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Characterization of Murine E-selectin Expression *In Vitro* Using Novel Anti-Mouse E-selectin Monoclonal Antibodies

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ABSTRACT: A recombinant phage containing the structural exons for mouse E-selectin has been isolated and characterized. Utilizing PCR techniques the lectin and egf domains were fused to form an artificial cDNA for expression in eukaryotic cells. Transient expression in COS cells demonstrated the lectin and egf domains were sufficient to mediate the binding of mouse and human neutrophils as well as HL60 cells. Recombinant soluble mouse E-selectin was purified and used to immunize rats to generate mAbs specific to mouse E-selectin. A panel of mAbs directed against mouse E-selectin was characterized including five that inhibit the adhesion of HL60 cells or mouse neutrophils to COS cells expressing the mouse lectin/egf domains. These mAbs have been used to characterize the expression and function of E-selectin on cytokine stimulated eEnd.2 murine endothelial cells.

E-selectin is a cytokine inducible endothelial cell surface glycoprotein capable of mediating the adhesion of neutrophils, eosinophils, monocytes, a subset of skin homing T cells, and a number of carcinoma cell lines (reviewd in 1). The expression of E-selectin in response to IL-1, TNF, or LPS stimulation requires transcription and translation and has been reported in a variety of inflammatory settings including delayed type skin reactions, psoriasis and contact dermatitis, inflammatory bowel disease, synovium of patients with arthritis, and in cardiac and renal tissue associated with allograft and xenograft rejection (2-4). Studies utilizing mAbs that block adhesion to E-selectin have demonstrated a role for E-selectin in neutrophil infiltration and tissue injury in a limited number of animal models of inflammation (5,6). However, these studies have been significantly hindered by the lack of cross-reactivity of anti-human E-selectin monoclonal antibodies into species with well defined *in vivo* models of inflammation. To establish the role of E-selectin in murine models of inflammation we

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have developed both nucleic acid and immunological reagents for mouse E-selectin. A genomic DNA clone encoding mouse E-selectin was isolated and characterized, and used to engineer functional recombinant protein. A panel of mAbs was generated to recombinant mouse E-selectin including five that are capable of blocking murine E-selectin dependent adhesion *in vitro*.

MATERIALS AND METHODS

Cell Culture and Antibodies. Mouse endothelioma cell line eEnd.2 was kindly provided by Dr. E. Wagner (Institute for Molecular Pathology, Vienna, Austria) and Dr. Beat Imhoff (Basel Institute for Immunology, Basel, Switzerland) and grown in Dulbecco's minimal essential media (DMEM) with 10% FCS. HL60 and COS 7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI Medium 1640 with 10% FCS and Iscove's Modified Dulbecco's media (IMDM) with 10% FCS, respectively. Human neutrophils (hPMNs) were isolated from freshly isolated whole blood as described previously (7). Mouse neutrophils (mPMNs) were isolated from the femurs of 8-week old female C57BL/6J mice following treatment with cyclophosphamide (150 mg/kg) and IL-1 as described by Benjamin et al. (8). Anti-murine VCAM-1 mAb M/K 2.7 was a generous gift from Dr. Paul Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK.). Anti- murine ICAM-1 mAb YN1/1.7.4 was produced from ATCC rat hybridoma CRL 1878.

Cloning and Expression of Mouse E-selectin. Two oligonucleotides Lec3s and Lec1a previously described (7) were used to amplify a portion of the mouse E-selectin lectin domain. Mouse genomic DNA was subjected to 25 cycles of in vitro amplification utilizing Taq polymerase (Cetus/Perkin-Elmer, Emeryville, CA). The amplicon was cloned into pBS, propogated in *E. coli* and sequenced. This mouse E-selectin fragment was used to screen a mouse genomic library (129SV, Strategene Inc.) under stringent conditions and a single recombinant purified and characterized. An artificial cDNA containing the lectin and egf domains was constructed using a two step PCR technique as outlined in Fig. 1 with appropriate overlapping primers. The fused domains were cloned into the vector pBJ1905 (9), provided by Dr. J. Kochan (Hoffmann-La Roche). The first and second cr elements of human E-selectin were fused to the mouse sequences (MuHu-E-selectin) using the two step PCR method as both a membrane anchored and secreted form. Both forms of the chimera were cloned into the plasmid pBJ1905. Constructs were tested for cell adhesion by transient expression in COS cells. The DNA sequence of all constructs was determined utilizing the ABI model 373A sequencer with fluoresecent chain terminators.

