

# The Role of Integrins in the Maintenance of Endothelial Monolayer Integrity

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**Abstract.** This paper shows that, in confluent human umbilical vein endothelial cell (EC) monolayers, the integrin heterodimers  $\alpha_2\beta_1$  and  $\alpha_5\beta_1$ , but not other members of the  $\beta_1$  subfamily, are located at cell-cell contact borders and not at cellular free edges. Also the  $\alpha$  chain, but not its most common partner  $\beta_3$ , that is widely expressed in EC cell-matrix junctions, is found at cell-cell borders. In EC monolayers, the putative ligands of  $\alpha_2\beta_1$  and  $\alpha_5\beta_1$  receptors, i.e., laminin, collagen type IV, and fibronectin, are also organized in strands corresponding to cell-cell borders. The location of the above integrin receptors is not an artifact of in vitro culture since it has been noted also in explanted islets of the native umbilical vein endothelium. The integrins  $\alpha_2\beta_1$  and  $\alpha_5\beta_1$  play a role in the maintenance of endothelial monolayer continuity in vitro. Indeed, specific antibodies to  $\alpha_2\beta_1$ ,  $\alpha_5\beta_1$ , and the

synthetic peptide GRGDSP alter its continuity without any initial cell detachment. Moreover, antibodies to  $\alpha_5\beta_1$  increase the permeation of macromolecules across confluent EC monolayers. In contrast  $\beta_3$  antibodies were ineffective. It is suggested that the relocation of integrins to cell-cell borders is a feature of cells programmed to form polarized monolayers since integrins have a different distribution in nonpolar confluent dermal fibroblasts. The conclusion is that some members of the integrin superfamily collaborate with other intercellular molecules to form lateral junctions and to control both the monolayer integrity and the permeability properties of the vascular endothelial lining. This also suggests that integrins are adhesion molecules provided with a unique biochemical adaptability to different biological functions.

THE vascular endothelium is a morphologically simple but functionally complex tissue that forms an active boundary between bloodstream and underlying tissues. The integrity of the endothelial monolayer is an essential requirement for controlled vascular permeability and for preventing the vessel wall from platelet deposition and thrombus formation. Monolayer integrity requires that both cell-cell and cell-matrix contacts are established and maintained during the lifespan of endothelia. Morphological studies in vivo and in situ have shown the presence of tight junctions, adherens junctions, and gap junctions between adjacent endothelial cells (EC)<sup>1</sup> (Schneeberger and Lynch, 1984; Francke et al., 1988); also, the anchoring of EC to subendothelial matrix proteins requires the organization, both in vivo (Hüttner et al., 1985; Drenckhahn and Wagner, 1986; White and Fujiwara, 1986) and in vitro, of firm adhesive structures on the basal side of the membrane called adhesion plaques (Dejana et al., 1987; for review see Burridge et al., 1988). Both cell-cell and cell-matrix adhesion structures are organized by transmembrane glycoproteins

which are linked to cytoskeletal structures (for review see Burridge et al., 1988).

In epithelial cells, cell-cell adhesions have been widely studied (Farquhar and Palade, 1963) and specific proteins belonging to the CAM superfamily have been identified (for review see Edelman, 1986, 1988; Takeichi, 1988). In EC,  $\text{Ca}^{2+}$ -dependent and -independent structures involved in intercellular adhesion have been characterized (Bavisotto et al., 1990) and some additional molecules responsible for the organization of junctional complexes have been identified (Müller et al., 1989; Albelda et al., 1990; Heimark et al., 1990; Newman et al. 1990; Simmons et al., 1990).

Most EC receptors for extracellular matrix proteins belong to the integrin superfamily (Hynes, 1987). These proteins are integral membrane glycoproteins provided with extensive amino acid sequence homology and formed by two noncovalently linked subunits denominated  $\alpha$  and  $\beta$  chains. The major integrins expressed by EC characterized so far are  $\alpha_2\beta_1$ , which is responsible for their adhesion to laminin (LM) and, with lower affinity, to collagen and fibronectin (FN) (Languino et al., 1989; Kirchhofer et al., 1990),  $\alpha_5\beta_1$  that promotes their adhesion to FN (Pytela et al., 1985a; Argraves et al., 1987; Conforti et al., 1989) and  $\alpha_v\beta_3$  (Pytela et al., 1985b; Suzuki et al., 1987) which binds

1. *Abbreviations used in this paper:* EC, endothelial cells; FN, fibronectin; LM, laminin.

to vitronectin (VN) and other substrata (Charo et al., 1987; Cheresch, 1987; Dejana et al., 1989, 1990). We have reported that  $\alpha_5\beta_1$  and  $\alpha_v\beta_3$  are organized in focal contacts following EC interaction with their respective ligand proteins (Dejana et al., 1988a). In this paper we report that integrins  $\alpha_2\beta_1$  and  $\alpha_3\beta_1$ , but not other integrins of the  $\beta_1$  family, are localized at EC cell-cell contacts and that specific antibodies directed to these proteins are able to affect EC monolayer integrity and to alter its property of permeability barrier. These data contribute to the identification of molecular components of EC junctional complexes and to the elucidation of their role in vascular physiology.

## Materials and Methods

### Cell Cultures

Human endothelial cells (EC) were isolated from umbilical vein (Barbieri et al., 1981) by treatment with collagenase (0.1% from *Clostridium histolyticum*, Boehringer, Mannheim, FRG) for 20 min at 37°C. This treatment resulted in detachment islets of EC from the vein wall. In a few experiments these EC islets were seeded directly on gelatin-coated coverslips as indicated. The culture medium for EC was medium 199 (Gibco Laboratories, Grand Island, NY) with 20% newborn calf serum (NCS; Gibco Laboratories), 100  $\mu\text{g/ml}$  endothelial cell growth supplement (ECGS, prepared from bovine brain), 100  $\mu\text{g/ml}$  heparin (from porcine intestinal mucosa, Sigma Chemical Co., St. Louis, MO), 50 U/ml penicillin, 50  $\mu\text{g/ml}$  streptomycin, 2.5  $\mu\text{g/ml}$  fungizone at 37°C. Cells within two in vitro passages were used. Human dermal fibroblasts were cultured in medium 199 with 20% NCS.

### Matrix Proteins

Human plasma FN was purified from freshly drawn citrated blood plasma by affinity chromatography (Engvall and Ruoslahti, 1977) on gelatin-Sepharose (Pharmacia, Uppsala, Sweden). Human plasma VN was purified from human plasma by affinity chromatography on heparin-Sepharose as described (Yatohgo et al., 1988). LM from EHS mouse tumor was a kind gift of Dr. G. Tarabozetti (Istituto Mario Negri Bergamo, Bergamo, Italy) and was purified as described (Timpl et al., 1979). Gelatin was from DIFCO Laboratories (Detroit, MI).

