were "flicked" dry between washes. Fluorescence, corresponding to cells bound in DPBS, was measured by a Fluoroskan II reader (Titertek, Elfab Oy, Finland) (Imhof et al., 1990). The binding index is defined as the ratio of the number of cells bound to endothelium in the presence of blocking antibody to the number of cells bound in control medium.

Polymorphonuclear Leukocytes, Peripheral T Lymphocytes, and Blood Platelets

For the isolation of leukocytes, we followed the protocol described by Lewinsohn et al. (1987). Briefly, blood was centrifuged onto a Percoll cushion (83.6% Percoll, 1.6% H_2O, 14.8% 10× HBSS, and 5 mM EDTA). Cells from the interface were collected, stained with May-Grünwald-Giemsa, and used for binding assays.

Peripheral T lymphocytes were obtained from mouse spleens by a negative sorting procedure. Briefly, to remove macrophages and B lymphocytes, spleen cells were incubated for 30 min with antibodies specific for macrophages (Mac-1) and B cells (B-220) followed by sheep anti-mouse IgG coated Dynabeads (40 beads/1 cell; Dynal A.S., Oslo, Norway). Bound cells were separated with the MACS magnet (Biotechnische Geräte, Gladbach, Germany).

Blood platelets were prepared by differential centrifugation as described (Catalfamo and Dodds, 1989).

Antibodies

Rabbit anti factor VIII serum was purchased from Behringwerke (Marburg, Germany). Antibody H 154.163 (anti-LFA-1) was kindly provided by Dr. P. Naquet (Centre d'Immunologie de Marseille Luminy, Marseille, France), YN1/1.7 (anti-ICAM-1) by Dr. F. Takei (Karolinska Hospital, Stockholm, Sweden). I 42/5 (anti CD44) from Dr. I. S. Trowbridge (The Salk Institute, San Diego, CA), and polyclonal anti-EndoCAM by Dr. C. Buck (The Wistar Institute, Philadelphia). Antibody Mel 14 was obtained from ATCC. Secondary antibodies were RITC-labeled goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA), FITC-labeled goat anti-rat IgG (Jackson Immuno Research Laboratories Inc., Avondale, PA), FITC-labeled anti-rat IgG_{2a}, and Biotin anti-rat IgG_{2b} (both from The Binding Site LTD, Birmingham, UK). Biotin-labeled streptavidin was from Amersham International (Amersham, UK).

The rat mAb EA-1, a IgG_{2a}, was prepared according to an immunization and cell fusion protocol described by Dr. M. Kosco (manuscript in preparation). Confluent endothelial cells (eEnd.2) from a 150 cm^2 culture flask were irradiated with 10,000 rad and harvested with cell scrapers (Costar Data Packaging). These cells were then washed with DPBS, mixed 1:1 with complete Freund's adjuvant for a final volume of 300 μl, and injected subcutaneously into the dorsal surface of the hind foot of a 2-mo-old PVG rat. Injections with cells in DPBS only were repeated after 7 and 14 d. At day 17, the draining popliteal lymph node was dissected from the rat. The tissue was enzymatically digested using the following enzyme stock solutions: 150 mg/ml protease type IX (P-6141; Sigma Chemical Co.); 8 mg/ml collagenase CLS 4 (Worthington Biochemical Corp., Freehold, NJ); 10 mg/ml DNAse I (D-0876; Sigma Chemical Co.). The enzyme solutions were mixed to a final volume of 2 ml (0.5 ml Collagenase, 0.1 ml Protease, 0.1 ml DNAse, 1.3 ml IMDM [Iscoves modified MEM; Gibco BRL]). A lymph node was opened by two slight crosscuts using a 25-gauge needle. Stroma were then digested at 37°C for two 30-min periods each with 1 ml enzyme cocktail. The cells were then carefully released into IMDM with Pasteur pipettes, washed in 50 ml IMDM, and counted. One part lymph node cells was then mixed with five parts mouse Sp2/0 myeloma cells, centrifuged, and fused with PEG 4,000 (E. Merck, Darmstadt, Germany) as described in Harlow and Lane (1988). The cells were then plated (100 μl) into conditioned medium in microtiter plates (96 wells; Costar Data Packaging) at a density of 5×10^4 cells/well in IMDM selection medium containing HAT (Gibco BRL), 10% FCS (Boehringer Mannheim GmbH, Mannheim, Germany), 50 μM β-mercaptoethanol, penicillin/streptomycin, and glutamine. Conditioned medium was produced by culturing 10^4 PVG rat thymocytes (100 μl/well) in selection medium for three days before fusion. The IgG was purified from hybridoma supernatant on protein G affinity columns (Pharmacia Fine Chemicals, Uppsala, Sweden).

