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Functional Analysis of Mononuclear Cells Infiltrating into Tumors: Establishment of T Cell Hybridomas Exhibiting Distinct Interacting Abilities with Endothelial Cells and Extracellular Matrix Components

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We have established eleven T cell hybridoma cell lines to investigate mechanisms controlling interaction of T lymphocytes with endothelial cells as well as extracellular matrix (ECM) proteins at the clonal level. T cell hybridomas were characterized and subdivided into four groups on the basis of their interaction behavior with high endothelial venules (HEV). Group 1 (G1) exhibited strong adhesiveness. The binding was temperature- and divalent cation-dependent. Group 2 exhibited both adhesiveness and transendothelial migration (TEM, i.e., transmigration beneath the cytoplasm of endothelial cells). Group 3 exhibited strong TEM. G2 and G3 hybridomas exhibited temperature-independent and divalent cation-independent binding to HEV. Group 4 exhibited nonspecific adhesiveness to the surface of a slide glass. BW 5147, a parent of T cell hybridomas, was classified as G4. TEM was dependent on both the nature of T cell hybridomas and endothelial cells. TEM was completely temperature-dependent. TEM of G3 hybridomas was not divalent cation-dependent. Each group of T cell hybridomas interacted with various ECM components.

Key words: Adhesion molecule — T cell hybridoma — High endothelial venule — Aorta endothelial cell — Extracellular matrix

We have previously demonstrated that tumor-infiltrating T lymphocytes play an important role in the regulation of tumor cell growth and that the intensity of T cell infiltration into tumor tissues correlates very well with the prognosis of cancer patients.[1-8] Therefore, it is important to analyze the mechanisms by which the infiltration of T cell into tumor tissues is controlled. We reported that lymphocyte chemotactic factors produced at the tumor tissues were responsible for the migration of T lymphocytes into tumor tissues. Tumor-infiltrating neutrophils produced a soluble facter, lymphocytes migration factor-a (LMF-a), that attracted CD4(+) T lymphocytes into tumor tissues, and then tumor-infiltrating CD4(+) lymphocytes produced another lymphocyte chemotactic factor, LMF-b, specific for CD8(+) T lymphocytes.[7,8] Thus, the infiltration of T cells into tumor tissues can be explained by these chemotactic factors. However, lymphocytes must confront various barriers *in vivo*, even if they respond to chemotactic factors. Among them, the interaction with endothelial cells might be the most critical process for extravasation of T cells. After the initial attachment of T cells to the endothelial cell surface, several important processes must take place for infiltration of T cells into tumor tissues.[9] Those are transmigration of T cells through endothelial cells, attachment to basement membrane, and interaction with various extracellular matrix (ECM) components, such as proteins and glycosaminoglycans. Recently we found that LMF-b not only acted as a chemotactic factor for CD8(+) T lymphocytes, but also enhanced adhesiveness of CD8(+) T lymphocytes specifically to high endothelial venules (HEV).[10] However, we used splenic lymphocytes in those studies. Because of their heterogeneity, detailed functional analysis of how T cells interact with HEV and/or ECM components was difficult. Therefore, in this study we wished to develop a system to investigate the interaction of T cells with endothelial cells, HEV and ECM componenets. Thus, we established distinct types of T cell hybridomas which interact with HEV and ECM components in different ways.

MATERIALS AND METHODS

Medium Cell culture and binding studies were performed in RPMI-1640 medium supplemented with L-glutamine (2×10^{-3} *M*), penicillin (100 U/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (5×10^{-5} *M*), and 10% fetal calf serum (FCS) (referred to as complete medium). For the adhesion assay with ECM components, complete medium with 1% bovine serum albumin (BSA) instead of FCS was used (referred to as 1% BSA RPMI).

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Reagents Human fibronectin (FN) was purchased from Iwaki Glass, Tokyo. Rat collagen type I and heparin were obtained from Collaborative Research Inc., Bedford, MA and Novo Nordisk Pharma Limited, Bagsvaerd, Denmark, respectively. Hyaluronate, heparan sulfate and chondroitin sulfate A & C were purchased from Seikagaku Kogyo, Tokyo. All ECM components were dissolved in phosphate-buffered saline (PBS) before use. Fibronection-related synthetic peptides were prepared as described previously.[11-13] Poly (RGD) contains repetitive RGD structure. H271 contains a heparin-binding fragment of fibronectin. CH-271 is a fusion protein of C-274 and H271. CS-1 contains DELPQLVTLPHPNLHGPEILDVPST sequence.