### Antibodies (Immunofluorescence Studies)

For localization purposes we used antibodies recognizing either the  $\beta$  ( $\beta_1$  or  $\beta_3$ ) or the  $\alpha$  ( $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_5$ ,  $\alpha_6$ ,  $\alpha_v$ ) chains of integrin receptors. Antibodies to the  $\beta$  chains were:  $\beta_1$ : a mouse mAb, clone ALA5 (Hemler et al., 1983) obtained from Dr. M. Hemler (Dana Farber Cancer Institute, Boston, MA);  $\beta_3$ : a mouse mAb (clone VIPI-2) to human platelet GpIIb obtained from Dr. W. Knapp (Institute of Immunology, University of Vienna, Austria). Antibodies to the  $\alpha$  chains of the  $\beta_1$  integrin subgroup were: a mAb to  $\alpha_2$  (12F1; Pischel et al., 1987) kindly provided by Dr. V. L. Woods (University of California Medical Center, San Diego, CA); mAb to  $\alpha_3$  (J143; Fradet et al., 1984) from the laboratory of Dr. L. J. Old (Memorial Sloan-Kettering Cancer Center, New York); a rabbit antiserum to the  $\alpha_5$  cytoplasmic domain (Roman et al., 1989) kindly donated by Dr. J. A. McDonald (Washington University School of Medicine, St. Louis, MO) and, for control purposes, the mAb PIF8 (Wayner and Carter, 1987); an mAb anti- $\alpha_6$  (GoH3; Sonnenberg et al., 1987) from Dr. A. Sonnenberg (Central Laboratory of the Netherlands Red Cross, Amsterdam, The Netherlands). The mAb LM142 (Cheresch et al., 1987) directed to the  $\alpha_v$  chain of the VN receptor was kindly donated by Dr. D. Cheresch (Research Institute of Scripps Clinic, Department of Immunology, La Jolla, CA, USA).

The mouse mAb to CD31 (clone 9G11; Simmons et al., 1990) was provided by Dr. J. Gordon (British Biotechnology Ltd., Cowley, Oxford, UK). A mouse mAb (IST-9) to the extra domain specifically expressed in cell-assembled FN and not in plasma FN (Borsi et al., 1987; Carnemolla et al., 1987) was donated by Dr. L. Zardi (Istituto Scientifico per lo Studio e la Cura dei Tumori, Genova, Italy). Rabbit antisera to LM and to collagen type IV were respectively from Gibco Laboratories (cat. no. 680-3019) and from Heyl GmbH, Berlin, FRG.

The localization of vinculin and talin were respectively assayed with a

mAb to vinculin from Bio Makor, Rehovot, Israel (VIN 11-5, cat. no. 6501) and a mAb cross-reacting with human talin (clone 8D4) obtained from Dr. K. Burridge (University of North Carolina at Chapel Hill, Chapel Hill, NC).

All the antibodies were used in immunofluorescence microscopy at Ig concentrations ranging between 15 and 30  $\mu\text{g/ml}$ . The best Ig concentration was selected upon testing a range of dilutions.

### Antibodies and Peptides (Functional Studies)

In functional assays we used a human placenta  $\alpha_5\beta_1$  goat antiserum prepared in our laboratory and characterized elsewhere (Conforti et al., 1989). This antiserum recognizes both the  $\alpha_5$  and the  $\beta_1$  chain. The antibodies to  $\alpha$  chains of the  $\beta_1$  integrin subfamily were: a mouse mAb to  $\alpha_5$  (BIE5; Werb et al., 1989) kindly provided by Dr. C. Damsky (University of California, San Francisco, CA); a mouse mAb to  $\alpha_2$  (CDW49b; Giltay et al., 1989) kindly provided by Dr. J. van Mourik (Central Laboratory of the Netherlands Red Cross, Amsterdam, The Netherlands). A mAb to the  $\beta_1$  chain (AIB2, Werb et al., 1989), kindly provided by Dr. C. Damsky was also used. Antibodies to the  $\beta_3$  chain were: a human platelet GpIIb-IIIa rabbit antiserum prepared in our laboratory and previously described (Dejana et al., 1988b); a human platelet GpIIb-IIIa mAb purified immunoglobulin IgG (7E3; Charo et al., 1987) kindly provided by Dr. B. S. Collier (State University of New York at Stony Brook, NY). A rabbit antiserum to human plasma FN was provided by Dr. G. Tarone (University of Torino, Torino, Italy) and a rabbit antiserum to LM by Dr. G. Tarabozetti. Preimmune goat or rabbit sera and mouse IgGs were used as negative controls.

The synthetic hexapeptides Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) were synthesized and donated by Dr. P. Neri (Dipartimento di Chimica, University of Siena, Siena, Italy) using an automated peptide synthesizer (model no. 430; Applied Biosystems, Inc., Foster City, CA) (Conforti et al., 1989).

### Fluorescence Studies

Cells to be examined by immunofluorescence microscopy were grown on glass coverslips. Glass coverslips (13 mm diameter) were prepared as described elsewhere (Dejana et al., 1988b). Briefly, they were coated overnight at 4°C with the indicated matrix proteins, rinsed with serum-free medium 199 before seeding with  $3.5 \times 10^4$  cells/well in 0.4 ml culture medium, and grown to confluency for 72 h as indicated. When antibodies or peptides were used confluent EC were refed with fresh culture medium and antibodies or peptides added at the concentrations and for the time intervals indicated. Coverslip-attached cells were then fixed with paraformaldehyde and processed for immunofluorescence microscopy as earlier described (e.g., Dejana et al., 1987).

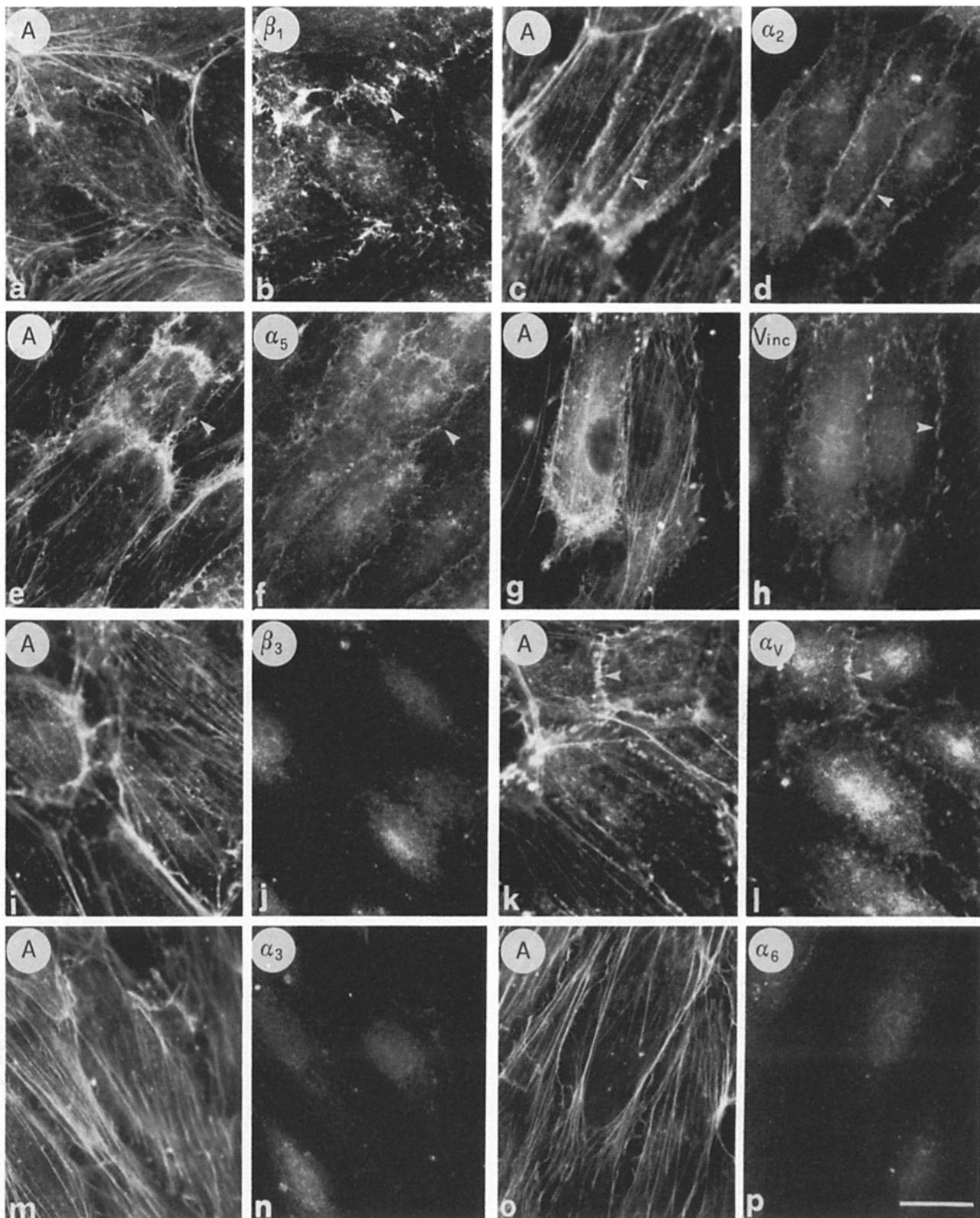
To show F-actin in the process of cell spreading and microfilament organization, fixed and permeabilized cells were stained with 2  $\mu\text{g/ml}$  rhodamine-labeled phalloidin (R-PHD; Sigma Chemical Co.) for 30 min at 37°C.