Immunofluorescence

Frozen sections were fixed with acetone and labeled with supernatant of the EA-1 hybridoma raised against eEnd.2 cells, followed by an FITC-conjugated goat anti-rat IgG antibody (Jackson Immuno Research Laboratories Inc.). To evaluate staining of adherent endothelial cells by flow fluorocytometry analysis (FACScan; FACs is a registered trademark of Becton Dickinson and Company, Mountain View, CA), the cells were first removed from the culture flasks by a very rapid trypsin treatment which does not affect EA-1 antigen. Addition of complete medium stopped the reaction. The cells in suspension were stained by unlabeled EA-1 followed by the same FITC-conjugated goat anti-rat IgG described above.

Immunoprecipitation

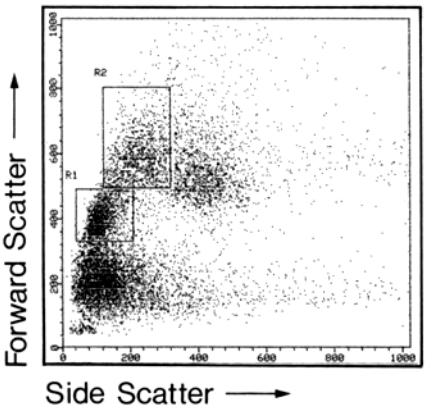
To determine the molecular weight of the protein recognized by EA-1, confluent endothelial cells grown in a 150-cm^2, culture flask were surface iodinated by the lactoperoxidase/glucoseoxidase method described elsewhere (Marchalonis, 1969). Briefly, cells were washed twice with DPBS and incubated with 1.5 mCi ^{125}Iodine, 2 ml DPBS containing 1 U/ml lactoperoxidase, 2 U/ml Glucoseoxidase, and 0.2% β-D-glucose. After 30-min incubation at room temperature, the cells were washed eight times with complete medium. Radioactive cells in DPBS containing 2 mM PMSF (Sigma Chemical Co.) and 1 TIU/ml Aprotinin (Sigma Chemical Co.) were harvested from the flask with a cell scraper (Costar Data Packaging). The cells were suspended in lysis buffer (2% NP-40 [Fluka AG], 150 mM NaCl, 50 mM Tris, pH 8, 2 mM PMSF, and 1 TIU/ml Aprotinin) and centrifuged for 10 min at 10,000 *g*. The supernatant was cleared twice with 100 μl Sansorbin (Calbiochem-Behring Corp., San Diego, CA) and a rabbit anti-rat serum (Jackson Laboratory, Bar Harbor, ME) for 30 min each. Hybridoma supernatant and a rabbit anti-rat serum (1:20) was added to an aliquot of the cleared lysate and precipitation was performed using 30 μl Pansorbin (Calbiochem-Behring Corp.). Pellets were washed three times with lysis buffer, boiled in SDS-dissociation buffer, and run on SDS-PAGE, 5-15% acrylamide.