Large scale transfection of COS cells was used for the generation of soluble MuHu-E-selectin. COS cells (5 X 10^7/0.8 ml in Dulbecco's Ca^{++} and Mg^{++} free PBS) were transfected by electroporation (350 V, 250 μF, Bio-Rad Gene Pulser) with 20 μg plasmid DNA, incubated on ice for 10 min, resuspended in IMDM with 10% FCS and plated at 10^7 cells per 225-cm^2-tissue culture flask. Conditioned media was harvested at 40-72 h, and MuHu-E-selectin was purified by affinity chromatography on anti-human E-selectin cr1/cr2 mAb 9A1 coupled to CNBr-sepharose (3.0 mg mAb/ml resin) with a typical yield of 0.2 - 1.0 mg/liter of transfected cell supernatant. Transient transfection of COS cells by the DEAE-dextran method was done as previously described (7) and used for adhesion assays and immunofluorescence.

Production of Monoclonal Antibodies. Hybridomas were produced by fusion of immunized rat splenocyte with SP2/0 cells using standard techniques (11). Lewis rats were initially immunized by foot pad injections of 1.5 X 10^7 COS cells transfected with the membrane bound murine E-selectin (lec/egf) and were boosted at 1 month intervals by intraperitoneal injection of 30 μg purified MuHu-E-selectin. Hybridoma supernatants were

screened by ELISA on microtiter plates coated with 200 ng/well MuHu-E-selectin and detected with peroxidase conjugated goat anti-rat IgG (Boehringer Mannheim, Indianapolis, IN.). Specificity for lectin/egf domain was determined by indirect immunofluorescence mouse E-selectin transfected COS cells, and selected cultures screened for inhibition of adhesion. Ascites was produced in nude mice, and mAbs purified by the caprylic acid precipitation method (12).

Adhesion Assays. Transfected COS cell monolayers (5 X 10^5 per 35 mm-diameter well) were incubated with 5 X 10^6 HL60 cells, human PMNs or murine PMNs for 30 min at 25°C. In blocking studies COS cells were preincubated with 20 μg/ml mAb at 25°C prior to the addition of HL60 cells. Wells were washed three times with RPMI, and bound cells visualized and photographed. Confluent monolayers of eEnd.2 cells on 96 well tissue culture plates were treated for 4.0 h at 37°C with IL-1 (550 pg/ml), TNF (40,000 U/ml), and LPS (1 μg/ml), washed and incubated with 50 μg/ml anti-mouse E-selectin for 1.0 h prior to addition of HL60 cells (2 x 10^5/well). Following a 15 min period of adhesion, wells were filled with buffer, the plates were sealed, inverted, and centrifuged (6 min at 500 g) to remove unbound cells. For quantitative assays, HL60 cells were labeled with 6-carboxyfluorescein (6-CFDA) as described (10) and the fluorescence associated with adherent cells determined in a CytoFluor 2300 plate reader (Millipore Corp. Bedford, MA.).

RESULTS

Cloning and production of recombinant mouse E-selectin

Oligonucleotide primers were made to regions of the lectin domain of E-selectin that exhibit conservation between human and rabbit E-selectin corresponding to amino acids 24 - 32 and 104 - 112 (7). Amplification using mouse genomic DNA as template yielded a 214 bp fragment. DNA sequence analysis indicated a 80% conservation with both human and rabbit E-selectin. The other members of the selectin family displayed significantly lower conservation with the amplicon, supporting the identification of the sequence as the mouse homolog of E-selectin. A mouse genomic DNA library was screened under stringent conditions with the mouse E-selectin amplicon as probe. From $3x10^5$ recombinants screened, a single positive clone with an insert of 14.5 kbp was identified and characterized. DNA sequence analysis indicated that the mouse genomic structure resembled the protein domain/exon organization common to other members of the selectin family (data not shown) (13-15). Utilizing PCR techniques, the exons encoding the mouse E-selectin lectin and egf domains were fused to create an artificial cDNA for expression in eukaryotic cells. The human E-selectin signal sequence was used to assure secretion and the mouse lectin and egf exons were joined to sequences of CD16 sufficient for anchoring the expressed protein to the cell surface via a glycosyl-inositol phosphate linkage (GPI) (Fig. 1A). Transient expression in COS cells indicated that the fused mouse exons were sufficient to mediate the adhesion of both human and mouse neutrophils (PMNs) (Fig. 2). None of the mAbs previously prepared against human or rabbit E-selectin (10) cross-reacted to the murine lectin/egf domains as judged by immunofluorescence on transfected COS cells. In order to produce mouse E-

