INDUCIBLE CELL ADHESION MOLECULE 110 (INCAM-110) IS AN ENDOTHELIAL RECEPTOR FOR LYMPHOCYTES
A CD11/CD18-independent Adhesion Mechanism

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Inflammatory/immune cytokines induce expression of endothelial cell surface structures that bind leukocytes. Endothelial-leukocyte adhesion molecule 1 (ELAM-1) (1, 2) primarily supports adhesion of polymorphonuclear leukocytes (PMN). Intercellular adhesion molecule 1 (ICAM-1) (3, 4) contributes to the adhesion of PMN, monocytes, and lymphocytes through interaction with leukocyte cell surface molecules of the CD11/CD18 complex (5–8). mAbs directed against CD11/CD18 molecules or ICAM-1 only partially inhibit lymphocyte or monocyte binding to activated endothelium (6, 7), suggesting the presence of additional adhesive mechanisms in inflamed endothelium (6).

A novel, cytokine-inducible endothelial cell surface glycoprotein of 110 kD, termed inducible cell adhesion molecule 110 (INCAM-110), was recently identified as a mediator of melanoma cell–endothelial adhesion (9, 10). We now report that INCAM-110 is involved in the adhesion of lymphocytes, monocytes, and certain leukocyte cell lines to endothelium in vitro, and is upregulated in inflammatory reactions in vivo.

Materials and Methods

Cell Culture. Human umbilical vein endothelial cells (HEC) were grown in microtiter wells (Costar, Cambridge, MA) (11). Ramos (human Burkitt's lymphoma), CCRF-CEM (human T cell lymphoma; American Type Culture Collection, Rockville, MD), and an EBV-transformed B lymphoblastoid cell line (IB-4; gift of M. Kurilla, Boston, MA) were grown in RPMI 1640 medium with 10% FCS (Gibco Laboratories, Grand Island, NY). Human dermal fibroblasts (AlF21) were maintained in M199 with 15% FCS.

mAbs. mAbs El/6 (IgG1) and H18/7 (IgG2a) recognize INCAM-110 (10) and ELAM-1 (1), respectively. mAb El/7 (IgG2a), raised against TNF-activated HEC, recognizes ICAM-1, as demonstrated by immunoprecipitation and gel electrophoresis, and by immunobinding to COS cells transfected with a cDNA encoding ICAM-1 (provided by B. Seed, Boston, MA). mAbs El/6 and El/7 were utilized as pooled hybridoma culture supernatants (RPMI 1640 with 10% horse serum) with maximal HEC surface binding and adhesion blocking activity (as determined by titration studies). mAbs RR1/1 (IgG1, anti-ICAM-1), TSI/2 (IgG1, anti-CD11a), and TSI/18 (IgG1, anti-CD18) were provided by T. Springer, Boston, MA. Anti-leu-M3 (monocyte marker) was obtained from Becton Dickinson & Co., Mountain View, CA.
Blood Cell Preparation. PMN were obtained from peripheral blood as described (11). Mononuclear cell fractions were washed in HBSS with 0.1% BSA (Sigma Chemical Co., St. Louis, MO) to remove platelets, and resuspended into RPMI with 0.1% BSA. Monocytes were isolated by two rounds of adhesion (30–60 min, 37°C) to gelatin-coated tissue culture plastic (Costar) that had been exposed to autologous platelet-free plasma for 30 min at 37°C. Adherent cells were collected with EDTA (2–4 mM, 15 min, 37°C). Lymphocyte- and monocyte-enriched fractions were also prepared by countercurrent flow elutriation (11).

Adhesion Assays. Leukocytes were labeled with 2', 7-bis(-2-carboxyethyl)-5 (and -6) carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Molecular Probes Inc., Eugene, OR) (2-5 mM, diluted from 1-M stock in DMSO; 20 min at 37°C) and resuspended in RPMI with 10% FCS. In some experiments, leukocytes were treated (20–30 min, 4°C) with heat-aggregated rabbit Ig (Pel-Freeze Biologicals, Rogers, AR; 10–20 mg/ml final concentration) to block potential Fc receptor interactions (1). HEC were incubated with mAbs (100 µl/well, culture supernatant or dilutions of ascites protein at 10 µg/ml) for 30 min at 37°C before addition of 0.5–2 x 10⁵ leukocytes per well. mAbs were not routinely removed. After 30 min at 25°C, assay plates were inverted and centrifuged (1); the number of bound leukocytes was determined using an automated fluorescence concentration analyzer (Pandex Laboratories, Inc., Mundelein, IL). In certain experiments, leukocytes were treated with anti-CD11/CD18 mAbs (dilution of ascites, 1:200 or 1:500) at 4°C for 20–30 min before assay.

Flow Cytometry, RIA, and Immunostaining. For flow cytometry, 5 × 10⁵ cells were exposed to primary mAbs (4°C, 30 min) followed by fluorescein-conjugated goat anti-mouse Ig (Boehringer Mannheim Biochemicals, Indianapolis, IN) (1:50 dilution in HBSS with 0.1% BSA; 4°C, 20 min), and fixed in 1% paraformaldehyde in PBS. Cell surface RIAs were performed using 1²⁵I sheep anti-mouse Ig (New England Nuclear, Cambridge, MA) (1). For immunoperoxidase studies, cryostat sections of human tissues were exposed to mAbs (16 h at 4°C) and developed using aminoethylcarbazole or diaminobenzidine as chromagens.