Results

FTF1 pro-T Cells Bind to Thymic Tissue

The FTF1 cell line has cellular, molecular, and functional properties of pro-T cells, the progenitors that are the earliest stage of T cell development (Pelkonen et al., 1987; Palacios et al., 1989). FTF1 cells express the pro-T cell specific sur-

Figure 5. Immunofluorescence labeling of EA-1 on various mouse tissues. Frozen sections of neonatal or adult mouse tissue were used for staining. (*A* and *B*) Double staining using EA-1 followed by labeled (Texas red) anti-rat IgG and rabbit anti-factor VIII followed by FITC labeled anti-rabbit IgG. (*A*) Cortical region of the thymus stained for EA-1. (*B*) Double staining for endothelial marker factor VIII. (*C*) EA-1 staining of the thymic cortico-medullary junction, apical surface, and cell-cell contact of endothelial cells are stained. (*D*) Corresponding phase contrast image, note endothelial adherent hemopoietic cells in the large capillary. (*E*) EA-1 staining of neonatal liver; the capillaries are positive. (*F*) EA-1 staining of adult spleen, all endothelia are positive. (*G*) Adult mouse blood platelets stained for EA-1. (*H*) Corresponding interference contrast image. Bars: (*A-F*) 100 μm; (*H*) 10 μm.



Antigen	Mature Lymphocytes [a]	Immature Cells [a]
EA-1+	12	57
Thy-1+	8	16
EA-1+Thy-1+	4	10
B220+	42	22
EA-1+B220+	0	14
Mac-1+	0	32
EA-1+Mac-1+	0	32

Figure 6. Double staining of adult bone marrow cells using EA-1 and typing antibodies for hemopoietic lineages. Total bone marrow cells were either single (EA-1, thy-1, B220, or Mac-1) or double stained (EA-1+thy-1, EA-1+B220, EA-1+Mac-1). EA-1 labeling was detected by a FITC-coupled anti-IgG_{2a} antibody and the hemopoietic markers by Biotin-coupled anti-IgG_{2b} antibody followed by phycoerythrin coupled avidin. Subpopulations of immature T and B lineage cells and immature myeloid cells were EA-1 positive. (*a*) Percent of cells in foreward vs side scatter, FACS window R1 corresponds to mature lymphocytes, FACS window R2 to immature cells.

face markers, Joro 75 and Joro 37-5; progenitor cells bearing these markers have been shown to migrate into and colonize the thymus both in the developing embryo and in adult mice (Palacios et al., 1990; Palacios and Samaridis, 1991). Therefore, we thought that the behavior of FTF1 pro-T cells in functional thymus homing assays in vitro would be a measure of interactions between pro-T cells and thymic tissue in vivo (Stamper and Woodruff, 1976).

We find that FTF1 cells bind to frozen sections of thymuses from newborn mice. They are concentrated on the thymic capillaries and especially to the larger blood vessels of the thymic cortico-medullary junction (Fig. 1 *A*). They also adhere to areas in the outer cortex and in the capsule of the isolated thymus, but less frequently. The FTF1 pro-T cells also bind to frozen sections of liver from newborn mouse, but they did not adhere to kidney, lung, or heart tissues. Twice as many FTF1 cells adhered to the thymus than to the liver (Fig. 1 *B* and Table I *A*). Thus, in contrast to other cell lines tested, as would be expected of a pro-T cell, FTF1 shows a clear preference for adhering to hemopoietic organs.

mAb EA-1 Inhibits pro-T Cell Adhesion to Thymic Endothelium

In an attempt to identify adhesion molecules involved in homing to the thymus, we prepared rat mAbs against the endothelial cell line eEnd.2 (derived from mouse embryonic tissue; Williams et al., 1989) and tested them in the frozen section binding assay. The antibody EA-1 totally blocked the binding of FTF1, partially a carcinoma but not the other hemopoietic cell lines to neonatal thymus (Fig. 2 and Table I *B*). The limited binding of FTF1 pro-T cells to neonatal liver was only partially blocked. Thus the antigen recognized by EA-1 antibody seems to be more important for the adhesion of pro-T cells to thymic tissue than it is for their adhesion to liver.