Antibodies Three monoclonal antibodies (mAb), MRC OX34 (murine IgG2a),[14] MRC OX54 (murine IgG1) and MRC OX55 (murine IgG1),[15] reacting with distinct epitopes of rat CD2 antigen, and a mAb, MRC OX50 (murine IgG1),[16] which detects rat CD44 antigen, were kindly supplied by Dr A. F. Williams, University of Oxford, Oxford, GB. 1F4 (anti-rat CD3; murine IgMκ) was purchased from Seikagaku Kogyo.[17] R1-3B3 (anti-rat CD5; murine IgG2a), RTH-7 (anti-rat CD4; murine IgG2a), R1-10B5 (anti-rat CD8; murine IgG2a), R4-8B1 (anti-rat MHC Class I; murine IgG2a) and RLN-9D3 (anti-rat pan B cell; murine IgG2a), which were all established at the Department of Pathology, Sapporo Medical University were also used in this study.[18-21] 1A29 (anti-rat ICAM-1; murine IgG1) and WT.1 (anti-rat CD11a; murine IgG2a) mAb were kindly donated by Drs. Masayuki Miyasaka and Takuya Tamatani, Department of Immunology, Tokyo Metropolitan Institute of Medical Science, Tokyo.[22, 23] 7D3 is a murine mAb which inhibits rat thymocyte adhesion to thymic epithelial cells.[24]

HEV and aorta endothelial cells For adhesion assay, we used two kinds of endothelial cells, namely, HEV and REC20. HEV were isolated from cervical lymph node of Fischer rats according to the method described by Ager.[25] The isolated cells were confirmed to be HEV as described previously.[26] REC20, aorta endothelial cells, were isolated from a Fischer rat in our laboratory by the method described elsewhere,[27] and used in this study. These cells express ICAM-1 antigen on their cell surface.

Generation of T cell hybridomas Sensitization of Fischer rats with tumor cells and isolation of regional peripheral lymph node cells for production of T cell hybridomas were done as follows. T-9 cells (N-nitrosourea-induced rat gliosarcoma) were injected subcutaneously into the dorsal area of syngeneic Fischer rats. Ten days after the inoculation, tumor masses were surgically removed and 1×10^7 mitomycin C-treated T-9 cells were injected subcutaneously once a week, 6 times. After this treatment, the immunized rats completely rejected 1×10^7 viable T-9 cells.[5] Sensitized Fischer rats were subcutaneously inoculated with 1×10^7 viable T-9 cells and regional popliteal lymph nodes were removed 4 days thereafter. Cell suspension was prepared from these lymph nodes. The lymph node cells were washed three times with RPMI-1640 containing 5% FCS, then fused with BW5147 using the method described by Köller and Milstein with minor modifications.[6, 28, 29] After fusion, cells were resuspended in HAT (1×10^{-4} *M* hypoxanthine, 4×10^{-7} *M* aminopterin, 1.6×10^{-5} *M* thymidine)-containing complete medium and were seeded in each well of 96-well plates. Cells were kept in HAT-complete medium for 2 to 3 weeks until clones appeared, and hybrid cells were later maintained in complete medium. Hybrid cells were cloned and their mode of interaction with HEV was examined. Representative clones with distinct interactive behavior towards HEV were used in this study.

Adhesion of T cell hybridomas to HEV and aorta endothelial cells HEV and REC20 were detached from 75 cm^2 plastic culture bottles (3024, Falcon, Becton Dickinson, Mountain View, CA) with PBS containing 0.05% trypsin (T8253, Sigma Chemical Co., St. Louis, MO) and 0.02% EDTA, and resuspended in complete medium at a cell density of 4×10^4 and 6×10^4 cells/ml, respectively. Then, 500 μl of each cell suspension was seeded in each well of 8-well Lab-TeK chamber slides (4808, Nunc, Naperville, IL) precoated with collagen type I and held overnight at 4°C. After cultivation overnight, a confluent monolayer was obtained. Each well was washed 3 times with complete medium, and 2×10^5 T cell hybridomas suspended in 300 μl of complete medium were added. In some assays, the binding inhibition assays were performed in the presence of various fibronectin-related synthetic peptides at a concentration of 100 μg/ml. After incubation for an adequate time (1–2 h), the upper structure of the chamber slide was removed and the slide glass with silicone gasket was dipped in an inverted position into 50 ml of 1% FCS RPMI 1640, prewarmed to 37°C, in a 100 mm diameter plastic dish (25020, Corning, New York, NY), and washed by gentle agitation for 5 min under inverted microscopic observation (CK2, Olympus, Tokyo). Then, the slide glass was dried and fixed with methanol, followed by Giemsa staining. Interactions between T cell hybridomas and endothelial cells were examined by light microscopic observation. To investigate whether or not binding and/or transendothelial migration processes are energy-dependent, assay was performed at 4°C. In some experiments, T cell hybridomas and/or HEV were pretreated with periodate-lysine-paraformaldehyde (PLP) fixatives.[30] The intensity of cell binding to endothelial cells was expressed as −, +, 2+, 3+ or 4+; "−" indicates that less than 50 T cell hybridomas adhered to HEV per field under microscopic observation (×400), "+" indicates between 50 and 100,