Indirect immunofluorescence experiments were performed as reported (e.g., Dejana et al., 1987, 1988b, 1990). Briefly, the primary antibody, at Ig concentrations ranging between 15 and 30  $\mu\text{g/ml}$ , was layered on fixed and permeabilized cells and incubated in a humid chamber for 30 min. After rinsing in 0.1% BSA in TBS, coverslips were incubated in the appropriate rhodamine-tagged secondary antibody (50  $\mu\text{g/ml}$ ; Dakopatts, Glostrup, Denmark) for 30 min at 37°C in the presence of 2  $\mu\text{g/ml}$  of fluorescein-labeled phalloidin (F-PHD; Sigma). Coverslips were then mounted either in Mowiol 4-88 (Hoechst, Frankfurt, FRG) or in 50% glycerol-PBS.

Observations were carried out in a Zeiss Axiophot photomicroscope equipped for epifluorescence and interference reflection microscopy. Fluorescence images were recorded on Kodak T-Max 400 films exposed at 1,000 ISO and developed in Kodak T-Max developer for 10 min at 20°C.

### Measure of EC Barrier Properties

The Transwell cell culture chambers (polycarbonate filters, 3  $\mu\text{m}$  pore size, Costar Corp.) were used as described by Langeler and van Hinsbergh (1988), with some modifications. The polycarbonate filters were coated with 10  $\mu\text{g/ml}$  human FN for 1 h at room temperature, rinsed with serum-free medium before seeding  $5 \times 10^4$  cells in 100  $\mu\text{l}$  culture medium in the upper compartment. Culture medium (600  $\mu\text{l}$ ) filled the lower compartment. Culture was continued for 5 d with daily refeeding. Before the experiment, the culture medium of both the upper and lower compartments was replaced by fresh culture medium. Horseradish peroxidase (0.126  $\mu\text{M}$ ; HRP, VI-A type, 44,000  $M_r$ , 1,280 U/mg, Sigma) was added to the upper



**Figure 1.** Immunocytochemical topography of integrins, vinculin, and F-actin in confluent EC monolayers upon initial adhesion to a purified LM substratum. The distribution of  $\beta_1$  (*b*),  $\alpha_2$  (*d*),  $\alpha_5$  (*f*), vinculin (*h*),  $\beta_3$  (*j*),  $\alpha_V$  (*l*),  $\alpha_3$  (*n*), and  $\alpha_6$  (*p*) has been shown by the corresponding mAbs used at Ig concentrations ranging between 15 and 30  $\mu\text{g/ml}$  (see Materials and Methods). *a*, *c*, *e*, *g*, *i*, *k*, *m*, and *o* show F-PHD staining of F-actin of the same fields. Arrowheads show the location of some integrins at intercellular contacts;  $\beta_3$  (*j*),  $\alpha_3$  (*n*), and  $\alpha_6$  (*p*) integrin chains were not detected at intercellular contracts. Bar, 5  $\mu\text{m}$ .

compartment. After the indicated time intervals at 37°C, the medium in the lower compartment was collected and kept on ice until the enzymatic activity of HRP was assayed. To assay HRP enzymatic activity (Ortiz de Montelano et al., 1988) 60  $\mu$ l of the culture medium, collected from the lower compartment, were added to 860  $\mu$ l of a reaction buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub> with 5 mM guaiacol) and the reaction as started by adding 100  $\mu$ l H<sub>2</sub>O<sub>2</sub> (0.6 mM in H<sub>2</sub>O, freshly made solution). The reaction was allowed to proceed for 25 min at room temperature before measuring the absorbance at 470 nm. Purified human  $\alpha$ -thrombin (1,665 U/ml specific activity) used in this assay was a gift from Dr. J. W. Fenton II (State University of New York at Albany). Human  $\alpha$ -thrombin was used at 5 U/ml.

The cells adherent to the filter in the upper compartment were fixed as above described (Dejana et al., 1987, 1988b, 1990) and stained with R-PHD for fluorescence microscopy.