To study binding to endothelia in vitro, we have developed an automated assay which involved culturing FTF1 pro-T cells labeled with fluorescein isothiocyanate on a layer of the thymus derived endothelial cell line tEnd.1 (Williams et al., 1989; Imhof et al., 1990). A microtiter reader equipped with a fluorimeter was used to count fluorescent cells adhering to this monolayer. The antibody, EA-1, inhibited binding of FTF1 cells to tEnd.1 endothelia in control medium by 60% (Fig. 3). EA-1 did not affect the binding of mature peripheral T cells to the thymic endothelial cell line (Fig. 3), nor did it affect the adhesion of polymorphonuclear leukocytes (data not shown).

Using FACS (Becton Dickinson and Co.) analysis we established that EA-1 recognized a cell surface antigen on endothelial cell lines of either embryonic (eEnd.2) or thymic (tEnd.1) origin. FTF1 pro-T cells did not bind EA-1 (Fig. 4).

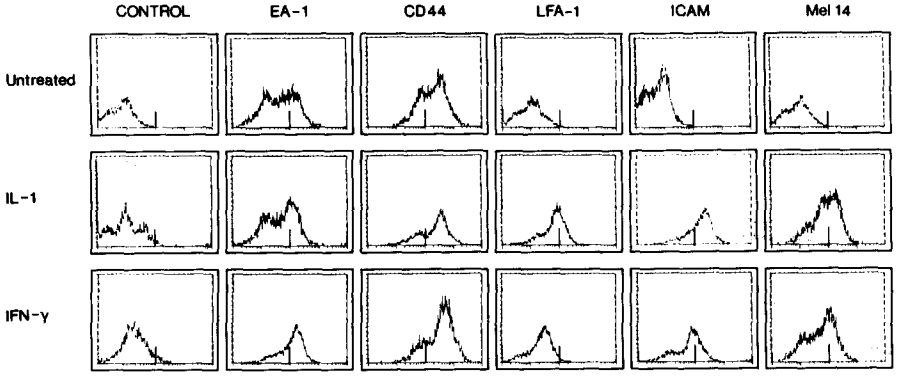


Figure 7. Regulation of the expression of adhesion molecules on eEnd.2 endothelial cells. Cells were treated with 100 U/ml IL-1 or IFN-γ overnight, rapidly detached from the culture dish, and stained in suspension with indicated antibodies. EA-1 and CD-44 showed no upregulation, LFA-1, ICAM-1, and Mel14 showed increased expression after cytokine treatment.

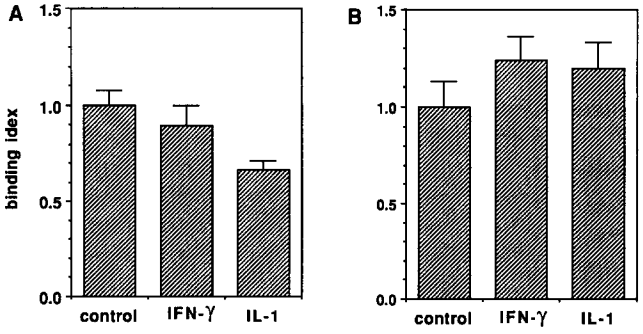


Figure 8. Ability of pro-T cells or polymorphonuclear leukocytes to bind to eEnd.2 endothelia following cytokine treatment of endothelium. (*A*) Endothelial cells were cultured over night with 100 U/ml IFN-γ or IL-1 and then used for binding assays with FTF1 cells. (*B*) Corresponding experiment using polymorphonuclear leukocytes. The number of bound FTF1 control cells was the same as in Fig. 3.

These findings suggest that the EA-1 antigen on endothelia recognizes an as yet undefined molecule on pro-T cells.