"2+" indicates between 100 and 150, "3+" indicates between 150 and 200, and "4+" indicates more than 200. The degree of TEM was expressed as −, ±, + or 2+; "−" indicates that less than 5 T cell hybridomas exhibiting TEM per field were found under microscopic observation (×400), "±" indicates between 5 and 25, "+" indicates between 25 and 45, and "2+" indicates more than 45. For each sample 10 microscopic fields were counted.

Adhesion of T cell hybridomas to ECM components For coating the tissue culture dishes, chondroitin sulfate A, chondroitin sulfate C, heparan sulfate and hyaluronate were dissolved in PBS (5 mg/ml). Collagen type I and fibronectin were dissolved in PBS at concentrations of 5 mg/ml and 10 μg/ml, respectively. Heparin was used without dilution (1000 U/ml). Each well in a 48-well culture plate (3548, Costar, Cambridge, MA) was pretreated overnight with 200 μl of glycosaminoglycans and proteins at 4°C, and washed 3 times with PBS. Residual uncoated sites were blocked by incubating the plate with 1% BSA RPMI 1640 for 30 min at 37°C. Just before assay, the medium was removed, and 2×10^5 T cell hybridomas suspended in 300 μl of 1% BSA RPMI 1640 were added to each well. After incubation for 3 h, unbound cells were removed by gentle aspiration, followed by washing three times with 1% BSA RPMI 1640 prewarmed to 37°C. Interactions between T cell hybridomas and ECM components were examined by phase-contrast microscopic observation (CK2, Olympus). The intensity of cell binding to ECM components was expressed as −, +, 2+, 3+ or 4+, defined in the same way as in the case of binding to endothelial cells (see previous section).

Immunofluorescence T cell hybridomas were incubated with appropriate dilutions of various mAbs for 30 min at 4°C, followed by incubation for 30 min at 4°C with goat anti-murine IgG+IgM-FITC (TAGO, Inc., Burlingame, CA) or by FITC-anti-rat kappa chain mAb (MAR18.5; murine IgG2a, American Type Culture Collection).[31] Each incubation procedure was followed by washing twice with 0.1% BSA PBS. Flow cytometric analysis was performed using a FACScan (Becton Dickinson). The intensity of fluorescence was expressed as −, ± or +; "−" means that less than 10% of cells were positive, "±" means 10–20% positive, and "+" means more than 20% positive.

RESULTS

Distinct adhesive behavior of T cell hybridomas to HEV We produced various T cell hybridomas cell lines, and on the basis of adhesion assay, we selected eleven clones which exhibited distinctive interaction with HEV. These T cell hybridomas were categorized into four groups according to their adhesive behavior towards HEV (Table I). BW5147 as well as RTT-9F2 had very weak binding ability to HEV, but adhered to slide glass. These clones were classified as G4. RTT-7D11 and RTT-7E7 clones adhered very strongly to the HEV cell surface, but did not show TEM even after 2 h incubation, and were classified as G1. RTT-2F10, -1B7, -5B4 and -6H4 clones adhered to HEV and transmigrated beneath the cytoplasm of HEV. These clones were classified as G2. RTT-3E6, -3G4, 6H2D9 and 6H2H2 also adhered to the HEV cell surface, exhibited strong TEM and were classified as G3. T cell hybridomas of G1, G2 and G3 did not bind to slide glass. Representative results from each group are depicted in Fig. 1. In order to clarify whether G2 and G3 reflect a kinetic difference of adhesion and TEM, kinetic studies of cell binding were performed. G2 and G3 hybridoma clones exhibited very weak TEM after 30 min incubation. TEM of G3 hybridoma clones at 2 h was stronger than TEM at 1 h, whereas TEM of G2 hybridoma clones was not significantly different between 1 h and 2 h (Fig. 2).