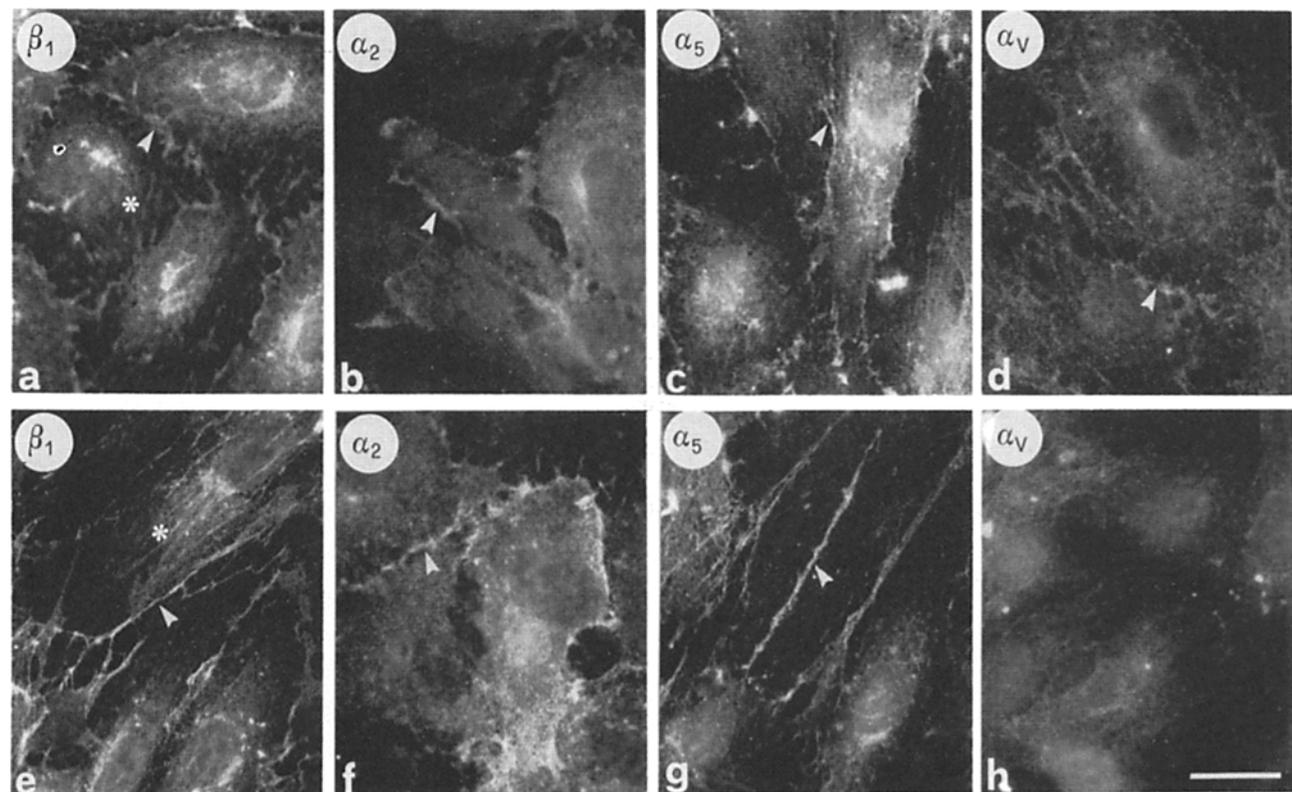
## Results

### Immunocytochemical Topography of Integrins in EC Monolayers

Integrin distribution as well as the F-actin distribution pattern in EC which had been plated on LM and grown to confluency is shown in Fig. 1. In general, the achievement of confluency was accompanied by a rearrangement of the microfilamentous pattern with a noticeable reduction of stress fibers and an increased presence of microfilaments organized as a paramarginal band (Fig. 1). The integrin chains  $\beta_1$  (Fig. 1, a and b),  $\alpha_2$  (Fig. 1, c and d),  $\alpha_5$  (Fig. 1, e and

f) are localized to EC outer boundaries in correspondence of sites of intercellular contact. The fluorescence signal along cell margins was not as continuous as that reported for other intercellular molecules like CD31 (Albelda et al., 1990; Newman et al., 1990; our unpublished observations) since it was somewhat intermittent along boundaries and more intense in some cell groups vs. others. The integrin chain  $\alpha_v$  (Fig. 1, k and l) showed a similar pattern of distribution but it was even more irregularly distributed. Conversely,  $\beta_3$  (Fig. 1, i and j),  $\alpha_3$  (Fig. 1, m and n) and  $\alpha_6$  (Fig. 1, o and p) integrin chains could never be detected at intercellular contacts of EC originally seeded on LM. Vinculin speckles were also observed at areas of cell-to-cell contact (Fig. 1, g and h) but talin, although observed in focal adhesions, was not found at intercellular borders (not shown). Focal contacts containing  $\beta_1$  and  $\alpha_5$  chains were observed along the route of stress fibers in typical extracellular matrix junctions (e.g., Roman et al., 1989; Dejana et al., 1988a, 1989) but were in general less prominent than in non-confluent EC (e.g., Dejana et al., 1988a); the  $\alpha_2$  chain was never found to form clusters at focal contacts even when EC were plated on LM or gelatin from the outset.

We have previously shown (Dejana et al., 1988a) that the composition of the matrix where EC are seeded determines which receptor is clustered at focal contacts within relatively early spreading times. Therefore, we verified whether this

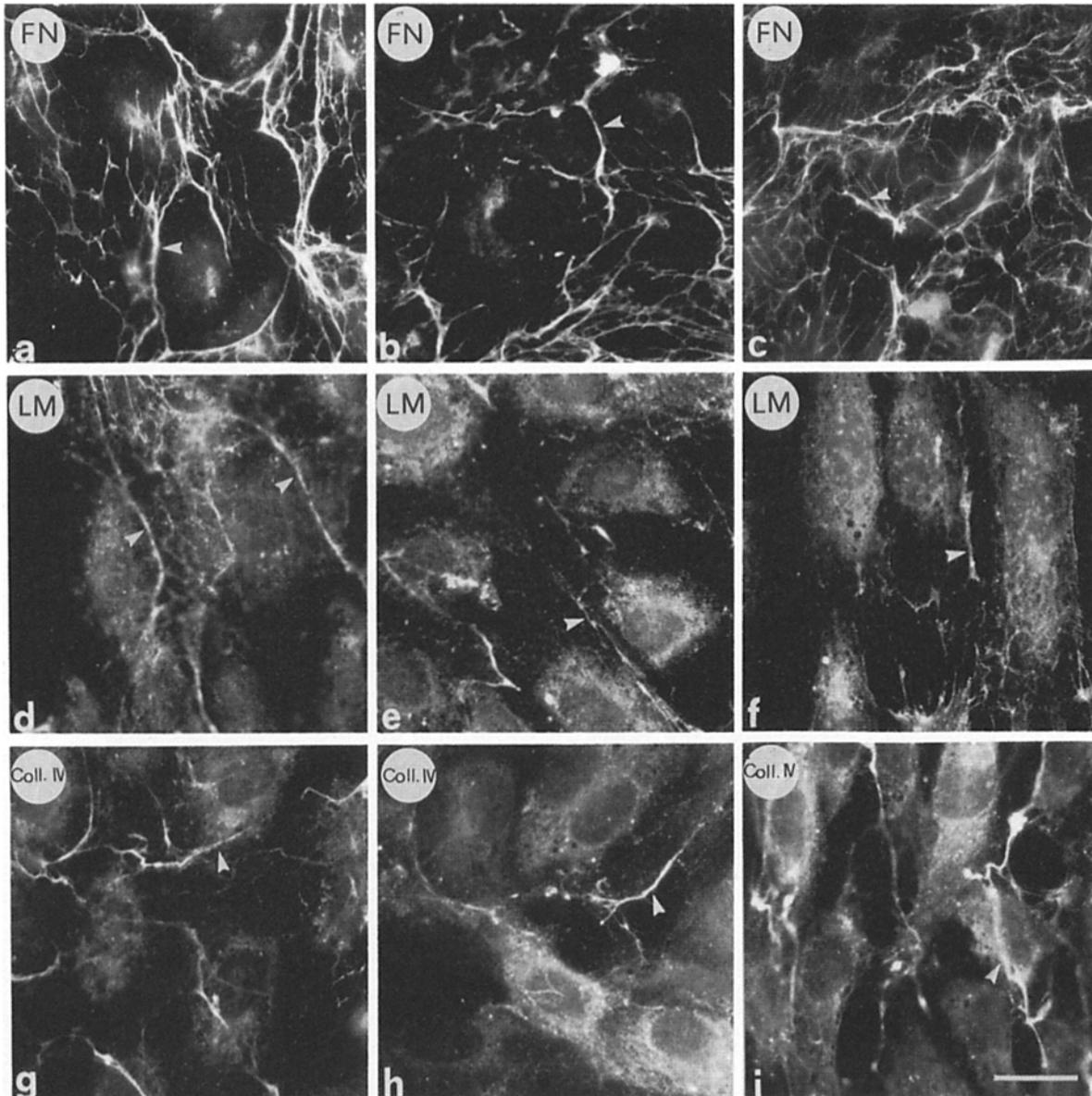


**Figure 2.** Immunocytochemical topography of integrins in confluent EC monolayers upon initial adhesion to FN (a–d) or gelatin (e–h) substrata. Distribution of  $\beta_1$  (a and e),  $\alpha_2$  (b and f),  $\alpha_5$  (c and g), and  $\alpha_v$  (d and h) has been detected by the corresponding monoclonal antibodies used at Ig concentrations ranging between 15 and 30  $\mu$ g/ml (see Materials and Methods). Arrowheads in selected panels show the location of some integrin chains at intercellular contacts;  $\alpha_v$  (h) was apparently absent from intercellular contacts only when initial adhesion took place on gelatin. The areas marked by asterisks in a and e show the simultaneous location of  $\beta_1$  at cell-to-substratum adhesions. Bar, 5  $\mu$ m.

also applies to integrin localization at intercellular contacts. As shown in Fig. 2,  $\beta_1$  (a and e),  $\alpha_2$  (b and f) and  $\alpha_5$  (c and g) integrin chains were enriched at intercellular boundaries in EC that had been plated on FN (a–d) or gelatin (e–h) and grown to confluency. Enrichment of  $\alpha_v$  at intercellular boundaries was observed in EC seeded on FN (d), but not in EC seeded on gelatin (h); never was  $\beta_3$  found at the cell periphery in EC plated on FN or gelatin. However,  $\beta_3$  was found, as expected, in focal contacts only in EC seeded on VN (data not shown).