Tissue Distribution of the Antigen Recognized by EA-1

Using an analysis by immunofluorescence staining and microscopy of frozen sections from newborn mouse tissue we showed that the EA-1 antibody stains blood vessels in the thymus. The endothelial nature of the cells positive for EA-1 was demonstrated by double labeling assays with EA-1 and anti-factor VIII antibodies as an endothelial marker (Fig. 5). The labeling pattern of EA-1 on the luminal side of endothelial cells was compared with that in regions of cell–cell contact. We found that EA-1 reacts with an antigen present on all thymic capillaries. However, the EA-1 antibody also bound to vascular endothelia in newborn liver, spleen, lung, kidney, and brain (Fig. 5; and data not shown). In adult tissues, the antigen recognized by EA-1 was detected on endothelia of many tissues (as shown in Fig. 5 for spleen), on a subpopulation of immature hemopoietic cells from the bone marrow (Fig. 6) and, on freshly isolated blood platelets (Fig. 5, *G* and *H*).

Relationship of EA-1 Antigen to Other Cell Adhesion Molecules

It is known that several adhesion molecules known to be expressed on endothelial cells can be upregulated by inflammatory reactions mediated by endotoxins or cytokines. IL-1 failed to induce a higher expression of the EA-1 antigen on the endothelial cell line eEnd.2, but INF-γ had a slight effect on a negative subpopulation (Fig. 7). Another adhesion molecule, CD44, showed no significant change. By contrast LFA-1, ICAM-1 and gp90^{Mel14} were upregulated upon cytokine treatment. Induction of LFA-1 and gp90^{Mel14} expression on eEnd.2 cells by IL-1 is a novel and interesting finding. It may be because of the fact that this particular cell line is of embryonic origin.

In analogy to the induction of adhesion molecules, inflammatory reactions increase the binding capacity of endothelium for myeloid cells or mature lymphocytes (Osborn, 1990). We found that treatment of eEnd.2 endothelium with IL-1 reduced pro-T cell binding (Fig. 8 *A*). By contrast, polymorphonuclear leukocytes bound slightly better to endothelia treated with IL-1 or IFN-γ (Fig. 8 *B*).

Antibodies directed against the known adhesion molecules, LFA-1, CD44, and gp90^{Mel14} did not interfere with pro-T cell adhesion to tEnd.1 endothelial cells (Fig. 9 *A*). Interestingly, antibodies against the integrin LFA-1 slightly increased the inhibitory effect of EA-1 antibodies (Fig. 9 *B*).

Immunoprecipitation of the Antigen Recognized by EA-1

After surface iodination, endothelial cell (eEnd.2) proteins were solubilized and immunoprecipitated with EA-1. SDS-PAGE, under reducing conditions, detected bands at a molecular weight of 110,000, 140,000, 160,000, and 200,000 (Fig. 10). Nonreducing conditions did not significantly change the pattern. However, depending on experimental conditions the intensity of the individual bands varied, probably because of limited digestion. The molecular weight of one band (140,000) is similar to that of PECAM/endoCAM/CD31 which is also expressed on endothelia and platelets. But using immunoclearing with a polyclonal anti-CD-31 antiserum, we could clearly show that the two molecules are different (Fig. 10).