Table I. T Cell Hybridomas Interact with High Endothelial Cells in Different Ways[a]

Group	T cell hybridomas	Adhesion	TEM
Group 1 (G1)	RTT-7D11	4+[b]	−
	-7E7	4+[c]	−
Group 2 (G2)	-2F10	2+	±
	-1B7	2+	±
	-5B4	2+	±
	-6H4	3+	+
Group 3 (G3)	-3E6	2+	2+
	-3G4	2+	2+
	6H2.D9	+[c]	2+
	6H2.H2	3+	2+
Group 4 (G4)	RTT-9F2	−	−
	BW-5147	−	−

a) T cell hibridomas were incubated with HEV for 1 h.
b) The intensity of adhesion and TEM was determined as described in "Materials and Methods."
c) Attached T cell hybridoma number (RTT-7E7) was counted in 10 different fields, and was 447.5±32.3, while TEM number was 0.32±0.4. Attached T cell hybridoma number (6H2.D9) was counted in 10 different fields, and was 77.2±15.2, while TEM number was 53.1±11.4.

The nature of endothelial cells is important for TEM of T cell hybridomas In order to test whether the ability of T cell hybridoma clones to transmigrate is determined by the nature of T cell hybridoma, T cell hybridomas were cultured on aorta endothelial cells, REC20 monolayers. The results are summarized in Table II. G3 T cell hybridoma clones such as RTT-3E6, -3G4, 6H2.D9, as well as 6H2.H2, exhibited very strong TEM against