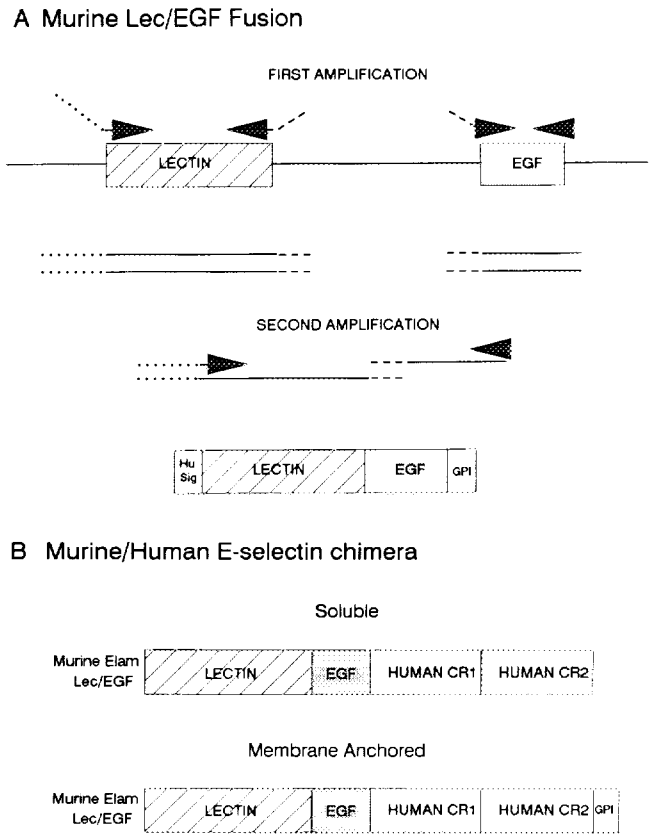

Figure 1. Expression of recombinant mouse E-selectin. A. Two step PCR strategy used to generate the articficial cDNA containing the lectin and egf domains. B. MuHu chimeras used to generate recombinant protein for use as immunogen for production of mAbs specific for mouse E-selectin.

selectin for use as an immunogen for the generation of mAbs, the first and second cr elements of human E-selectin were fused to the mouse lec/egf domains. The Mulec/egf-Hucr1/cr2 (MuHu-E-selectin) was generated as both a GPI membrane anchored and secreted construct (Fig. 1B). Transient expression of the membrane anchored form indicated this construct was also capable of mediating the adhesion of both human and mouse PMNs. Transient expression in COS cells of the secreted murine/human E-selectin chimera was performed to generate large quantities of material that was purified by immunoaffinity chromatography using anti-human E-selectin cr1-cr2 mAb 9A1.

Production and Characterization of Anti-Murine E-selectin Monoclonal Antibodies

Rats were immunized with soluble MuHu-E-selectin purified from transfected COS cell supernatants and hybridoma supernatants were screened by ELISA assay with MuHu E-selectin coated onto microtiter plates. To discriminate between antibodies reactive with the mouse lectin and egf domains from those recognizing the human cr domains, hybridomas