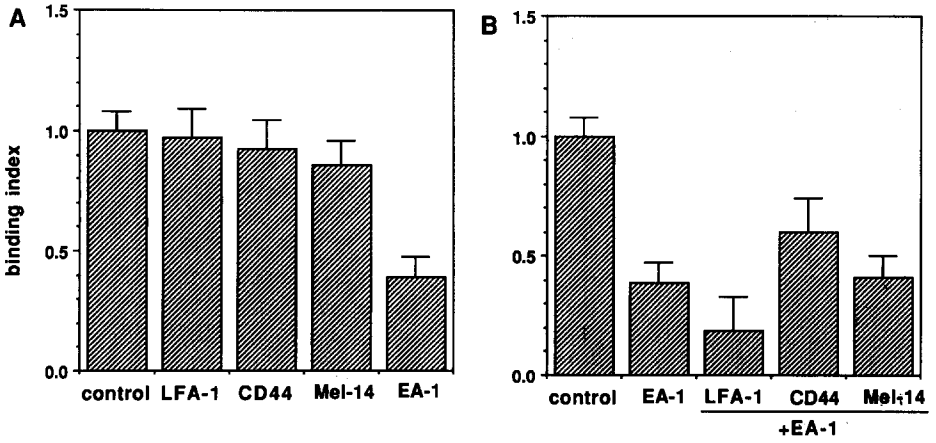


Figure 9. Adhesion molecules involved in inflammation do not interfere with pro-T cell adhesion. Binding of FTF1 cells on tEnd.1 endothelia was performed as described in Fig. 3. Antibodies against LFA-1, CD44, and gp90^{Mel-14}, and the antibody EA-1, were added as hybridoma supernatants in the FTF1 binding assay. Synergistic effects of the various adhesion molecules on FTF1 binding were studied by mixing the above antibodies with EA-1 in the adhesion assay. Only anti-LFA-1 showed a minor effect. The number of bound FTF1 control cells was the same as in Fig. 3.

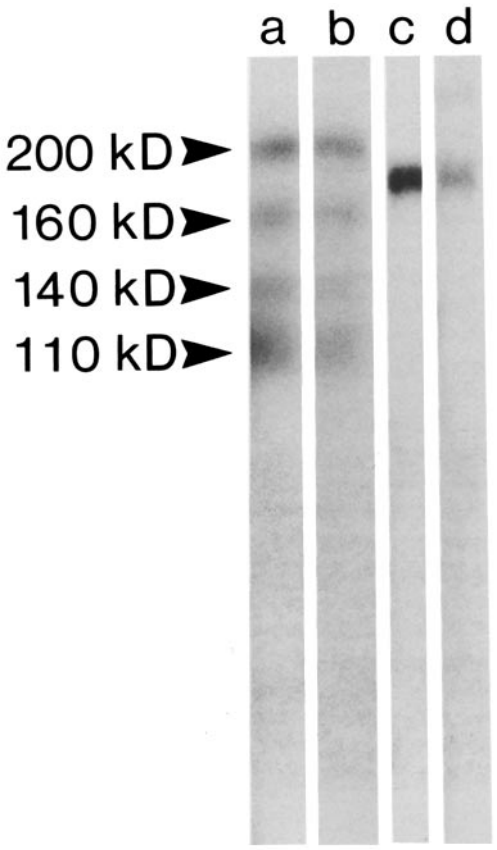


Figure 10. Immunoprecipitation and immunoclearing with EA-1 antibody and a polyclonal anti-CD31 antiserum. Surface ^{125}Iodinated eEnd.2 cells were prepared for immunoprecipitation using the antibodies and *Staphylococcus aureus*. The precipitate was analyzed by a reducing SDS-PAGE followed by autoradiography. (*a*) Precipitation with EA-1. (*b*) Precleared with anti-CD31 and precipitated with EA-1. (*c*) Precipitation with anti-CD31. (*d*) Precleared with EA-1 and precipitated with anti-CD31.

Discussion

pro-T Cells Interact Specifically with Thymic Endothelium

Here we have shown that pro-T lymphocytes bind to thymus and liver from newborn mice as well as to an endothelial cell line derived from thymus. This property of T-cell progenitors appears restricted to vessels in hemopoietic tissue; that is, they do not adhere to endothelia in other organs such as kidney, lung, or heart. The EA-1 antibody reacts with molecules of apparent molecular weights of 110,000, 140,000, 160,000, and 200,000, which are components of the plasma membrane of endothelial cells.