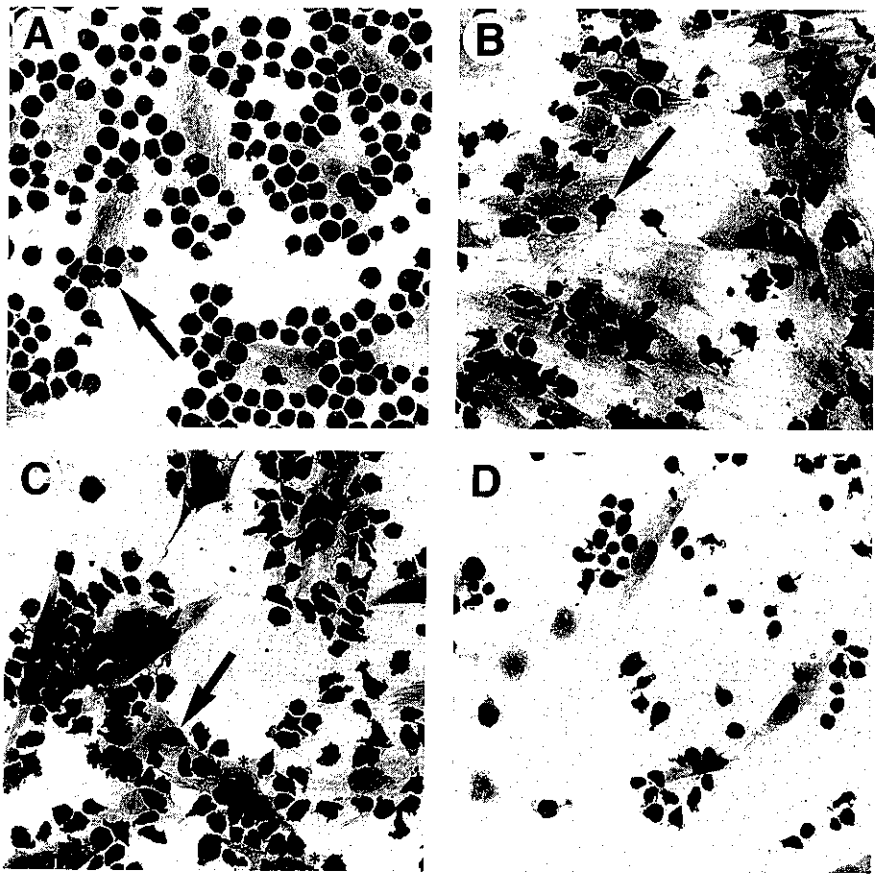

Fig. 1. The binding of T cell hybridomas to HEV. Various T cell hybridoma cells were cultured with HEV for 1 h. Cells were stained with Giemsa solution (×400). A: G1 T hybridoma cells (RTT-7D11). B: G2 hybridoma cells (RTT-6H4). C: G3 hybridoma cells (RTT-3G4). D: G4 hybridoma cells (RTT-9F2). T cell hybridomas which adhered to the HEV cell surface are indicated by closed arrows. T cell hybridomas exhibiting TEM possess flattened nuclei and halos around the nuclei are visible (as indicated by ☆). When TEM is evident, nuclei of HEV are shifted and deformed, indicating that T cell hybridomas compress the nuclei of HEV (as indicated by ＊).

HEV. However, these clones, as well as G2 T cell hybridoma clones, failed to transmigrate beneath the cytoplasm of REC20. In addition, G1 T cell hybridoma clones, RTT-7D11 and -7E7, did not exhibit TEM against REC20.

Effect of temperature, PLP fixation, divalent cations and synthetic peptides on T cell hybridoma-HEV interaction

We next examined the effect of low temperature and PLP fixation on T cell hybridoma-HEV interaction to determine whether or not binding and TEM of T cell hybridoma clones are energy-dependent (Table III). T cell hybridomas of G1 showed temperature-sensitive adhesion to HEV, whereas T cell hybridomas of G2 and G3 showed little temperature dependency. However, RTT-5B4 (G2) and 6H2.H2 (G3) exhibited moderate temperature dependency on binding to HEV.

Pretreatment of G1, G2 and G3 T cell hybridoma clones, or pretreatment of both T cell hybridoma clones and HEV with PLP, completely abolished adhesion. Furthermore, the PLP fixation of HEV alone partially inhibited cell adhesion of G1 T cell hybridoma clones to HEV. In contrast, PLP fixation of HEV alone had little effect on binding of G3 T cell hybridomas to HEV.

The absence of Mg^{2+} alone did not affect adhesion of RTT-7D11 (G1) to HEV, but this binding was partially inhibited under a Ca^{2+}-free condition, and almost completely abolished under an Mg^{2+}, Ca^{2+}-free condition. The absence of divalent cation did not affect adhesion of other clones (G2 and G3) to HEV.

Next, we determined the effect of temperature, fixation and divalent cations on TEM (Table IV). TEM of G2 and G3 hybridomas was completely temperature-depen-