### ***Extracellular Matrix Proteins Colocalize with Integrins to Intercellular Borders***

Then, integrin distribution at intercellular contacts appeared independent of the substrate where EC had been initially seeded. However, during the time required to achieve full confluency, EC synthesize and organize their own matrix proteins which can eventually modulate integrin distribution. Next, we verified the localization of some extracellular matrix proteins in EC originally seeded on various substrates



**Figure 3.** Distribution of FN (a–c), LM (d–f), and collagen IV (g–i) studied with specific antibodies used at Ig concentrations ranging between 15 and 30  $\mu\text{g/ml}$  (see Materials and Methods) in confluent EC monolayers upon initial adhesion to gelatin (a, d, and g), VN (b, e, and h), LM (c), or FN (f and i). The figure indicates that several molecules of the extracellular matrix are produced and organized during the formation of the monolayer, independently of the initial attachment substratum. Arrowheads show that fibrous strands of extracellular matrix proteins may be located also along the contour of confluent EC and may thus serve as potential ligands for the integrins found at intercellular contacts. Bar, 5  $\mu\text{m}$ .

and grown to confluency. As shown in Fig. 3, the FN produced and organized by EC in the time required to form a monolayer appeared at the cell periphery and as a network under individual cells, independently of the substrate initially presented to the cell (*a-c*). Also independently of the substrate, LM (Fig. 3, *d-f*) and collagen type IV (Fig. 3, *g-i*) appeared mostly organized at the cell periphery. Then, in confluent EC we observe a deposition of matrix strands at least in coincidence with cell peripheral rims. Such proteins represent potential ligands for the integrins we find at the same locations.

#### **Intercellular Localization of the $\alpha_5\beta_1$ Integrin in Polarized vs. Nonpolarized Cells**

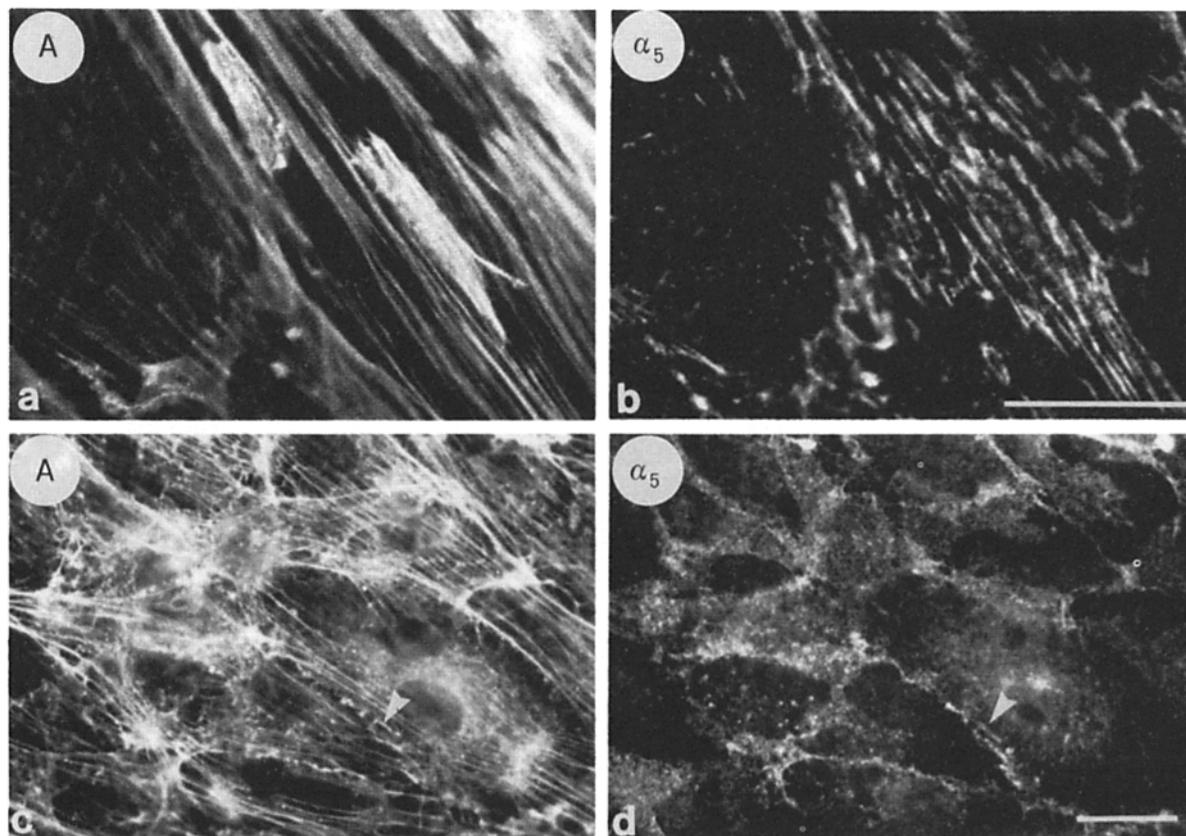
In nonpolarized cells such as human dermal fibroblasts originally seeded on FN,  $\alpha_5$  (Fig. 4) did not appear in coincidence with intercellular contacts as in EC (Fig. 4, *c* and *d*), but only in extracellular matrix adhesions distributed along stress fibers (Fig. 4, *a* and *b*). This pattern was observed in fibroblasts which had been kept in culture long enough to reach considerable density. Moreover, in dense fibroblasts, the  $\alpha_5$  chain was also located both at terminal adhesion plaques in association with  $\beta_3$  and along stress fibers without  $\beta_3$ , indicating that, in the latter sites there is a possible occurrence of the  $\alpha_5\beta_1$  combination (not shown).

#### **The Intercellular Localization of Integrins Is Triggered by Cell-Cell Contact**

The sorting out and eventually the enrichment of integrins at cell borders required the establishment of intercellular contacts. Indeed, in subconfluent EC, neither integrins nor the EC intercellular adhesion molecular CD31 were detected at the cell periphery (Fig. 5). Both  $\alpha_5\beta_1$  and CD31 were expressed at contacting cell borders and never along free cell edges. In each individual EC,  $\alpha_5\beta_1$  (Fig. 5 *b*) but not CD31 (Fig. 5 *d*), was also found at extracellular matrix junctions. Again, the integrin  $\alpha_2\beta_1$  was never found in association with discrete adhesion sites but was localized as  $\alpha_5\beta_1$  to cell-cell contact rims (not shown).