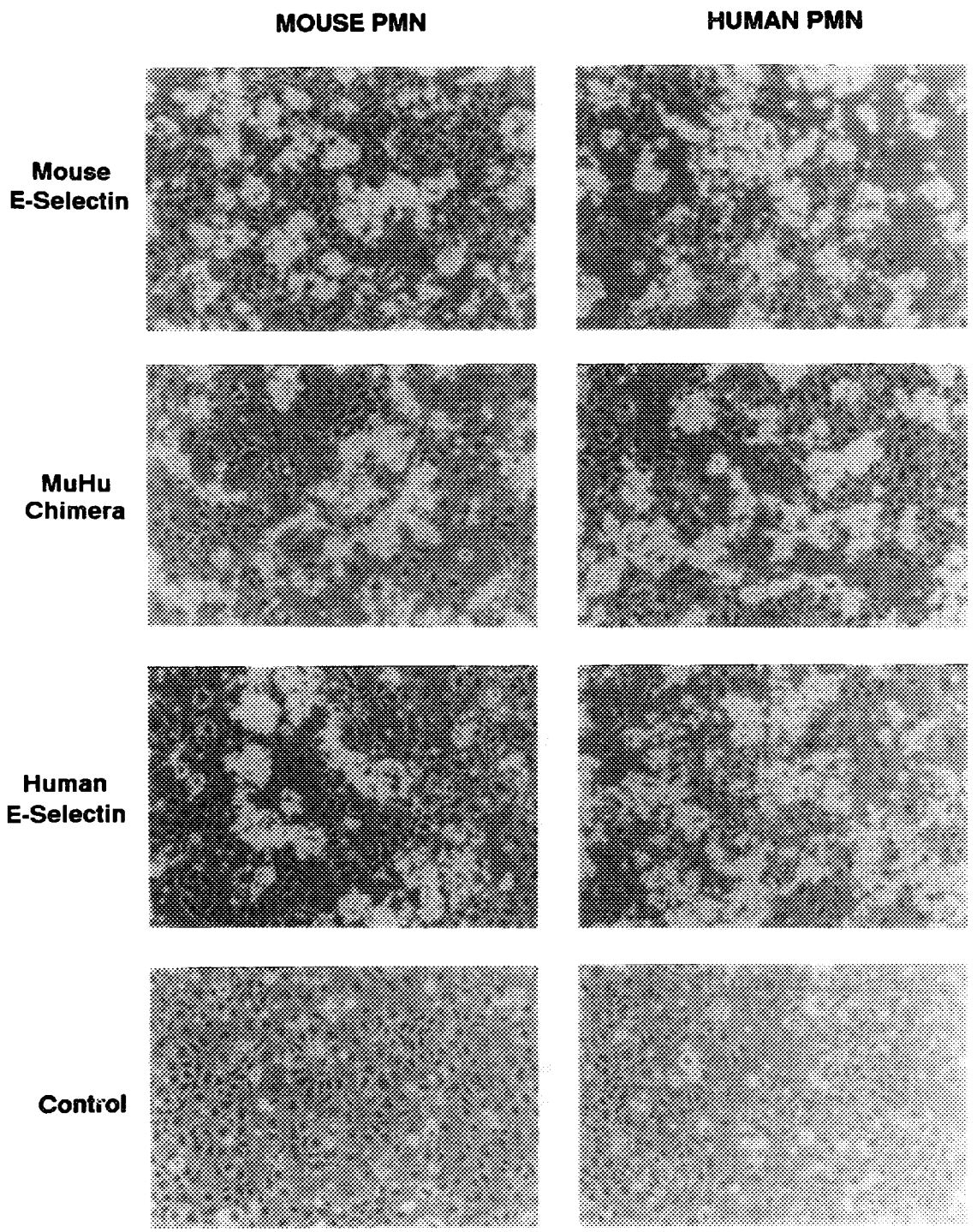

Figure 2. Neutrophil adhesion to recombinant mouse E-selectin cDNAs. COS cells were transfected with the membrane anchored forms of either mouse E-selectin lec/egf, the murine lec/egf-human cr1/cr2 chimera, or human E-selectin. Control represents cells transfected without DNA. Adhesion assays were performed 48 h post transfection with mouse or human neutrophils as described in "Materials and Methods".

were screened by indirect immunofluorescence on COS cells transfected with a membrane anchored construct containing only the mouse lectin and egf domains. We identified nine murine E-selectin specific hybridomas of which five were able to significantly inhibit HL60 adhesion to transfected COS cell monolayers (data not shown). Monoclonal antibodies 9A9, 2B11, 10E6, 13C8, and 13B8 were also able to block mouse PMN adhesion to transfected COS cells (data not shown).