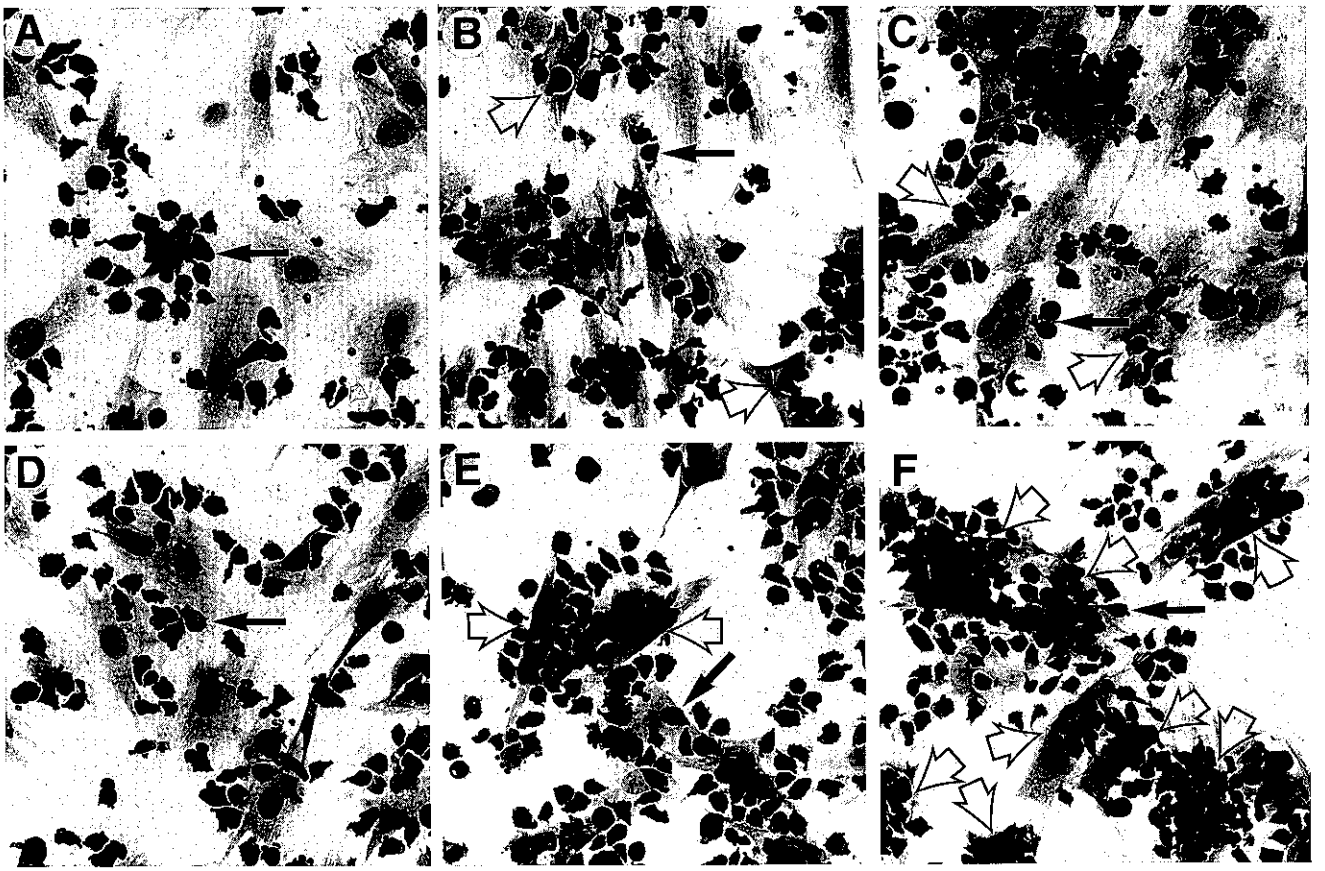

Fig. 2. Kinetic study of G2 and G3 hybridoma interactions with HEV. Giemsa staining ×400. G2 T cell hybridoma, RTT-6H4 (A) (B) (C) and G3 T cell hybridoma, 6H2.D9 (D) (E) (F), were cultured with HEV for 30 min (A and D), 1 h (B and E) or 2 h (C and F). Closed arrows and open arrows indicated adhesion and TEM of T cell hybridomas. Note that G2 and G3 clones exhibited very little TEM at 30 min incubation (A and D). The degree of TEM of G3 hybridoma became intense with longer incubation (E and F), whereas G2 hybridoma remained unchanged (B and C).

Table II. Transmigration of T Cell Hybridomas Was not Observed against Aorta Endothelial Cells[a)]

T cell hybridomas		Adhesion	TEM
G1	RTT-7D11	4+[b)]	−
	-7E7	4+	−
G2	-2F10	−	−
	-1B7	−	−
	-6H4	−	−
	-3E6	−	−
	-3G4	3+	−
	6H2.H2	3+	−

a) T cell hybridomas were incubated with HEV for 1 h.
b) The intensity of adhesion and TEM was determined as described in "Materials and Methods."

dent. TEM of G3 was not influenced by the absence of divalent cations. Prefixation of either T cell hybridomas or HEV completely abolished subsequent TEM of G3 hybridomas.

Binding of G1 T cell hybridoma, RTT-7D11, to HEV was strongly inhibited by a synthetic fibronectin-related peptide, CH271 (Table V). Other fibronectin-related peptides, poly(RGD), CS-1, H271 and the combination of poly(RGD) and H271 did not influence this adhesion.

Adhesive behavior or T cell hybridoma clones to ECM components T cell hybridoma clones revealed distinct adhesive behavior to ECM components as compared to BW5147 cells. In general, the binding of G1, G2 and G3 T cell hybridoma clones to fibronectin and collagen type I is stronger than that seen in G4 hybridoma. No binding ability of T cell hybridomas to hyaluronate was detected (Table VI).