#### **Integrins Are Located at Intercellular Borders also in Portions of Freshly Explanted Endothelium**

A further question that had to be answered was whether integrins were found at intercellular contacts also in the native endothelium of the human umbilical vein prior to dissociation of individual EC for culture purposes. To tackle this problem, we obtained freshly explanted islets of endothelium by brief collagenase treatment of the umbilical vein inner lining and let the islets attach to gelatin-coated coverslips for 4 h. The islets were of variable size, composed of



**Figure 4.** Immunocytochemical topography of the  $\alpha_5$  integrin chain in dense human dermal fibroblasts (*b*) or in confluent EC (*d*) both seeded on a FN substrate. Adjacent panels (*a* and *c*) show F-PHD staining of the same fields for F-actin. While  $\alpha_5$  is predominantly found at intercellular contact rims in confluent EC (*d*, e.g., at arrowhead), in dense fibroblasts,  $\alpha_5$  is found mostly at extracellular matrix adhesions following the direction of stress fibers (*b*). Bars, 5  $\mu$ m.

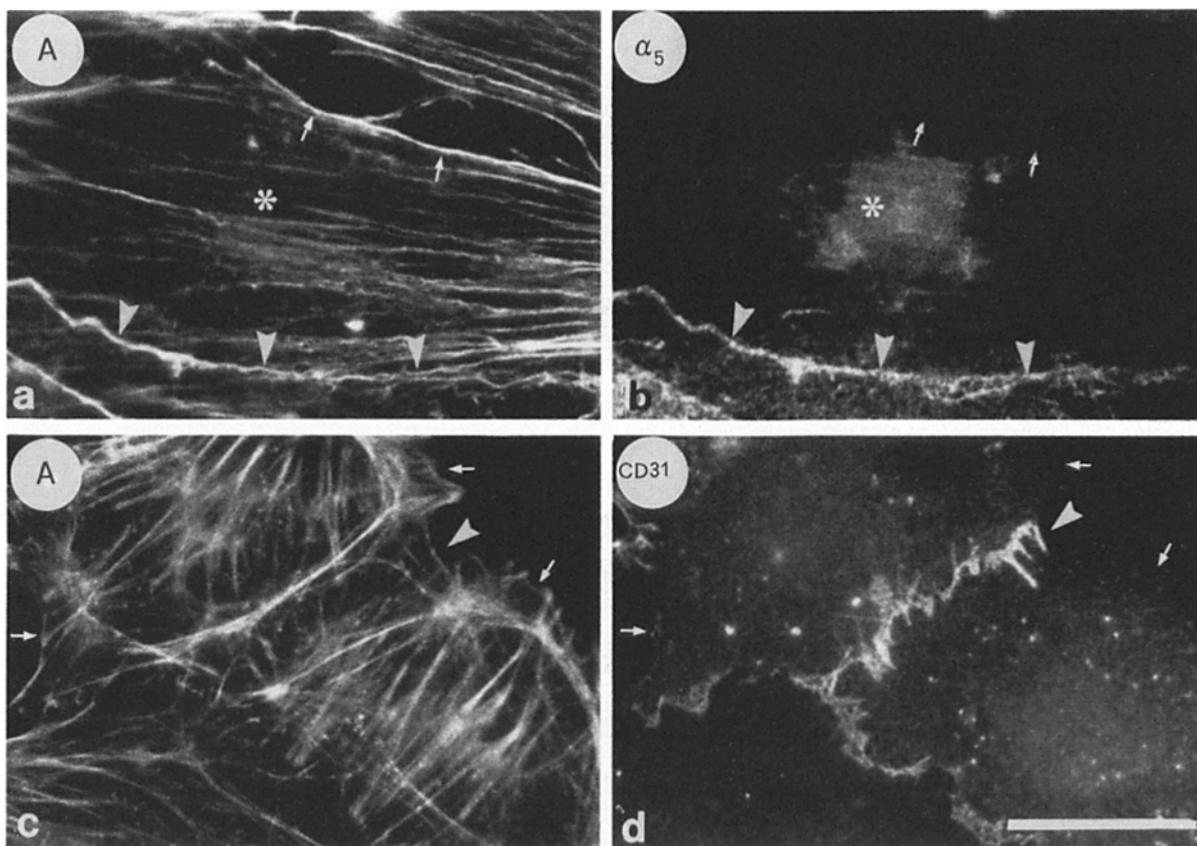
between 6 and  $\sim 100$  cells. Fig. 6 shows that  $\beta_1$  (a),  $\alpha_2$  (b),  $\alpha_5$  (c), and  $\alpha_v$  (d) were originally located to cell-to-cell borders of native endothelium. In the few cells that had begun spreading out of the islets in the short time elapsing from explant, both  $\alpha_v$  (Fig. 6, e and f) and  $\alpha_5\beta_1$ , but not  $\alpha_2\beta_1$ , were also found at newly formed focal contacts. Conceivably, this suggests that integrins are also physiologically located to intercellular contacts in the vascular endothelium in situ.

### Functional Role of Intercellular Integrins in Endothelia

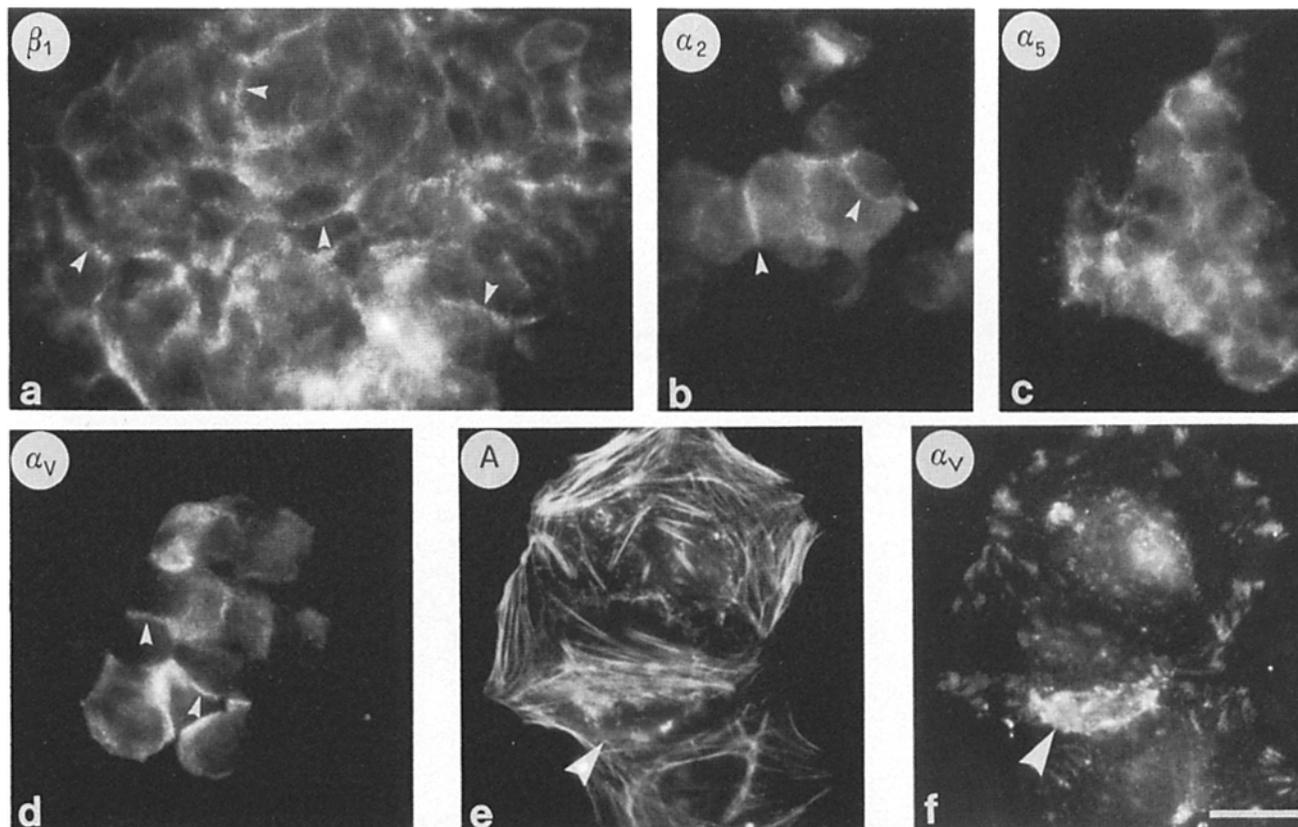
To gain information on the putative role of integrins at intercellular contacts we used functionally active antibodies against the  $\alpha_5\beta_1$ ,  $\alpha_2$ ,  $\alpha_5$ , and  $\beta_3$  chains and the GRGDSP peptide. When confluent EC monolayers were incubated with progressively higher concentrations of antibodies recognizing the  $\beta_1$ ,  $\alpha_2$  or  $\alpha_5$  chains, the first morphologically detectable effects were minute flaws in the continuity of the cellular pattern behaving like "stocking ladders". These tiny discontinuities were apparently determined by the dissociation of adjacent cells and not by the detachment of cells leaving behind an empty space (Fig. 7). No obvious decrease in cell number was detected but subtle changes in microfila-