Although it recognizes an antigen expressed on vascular endothelium from several tissues, EA-1 effectively blocks the binding of pro-T cells to thymus-derived endothelium, but it does not affect the adhesion of mature T lymphocytes or myeloid cells to any tissue. This result strongly suggests that the molecules recognized by the EA-1 antibody on endothelial cells selectively participate in the adhesion of pro-T cells to thymic endothelium. EA-1, located on other tissues may either not be functional or it could be involved in homotypic endothelial cell–cell contact. The specificity of EA-1 antigen for pro-T cells in the thymus might result from interaction of the endothelium with the thymus in a way that alters its binding affinity. Such a process is also thought to be involved in LFA-1–mediated adhesion and deadhesion of lymphocytes (Dustin and Springer, 1989; Figdor et al., 1990). Specificity might also result from posttranslational modifications of the antigen, as had been shown for the receptor molecule of ELAM-1 or PADGEM/GMP-140, lacto-*N*-fucopentaose III (Walz et al., 1990; Larsen et al., 1990; Lowe et al., 1990; Phillips et al., 1990). Moreover, different adhesion molecules may act together and tissue specificity is probably created by the proportional number of these molecules to each other (Hamann et al., 1988).

In addition to pro-T cells, peripheral mature T cells and polymorphonuclear leukocytes adhere to the endothelial cell lines tested here. But EA-1 antibody specifically blocked the adhesion of FTF1 pro-T cells without affecting that of other hemopoietic cell lineages. Also, IL-1, a cytokine involved in inflammatory reactions, stimulated the binding of myeloid cells but had a negative effect on pro-T cell binding. Thus, the binding of these various hemopoietic cells seems to depend on interaction with a different set of adhesion molecules. Moreover, these results suggest that the homing of pro-T cells to the thymus uses molecular mechanisms different from those described for inflammation mediated cell adhesion to endothelia.

The in vitro assay described here ought to prove valuable in studying thymic specific homing mechanisms involved in embryonic pattern formation.

The EA-1 Antigen Is A Novel Adhesion Protein

The question arises whether the EA-1 antigen is a novel molecule or whether pro-T cell binding to thymic endothelium is a function of a previously described adhesion molecule. PADGEM/GMP-140, a protein-mediating cell adhesion of hemopoietic cells to endothelia, has a similar tissue distribution and the same molecular weight as the EA-1 antigen (Johnston et al., 1989; Parmentier et al., 1990). However several experiments reveal major differences between the two molecules. EA-1 is mainly expressed on the cell surface, whereas GMP-140 colocalizes with factor VIII, in Weibel-Palade bodies in endothelial cells (Bonfanti et al., 1989). Only inflammatory activation of the cells brings GMP-140 to the cell surface (Osborn, 1990). EA-1 is constitutively expressed on endothelial cell surfaces and stimulation of these cells by IL-1 does not increase the expression of EA-1. PECAM-1/EndoCAM, another adhesion molecule found on endothelium is found on the apical plasma-membrane and in cell–cell junctions of endothelial cells. Its published molecular weight is 130 kd (Newman et al., 1990). Identity with EA-1 could be excluded by immunoclearing, which was possible because of bovine/mouse crossreactivity of a polyclonal anti-EndoCAM antibody (Albelda et al., 1990). The other adhesion molecules ELAM-1, PgP-1/CD44, I-CAM, gp90^{MEL14}, β_1, and β_4 integrins differed from EA-1 in molecular weight, tissue distribution pattern, and their regulation of cell surface expression (Pont et al., 1986; Simmons et al., 1988; Picker et al., 1989; Zhou et al., 1989; Siegelman et al., 1989; Albelda and Buck, 1990; Hogervost et al., 1990; Stoolman, 1990). Therefore, we conclude that the EA-1 antigen has not been previously described. Our data suggest that

it selectively plays a role in the adhesion of T lymphocyte progenitor cells to endothelium. Interestingly, the EA-1 antigen may also be involved in carcinoma cell adhesion to endothelium. Adhesion molecules used by cells of the immune system have already been found to play a role in cancer metastasis and they may open possibilities of applications in therapy (Rice et al., 1989; Günthert et al., 1991). We are currently attempting to isolate cDNA encoding the molecule recognized by EA-1 antibody to further define the nature, function, and relatedness of this protein with other adhesion molecules.

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