Expression of Mouse-E-selectin in eEnd.2 Endothelioma Cells

Confluent monolayers of mouse eEnd.2 endothelial cells were costimulated with hIL-1α (550 pg/ml), TNF (40,00 U/ml), and LPS (1 μg/ml) for 4.0 hours at 37°C and analyzed for binding of anti-murine E-selectin, VCAM-1 and ICAM-1 mAbs (Fig. 3A). Mouse eEnd.2 cells showed inducible cell surface expression of both murine E-selectin and VCAM-1, and constitutive and up-regulated expression of ICAM-1. MAbs 9A9, 2B11, and 14E4 immunoprecipitated a 100 kD protein from stimulated eEnd.2 cells (Fig. 3B). MAb M/K/2.7 specifically precipitates VCAM-1 from stimulated cells, while ICAM-1 was detected in both control and stimulated cells. HL60 cell adhesion was increased approximately 20 fold by costimulation of eEnd.2 cells with IL1α, TNF, and LPS (Fig. 4). Anti-murine E-selectin mAbs 9A9, 2B11, 13B8, 10E6, and 13C6 significantly inhibit the adhesion of HL60 cells to stimulated cells while mAb 14E4 had no effect. All five neutralizing mAbs showed similar dose responses (IC_{50}=0.5 μM) for inhibition of adhesion and failed to show any additive effect when used in combination, suggesting they recognize overlapping epitopes. The inability of these antibodies to completely block adhesion may be due to the interaction of VCAM-1 with its ligand VLA4, which is known to be present on HL60 cells.

DISCUSSION

With the exception of anti-human E-selectin mAb CL3's cross-reactivity to rat E-selectin (9), monoclonal antibodies have shown restricted cross-reactivity in non-primate species and have limited the ability to specifically investigate the function of E-selectin in animal models of inflammation or metastasis. We have previously described a panel of ten anti-human and anti-rabbit E-selectin monoclonal antibodies which recognize epitopes within the lectin/egf domains including both inhibitory and non-inhibitory antibodies (10). Because we were unable to show cross-reactivity of these antibodies to mouse E-selectin, we developed an alternative strategy for the production of mouse specific reagents utilizing chimeric mouse lec/egf-human cr1/cr2 E-selectin. We have utilized a novel PCR strategy to amplify coding sequences from the structural gene for murine E-selectin to produce fusion constructs containing the lectin and egf domains. The coding sequences are identical to the cDNA sequences recently published for murine E-selectin (16,17) and demonstrate that the N-