ment organization of individual cells were observed. Upon these initial lesions, progressively enlarging discontinuities of the EC monolayer followed, ending up with the appearance of real "holes" and resulting in the monolayer detachment from the substrate. The same effect was noted when the GRGDSP peptide was used but no effect was observed with the inactive analogue GRGESP. Conversely,  $\beta_3$  antibodies did not affect the monolayer integrity at exposure times and dilutions which were highly effective when the  $\alpha_5\beta_1$  antibodies were used (Fig. 7). A mAb to human platelet GpIIb-IIIa(7E3) gave results comparable to those observed after rabbit antiserum to platelet GpIIIa.

In conclusion, antibodies to  $\alpha_5\beta_1$  but not to  $\beta_3$  integrins, induce early dissociation of the monolayer continuity presumably by initially affecting intercellularly located  $\alpha_2\beta_1$  and  $\alpha_5\beta_1$  integrins. Later, i.e., at times longer than 2 h, when antibodies could gain access underneath the cells, a more marked effect was observed accompanied by frank cell detachment. At longer times, cell detachment was observed also using  $\beta_3$  antibodies. Rabbit antibodies to FN (1:10) or to LM (1:10) did not affect the integrity of the endothelial monolayer in vitro but were effective in suppressing any attachment to the relevant matrix of EC in suspension (data not shown).



**Figure 5.** Immunocytochemical topography of the  $\alpha_5$  integrin chain (b) and of the intercellular adhesion molecule CD31/PECAM-1 (d) in partially confluent EC. a and c show F-PHD staining of the same fields for F-actin. The localization of both  $\alpha_5$  and CD31 at intercellular contact rims is indicated by the large arrowheads while free cell borders, indicated by small arrows, do not show any enrichment in either molecules. The areas indicated by asterisks in a and b show that only  $\alpha_5$  is found at extracellular matrix adhesions oriented along the route of stress fibers. Bar, 5  $\mu\text{m}$ .



**Figure 6.** Immunocytochemical topography of integrins in islets of freshly explanted endothelium. Islets were let attach on gelatin-coated glass coverslips in the presence of serum. Distribution of  $\beta_1$  (a),  $\alpha_2$  (b),  $\alpha_5$  (c), and  $\alpha_v$  (d and f) has been detected by the corresponding mAbs used at Ig concentrations ranging between 15 and 30  $\mu\text{g/ml}$  (see Materials and Methods). Arrowheads in selected panels show the location of some integrin chains at intercellular contacts; in cells which had already partially spread,  $\alpha_v$  (f) was found in focal contacts located at stress fiber endings (e) as well as in a residual area of intercellular contact (f, large arrowhead). The same pattern was observed for  $\alpha_5$  and  $\beta_1$  but never for  $\alpha_2$  (not shown). e represents the F-actin pattern of the cells of f. Note that all the cells shown in this figure are reproduced at the same magnification. Bar, 5  $\mu\text{m}$ .

### Role of Integrins in Controlling the Permeability of EC Monolayers

Confluent EC monolayers covering the filter septum of Transwell chambers prevented the permeation of HRP ( $M_r$  44,000) between the upper and lower compartments while fibroblast monolayers displayed a significantly lower effect (Fig. 8). Alpha-Thrombin (5 U/ml), which has been reported to induce reversible EC retraction and increased vascular permeability (Langelier et al., 1989), indeed increased the permeation of HRP across the EC monolayer. Moreover, the passage of HRP across the filter increased dramatically when incubation was carried out without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  along with a progressive detachment of the EC monolayer.

When goat antibodies to the  $\alpha_5\beta_1$  receptor were added to the EC monolayer, increased diffusion of HRP was observed. This effect was specific, antibody concentration- and time-dependent but was almost negligible with rabbit  $\beta_3$  antibodies (Fig. 9). The integrity of EC monolayers was checked by staining filters with R-PHD in each experiment. Exposure of monolayers for 1 h to 1:50  $\alpha_5\beta_1$  goat antiserum increased diffusion of HRP but did not induce microscopically obvious discontinuities of the EC monolayer. However, microscopic lesions of the monolayers were observed, as

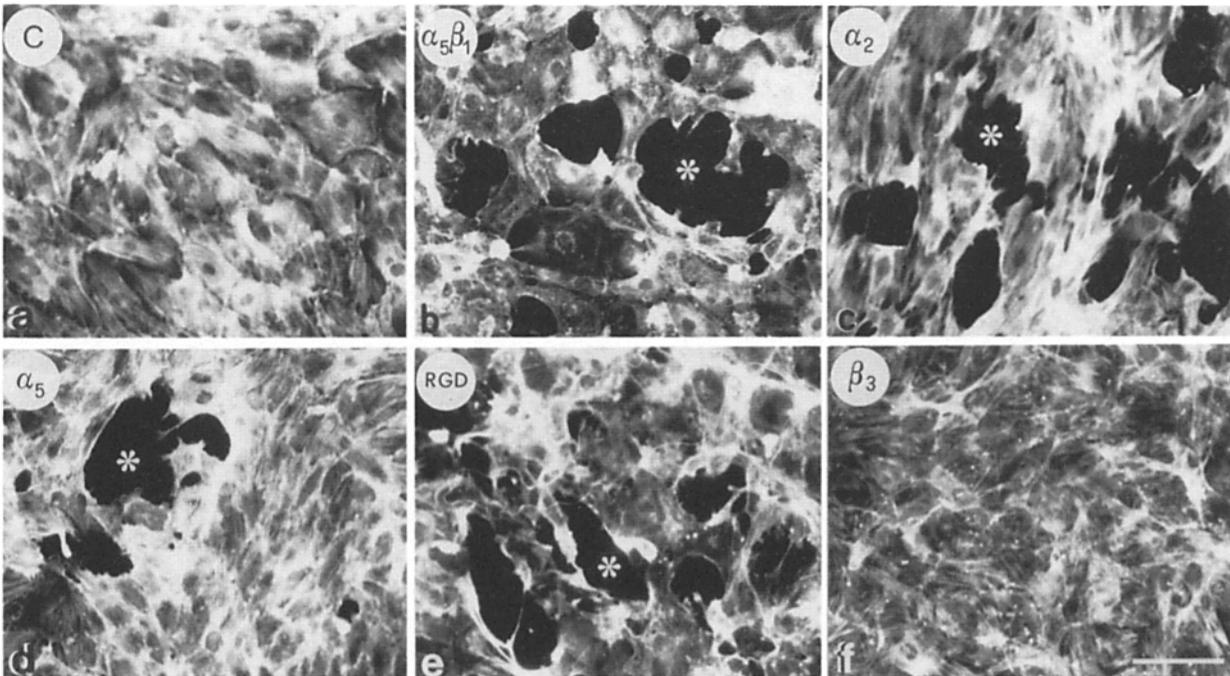
above described, upon incubation with higher antibody concentrations or at longer incubation times.