Results and Discussion

Expression of INCAM-110 In Vitro Fewer than 10% of unstimulated HEC bound mAb El/6 by flow cytometric analysis, consistent with the low level expression previously observed by cell surface RIA (10). mAb El/6 bound to >80% of IL-1- or TNF-treated HEC, with an increase in mean fluorescence intensity of ~20-fold. mAb El/6 did not bind to blood mononuclear cells (lymphocytes and monocytes), PMN, or human dermal fibroblasts (A1F21 cells; RIA).

INCAM-110 and ICAM-1 Independently Mediate Lymphocyte Adhesion to Endothelial Monolayers. Exposure of HEC to IL-1 (5 U/ml, 6–8 h) increased the adhesion of PBL by 4.8 ± 0.9 times (mean ± SEM; five experiments). Anti-INCAM-110 mAb El/6 inhibited adhesion of PBL to IL-1-activated HEC by 41 ± 5% (five experiments) (Fig. 1). Treatment of PBL with mAb El/6 (30 min, 4°C) followed by washing had no effect (data not shown). Anti-ICAM-1 mAb El/7 also inhibited PBL adhesion to HEC, but to a lesser degree (12 ± 3%; four experiments) (6). mAbs El/6 and El/7 in combination blocked PBL adhesion by 68 ± 3%, while two irrelevant, isotype-matched mAbs (which bind to both control and cytokine-activated HEC) had no effect. Similar results were observed using anti-ICAM-1 mAb RR1/1 (data not shown).

mAb El/6 blocked the adhesion of Ramos cells (60 ± 7%; four experiments) (Fig. 1), whereas anti-ICAM-1 mAb El/7 had no effect (−1 ± 5% inhibition). In contrast, adhesion of an EBV-transformed B lymphoblastoid cell line (IB-4) was not substantially blocked by mAb El/6 alone (<10%), but was inhibited by mAb El/7 (31 ± 4%; three experiments). Greater inhibition was observed with a combination...
of mAbs E1/6 and E1/7 (55 ± 6%) than with E1/7 alone, suggesting a role for INCAM-110 in IB-4 adhesion. CEM cells (T lymphoblastic lymphoma) were more affected by mAb E1/6 than by mAb E1/7 (Fig. 1). These data suggest that INCAM-110 and ICAM-1 function independently to mediate lymphocyte adhesion.

**INCAM-110 Mediates Lymphocyte Adhesion by a CD11aCD18-independent Mechanism.** mAb TSI/22 (anti-CD11a) inhibited the adhesion of PBL to IL-1-activated HEC by 30 ± 6% (mean ± SEM; four experiments) (Table I). mAb E1/6 in combination with mAb TSI/22 resulted in greater inhibition of PBL adhesion (69 ± 9% decrease; four experiments) than either mAb alone. Similarly, the combination of

### Table I

| HEC Treatment | Leukocytes bound per mm² |
|---------------|--------------------------|
|               | Lymphocytes              | Monocytes                |
| No mAb        | 962 ± 88                 | 895 ± 52                 |
| mAb E1/7 (ICAM-1) | 816 ± 65            | 726 ± 67                 |
| mAb E1/6 (INCAM-110) | 545 ± 36            | 186 ± 13*                |

Blood lymphocytes (<2% leu-M3 reactive) and monocytes (90% leu-M3 reactive) were incubated in media without mAb, or with mAb TSI/22 (1:200 or 1:500 dilution of ascites preparation) for 20 min at 4°C. The leukocyte preparations were warmed to 37°C, added to IL-1-activated HEC monolayers (which had been incubated without mAb, with anti-ICAM-1 mAb E1/7, or with anti-INCAM-110 mAb E1/6; 30 min, 37°C), and a 30-min adhesion assay was performed. Adhesion of lymphocytes and monocytes to unstimulated HEC was 248 ± 46 and 357 ± 20 cells/mm², respectively. Data presented are mean ± SD of quadruplicate microtiter wells.

* p < 0.001 compared with TSI/22 treatment alone, student's t test.

† p < 0.01 compared with TSI/22 treatment alone.
mAb E1/6 and an antibody directed against CD18 (mAb TS1/18) inhibited PBL adhesion by 78 ± 1% (two experiments). These data suggest that LFA-1 and INCAM-110 function independently in PBL adhesion. LFA-1 and ICAM-1 interact directly as a receptor/ligand pair (5). Combinations of mAb directed against ICAM-1 (E1/7, RR1/1) and LFA-1 (TS1/22) were no more effective in blocking adhesion (30 ± 10% inhibition; three experiments) than anti-LFA-1 alone. In addition, CD11a was not detected on Ramos cells (flow cytometric analysis; two experiments), which bind to activated HEC by an INCAM-110-dependent mechanism (Fig. 1). Finally, lymphoblasts deficient in CD11/CD18 (prepared by IL-2 stimulation of PBL from a patient with leukocyte adhesion deficiency; gift of D. Anderson, Houston, TX) were partially inhibited by mAb E1/6 (single experiment), but not by mAb E1/7. Taken together, these data strongly suggest that INCAM-110 binding of lymphoid cells does not require CD11/CD18.