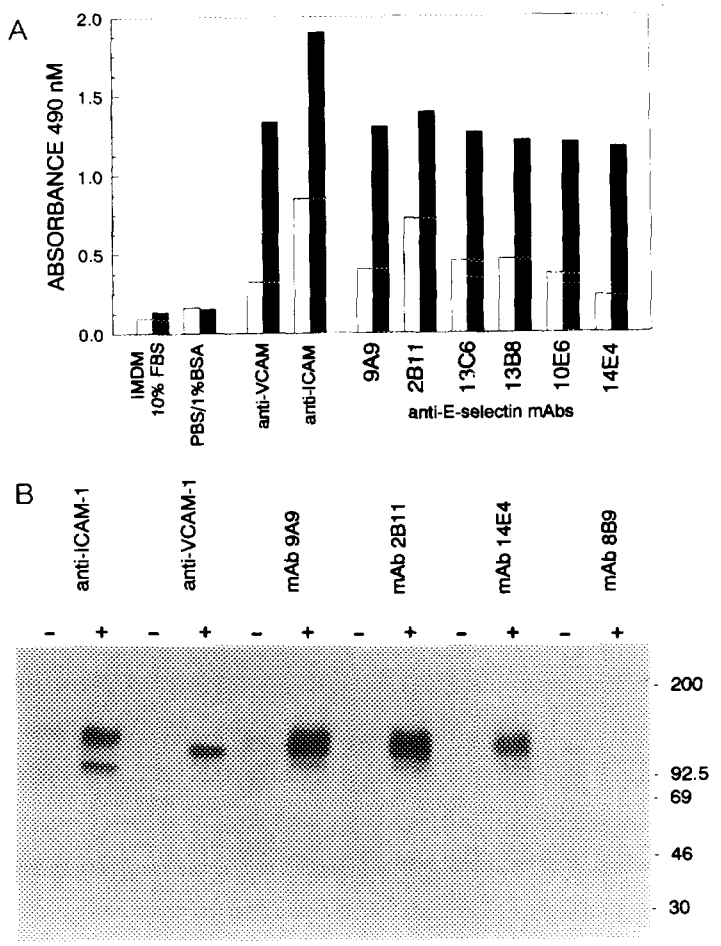

Figure 3. Expression of E-selectin, ICAM-1, and VCAM-1 on stimulated eEnd.2 Mouse endothelial cells. A. Confluent monolayers of eEnd.2 were costimulated with IL-1, TNF, and LPS for 4.0 h at 37°C. Cells were fixed for 15 min at 22°C with 2% paraformaldehyde. Cells were incubated with rat monoclonal antibodies (10 μg/ml) for 1 h, washed and incubated with HRP-conjugated goat-anti-rat antibody, washed and developed using standard protocols. B. Control and IL-1, TNF, and LPS stimulated eEnd.2 cells were labeled for 4.0 h with 150 μCi/ml ^{35}S-cysteine and solubilized in CHAPS lysis buffer (50 mM Tris, pH 8.0, 0.3M NaCl, 10 mM CHAPS, 1 mM PMSF, 10 mM idoacetamide). Cells extracts were incubated with purified rat mAbs to mouse ICAM-1 (YN1/1.7.4), mouse E-selectin (mAbs 9A9, 2B11, 14E4), conditioned media from anti-mouse VCAM-1 hybridoma (M/K 2.7), or rabbit E-selectin (mAb 8B9) as a negative control. Antigen-antibody complexes were precipitated by the addition of goat anti-rat agarose beads. Immunoprecipitates were run under reduced conditions on a 5-15% SDS polyacrylamide gel and analyzed by autoradiography.

terminal lectin and egf domains alone are sufficient to mediate the adhesion of HL60 cells, murine PMNs and human PMNs. The mouse E-selectin gene displays the characteristic exon/protein motif organization representative of the selectin family (13-15). This chimeric E-selectin was used to produce murine-E-selectin specific mAbs that are positive in ELISA, immunoprecipitation assays and immunohistochemical staining of murine tissue sections (data

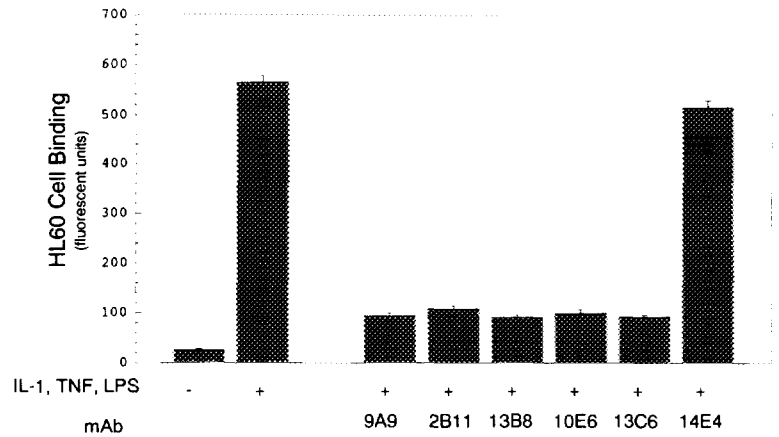

Figure 4. Inhibition of HL60 cell adhesion to stimulated eEnd.2 cells. Mouse eEnd.2 cells were stimulated for 4.0 h with IL-1, TNF, and LPS and were incubated for 1.0 h with 50 μg/ml anti-mouse E-selectin mAbs. HL60 cells labeled with 6-CFDA (2 X 10^5/well) were allowed to adhere for 15 min at 22°C. Wells were filled with buffer and plates were sealed, inverted and centrifuged to remove unbound cells.

not shown). Using these mAbs we demonstrated inducible E-selectin expression in the mouse e.End.2 endothelial cell lines. Our results are in agreement with those recently reported by Hahne et al. (18) on E-selectin expression in eEnd.2 cells, and support the utility of this endothelial cell line for *in vitro* studies of leukocyte adhesion. This panel of both blocking and non-blocking mAbs should be useful for evaluating the E-selectin expression and function in murine models of inflammation and metastisis and have the potential of defining the role of E-selectin in leukocyte migration *in vivo*.

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