Table III. Effect of Low Temperature, Periodate-lysine-paraformaldehyde Fixation and Absence of Divalent Cations on T Cell Hybridoma-High Endothelial Venule Binding[a]

Group	T cell hybridomas	Temperature		PLP pretreatment of			Divalent cations		
		37°C	4°C	Hybridomas	HEV	Both	$Mg^{2+}(-)$	$Ca^{2+}(-)$	$Ca^{2+}(-)Mg^{2+}(-)$
G1	RTT-7D11	4+[b]	−	−	2+	−	4+	2+	−
	-7E7	4+	−	−	3+	−	ND[c]	ND	ND
G2	-2F10	2+	ND	ND	ND	ND	2+	2+	2+
	-1B7	2+	+	ND	ND	ND	ND	ND	ND
	-5B4	2+	−	ND	ND	ND	ND	ND	ND
	-6H4	3+	2+	ND	ND	ND	ND	ND	ND
G3	6H2.D9	+	+	ND	ND	ND	+	+	+
	RTT-3E6	2+	−	−	2+	−	ND	ND	ND
	-3G4	2+	2+	−	2+	−	ND	ND	ND
	6H2.H2	3+	+	−	2+	−	ND	ND	ND

a) T cell hybridomas were incubated with HEV for 1 h.
b) The intensity of adhesion and TEM was determined as described in "Materials and Methods."
c) ND; not determined.

Table IV. Effect of Low Temperature, Periodate-lysine-paraformaldehyde Fixation and Absence of Divalent Cations on Transendothelial Migration of T Cell Hybridomas to High Endothelial Venules[a]

Group	T cell hybridomas	Temperature		PLP pretreatment of			Divalent cations		
		37°C	4°C	Hybridomas	HEV	Both	$Mg^{2+}(-)$	$Ca^{2+}(-)$	$Ca^{2+}(-)Mg^{2+}(-)$
G2	RTT-1B7	±[b]	−	ND[c]	ND	ND	ND	ND	ND
	-5B4	±	−	ND	ND	ND	ND	ND	ND
	-6H4	+	−	ND	ND	ND	ND	ND	ND
G3	6H2.D9	2+	−	ND	ND	ND	2+	2+	2+
	RTT-3E6	2+	−	−	−	−	ND	ND	ND
	-3G4	2+	−	−	−	−	ND	ND	ND
	6H2.H2	2+	−	−	−	−	ND	ND	ND

a) T cell hybridomas were incubated with HEV for 1 h.
b) The intensity of adhesion and TEM was determined as described in "Materials and Methods."
c) ND; not determined.

Table V. Effect of Fibronectin Peptides on RTT-7D11 Adhesion to High Endothelial Venules[a]

Peptides	Adhesion to HEV
None[b]	4+[c, d]
Poly(RGD)	4+
CS-1	4+
H271	4+
CH271	−[d]
Poly(RGD)+H271	4+

a) T cell hybridomas were incubated with HEV for 1 h.
b) Each fibronectin-related peptide was used at a concentration of 100 μg/ml.
c) The intensity of adhesion and TEM was determined as described in "Materials and Methods."
d) Attached T cell hybridoma number was 428.3±35.5 per 10 fields in the control, whereas it was 17.6±9.2 per 10 fields in the CH271-added group. The difference between the two groups is statistically significant ($P<0.001$).

Flow-cytometric analysis of T cell hybridomas Phenotypes of T cell hybridomas are shown in Table VII. T cell hybridomas of G3, which showed strong TEM, tended to express more LFA-1 and CD2 than other groups, and one of the adhesion molecules, 7D3 antigen, was expressed on hybridomas of G2 and G3. There seemed to be no relationship between expression of CD44 and degree of adhesion or TEM.

DISCUSSION

T cell hybridomas interacted with HEV in different ways, and were accordingly subdivided into four groups. By using these hybridomas, we were able to analyze the mechanisms involved in T cell binding to HEV and endothelial cells.

The binding of G1 hybridomas to HEV appears to be unique, being temperature-sensitive and requiring

Table VI. T Cell Hybridoma Clones Interact with Distinct Extracellular Matrix Components in Different Ways[a)]

Precoated with[b)]	G1	G2		G3		G4	
	-7D11	-2F10	-1B7	6H2.D9	6H2.H2	-9F2	BW5147
None	−[c)]	−	−	−	−	−	+
Fibronectin	4+	3+	2+	2+	2+	2+	2+
Collagen type I	3+	3+	3+	3+	4+	+	+
Hyaluronate	−	−	−	−	−	−	4+
Chondroitin sulfate A	2+	−	−	−	2+	−	2+
Chondroitin sulfate C	4+	2+	2+	2+	2+	2+	3+
Heparan sulfate	4+	3+	3+	3+	4+	2+	3+
Heparin	−	−	+	+	−	−	3+

a) T cell hybridomas were cultured on plates precoated with ECM for 1 h.
b) Each well in a 48-well culture plate was pretreated with various ECM proteins overnight at 4°C.
c) The intensity of cell binding was determined as described in "Materials and Methods."