The Role of INCAM-110 in Monocyte Adhesion. Anti-INCAM-110 mAb E1/6 blocked the adhesion of U937 cells to TNF-activated HEC by 49 ± 11% (two experiments), suggesting that monocytic cells recognize INCAM-110. Using unstimulated HEC monolayers, which express INCAM-110 (10) and ICAM-1 (3, 4) at low levels, mAbs E1/6 and E1/7 reduced the binding of isolated blood monocytes by 27 ± 7% and 43 ± 7%, respectively. On cytokine-activated HEC, mAb E1/6 alone had little effect on the adhesion of monocytes (8 ± 2% decrease; 10 experiments); mAb E1/7 blocked adhesion by 17 ± 3% (nine experiments). Combinations of mAbs E1/6 and E1/7 were more effective than either reagent alone (43 ± 5% inhibition; p <0.001), further suggesting a role for INCAM-110 in monocyte adhesion. Similar results were observed with combinations of mAbs E1/6 and anti-CD11a (Table 1).

PMN adhesion to IL-1-activated HEC exhibited little or no dependence on INCAM-110. mAb E1/6 alone did not block PMN adhesion (1 ± 6% inhibition; four experiments), nor did it further reduce the adhesion observed in the presence of blocking mAb against ICAM-1 or CD11a/CD18 (data not shown).

In Situ Expression of INCAM-110 in Lymphoid Tissues and Sites of Inflammation. Anti-INCAM-110 mAb E1/6 did not bind to endothelium of normal human skin (Fig. 2 A) (10), but strongly reacted with small vessels (predominantly venules) associated with florid perivascular inflammatory infiltration in a delayed hypersensitivity reaction to tuberculin (72 h) (Fig. 2 B). Similarly, mAb E1/6 bound to vascular endothelium in a cutaneous insect bite reaction. mAb E1/6 bound focally to venular endothelium in peripheral lymph node and tonsil (typically 5–20% of vessels), but did not to mark high endothelial venules selectively. mAb E1/6 also bound to cells within lymphoid follicles (Fig. 2 C) and certain interfollicular cells (Fig. 2 D) having the appearance of follicular dendritic and interdigitating dendritic cells, respectively. INCAM-110 on dendritic cells could influence lymphocyte localization or antigen presentation, which may involve direct adhesive interactions (12).

Increasing evidence suggests that activated vascular endothelium can affect the pattern and makeup of inflammatory cell infiltrates through expression of specific adhesion molecules (13). ELAM-1 appears to function primarily in the adhesion of PMN (1, 2), while ICAM-1 appears to mediate adhesion of most leukocyte types (6–8). The present studies indicate that INCAM-110 supports the adhesion of lymphocytes and monocytes, but not PMN. Expression of INCAM-110 on activated en-
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FIGURE 2. Photomicrographs of frozen sections of human tissues stained with an immunoperoxidase technique using mAb El/6. Normal human skin (A) demonstrated little or no binding of mAb El/6, whereas the endothelial lining of vessels in a delayed-type hypersensitivity reaction (tuberculin) induced in the same patient (B) was strongly positive. In human tonsil (C), mAb El/6 reacted with vascular endothelium in a minority of small vessels. mAb El/6 also bound extensively to lymphoid follicles (C), primarily decorating cells of dendritic morphology. Interfollicular cells with dendritic morphology were also marked (D). No significant reactivity was noted with control mAb K16/16 (IgG1).

dothelium in vitro is more sustained than that of ELAM-1, suggesting that it may act in concert with ICAM-1 in the development of chronic inflammatory processes.

Summary

Inducible cell adhesion molecule 110 (INCAM-110) is a 110-kD glycoprotein expressed on cytokine-activated human vascular endothelial cells. mAb blocking studies indicate that INCAM-110 and intercellular adhesion molecule 1 (ICAM-1) independently support the adhesion of lymphocytes to activated human umbilical vein endothelial cell monolayers. Anti-CD11a/CD18 antibodies with anti-INCAM-110 mAb El/6 produce greater inhibition of lymphocyte adhesion than either reagent alone, suggesting that INCAM-110 and LFA-1 are not an obligate receptor-ligand pair. Blood monocytes, but not polymorphonuclear leukocytes, also appear to bind endothelial INCAM-110. Endothelial expression of INCAM-110 is upregulated at sites of inflammation, suggesting a role in the recruitment of mononuclear leukocytes.
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Note added in proof: Unpublished studies (M. Bevilacqua and A. Aruffo) indicate that mAb EI/6, used to identify INCAM-110 (9, 10), recognizes an epitope encoded by the recently isolated cDNA designated VCAM-1 (14).

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