Table VII. Phenotypic Analysis of T Cell Hybridomas

T cell hybridomas		CD2	CD5	CD3	CD4	CD8	Class I	LFA-1	ICAM-1	7D3	CD44
G1	RTT-7D11	−[a)]	−	−	−	−	−	−	−	−	±
	-7E7	−	−	−	−	−	−	−	−	−	NT[b)]
G2	-2F10	−	−	−	−	−	−	±	−	±	−
	-1B7	−	−	−	−	−	+	±	−	+	−
	-5B4	±	+	−	−	−	+	±	±	+	NT
	-6H4	±	−	−	−	−	+	+	±	±	+
G3	-3E6	+	−	−	−	−	+	+	−	+	NT
	-3G4	+	±	−	±	−	+	+	−	+	+
	6H2.D9	+	−	±	−	−	+	+	−	+	−
	6H2.H2	±	+	+	−	−	+	+	±	+	−
G4	RTT-9F2	−	−	−	−	−	−	−	−	−	−

a) The intensity of cell staining was determined as described "Materials and Methods."
b) NT; not tested.

energy-dependent processes in both T cell hybridomas and HEV, since the fixation of either HEV or T cell hybridomas reduced cell binding. In addition, this binding is divalent cation-dependent. These data indicate that the binding of G1 hybridomas to HEV involves integrin molecule(s). Since G1 hybridomas failed to express LFA-1, a $\beta 2$ integrin, we examined whether $\beta 1$ integrins are involved in this binding. In fact, the binding of T cell hybridoma, RTT-7D11, to HEV was strongly inhibited by a fibronectin-related synthetic peptide, CH-271, which contains both heparin-binding and cell-binding (-RGDS) domain of fibronectin. It has been reported that the binding of T cells to fibronectin is mediated by VLA-4 and or VLA-5, both members of the integrin family.[32] Since CS-1 did not show any inhibitory effect, VLA-5 may be primarily involved in G1 T cell hybridoma binding to HEV. However, C274, which specifically binds to VLA-5, did not show any inhibition. Similar findings have already been reported: CH271 significantly inhibited liver metastasis of lymphoma, whereas C-274, which contains a cell-binding domain of fibronectin, RGDS, had no effect.[13] The heparin-binding domain of fibronectin significantly influences the binding of T cells through VLA-5. Another molecule that might be involved in this binding is L-selectin, but L-selectin-mediated binding is not temperature-dependent. Conversely, the binding of G2 and G3 hybridomas to HEV is not temperature-sensitive and is divalent cation-independent, indicating that some molecule(s) other than integrins is involved.

Several reports have suggested that the expression of LFA-1 on T cells and ICAM-1 on endothelial cells is related to the degree of TEM.[33-35] In accordance with those reports, all G3 T cell hybridomas (which exhibited strong TEM) expressed LFA-1 and CD2. Furthermore, LFA-1-negative as well as CD2-negative G1 and G4 T cell hibridomas failed to exhibit TEM. Some G2 hybridomas which also exhibit TEM failed to express CD2 but not LFA-1. Therefore, it is possible that LFA-1 regulates

the TEM of T cell hybridomas. One critical argument against this hypothesis is that G3 hybridomas exhibit TEM in the absence of divalent cations, indicating that TEM does not primarily involve integrins. We also demonstrated that G2 and G3 hybridomas failed to exhibit TEM to aorta endothelial cells, which express ICAM-1. Our working hypothesis is that the binding of G2 and G3 hybridomas to HEV and the subsequent TEM involve at least participation of non-integrin molecule(s) as well as LFA-1.

Oppenheimer-Marks *et al.* reported that CD44 also mediates lymphocyte adhesion and TEM.[34] However, there was no relationship between the expression of CD44 and the degree of TEM or adhesion in our assay. This finding is supported by the fact that three of four G2 and G3 T cell hybridomas did not bind to the CD44 ligand, hyaluronate. Although further analysis is required, the combination of hybridomas and endothelial cells reported here provides effective tools for analysis of the antigens involved in TEM.

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