### Discussion

In this paper we report that, in confluent EC, integrins  $\alpha_2\beta_1$  and  $\alpha_5\beta_1$  are localized to cell-cell borders and that corresponding antibodies alter EC monolayer integrity and affect its role of permeability barrier.

This pattern of localization appears to be specific for  $\alpha_2\beta_1$  and  $\alpha_5\beta_1$ , since other integrins such as  $\alpha_3\beta_1$ ,  $\alpha_6\beta_1$ , and those sharing the  $\beta_3$  chain were not present in cell-cell borders. However and in contrast with the consistent absence of  $\beta_3$ ,  $\alpha_v$  was found at cell-cell adhesion structures. These data suggest that alternative integrin(s) sharing the  $\alpha_v$  chain, such as the recently described  $\alpha_v\beta_5$  (Freed et al., 1989),  $\alpha_v\beta_3$  (Cheresh et al., 1989; Smith et al., 1990),  $\alpha_v\beta_1$  (Bodary and McLean, 1990; Vogel et al., 1990),  $\alpha_v\beta_n$  (Dedhar and Gray, 1990) or unknown combinations, may also be involved in intercellular contacts.

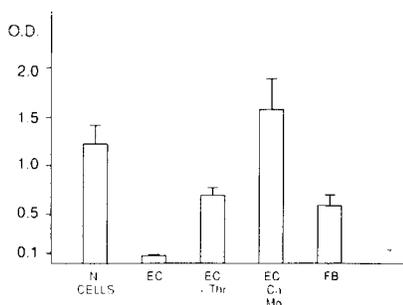
In contrast to integrin organization in cell-to-matrix adhesion that depends on the type of matrix ligand offered to EC at least at short times (Dejana et al., 1988a, 1989, 1990),



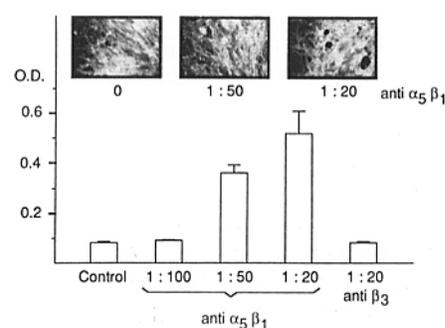
**Figure 7.** Effects of the incubation of confluent EC monolayers with antibodies to  $\alpha_5\beta_1$  (b),  $\alpha_2$  (c),  $\alpha_5$  (d), and  $\beta_3$  (f) as well as with the synthetic peptide GRGDSP (e). The control (a) was incubated with an irrelevant antibody. Antibodies to  $\alpha_5\beta_1$ ,  $\alpha_2$ ,  $\alpha_5$ , and GRGDSP cause the appearance of discontinuities in the monolayer (marked by white asterisks in b–e) while antibodies to  $\beta_3$  do not. EC had been originally seeded on gelatin. Goat antiserum to  $\alpha_5\beta_1$  (1:20), mAb to  $\alpha_2$  (CD49b; 1:5), mAb to  $\alpha_5$  (BIE5; 1:2), rabbit antiserum to human platelet GpIIIa (1:10) were kept on EC monolayer for 2 h. GRGDSP (0.85 mM) was kept on EC monolayer for 20 min. The EC monolayers represented in this figure have been fixed, permeabilized and stained with R-PHD for F-actin. Bar, 20  $\mu\text{m}$ .

the localization of integrin molecules to cell-cell borders does not depend on the substrate where EC were initially grown. If a matrix ligand is indeed required for the intercellular role of integrins, this may be produced and organized by EC during the relatively long time required for building up monolayers. Good candidates are matrix molecules such as FN, LM or collagen, all major ligands for  $\alpha_2\beta_1$  (Kunicki

et al., 1988; Languino et al., 1989) and  $\alpha_5\beta_1$  (Pytela et al., 1985a; Conforti et al., 1989). The latter matrix molecules are indeed produced by EC (Jaffe and Mosher, 1978; Madri and Williams, 1983; Kramer et al., 1985) and are concentrated in strands corresponding to cell-cell interaction rims. These data, even if suggestive, are not conclusive for a direct role of these molecules in mediating EC association. Anti-



**Figure 8.** Permeation of HRP across Transwell filters. No cells, empty filter. EC, filter with endothelial cells grown for 5 d. EC + Thr, filter with EC treated with 5 U/ml human  $\alpha$ -thrombin during the 2 h permeation interval. EC- Ca-Mg, filter with EC incubated in the absence of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  both in the upper and lower compartment during the 2 h permeation interval. FB, filter with fibroblasts grown for 5 d. HRP was added to the upper compartment and its enzymatic activity was measured in the lower compartment after 2 h incubation. Optical density (OD) at 470 nm is reported on the ordinate. The values shown are the mean of three independent observations  $\pm$  SD.



**Figure 9.** Permeation of HRP across Transwell filters. Effect of antibodies to integrins. EC were cultured on Transwell filters for 5 d. The indicated doses of goat antiserum to  $\alpha_5\beta_1$  or rabbit serum to  $\beta_3$  were added during the 2-h interval of HRP permeation. Control, EC with goat pre-immune serum (1:20). OD at 470 nm is reported on the ordinate. The values are the mean of three independent observations  $\pm$  SD. The insets show the endothelial cell monolayer on Transwell filters after 2 h incubation with: 0, goat preimmune serum (1:20); or 1:50 and 1:20 goat antiserum to  $\alpha_5\beta_1$ . F-actin was stained with R-PHD.  $\times 400$ .

bodies to FN and LM were inactive in inducing minute flaws in the monolayer and, thus, in inhibiting cell-cell interaction (data not shown) while they were potent in blocking cell-substrate adhesion (Dejana et al., 1989, 1990). This indicates either a poor accessibility of the antibodies to cell-cell adhesion structures or that these matrix proteins play a null or a marginal role in EC lateral association.

Integrin distribution in nonpolarized cells, such as dermal fibroblasts, is totally different when compared with that of EC. In fibroblasts,  $\alpha_5\beta_1$  is clustered in focal contacts mostly along microfilament bundles, whereas  $\alpha_2\beta_1$  remains diffusely distributed on any substrate. The fibroblastic  $\alpha_v$  chain is also distributed along stress fibers, where  $\beta_3$  is absent, indicating its possible association with  $\beta_1$  or with other  $\beta$  chains. In general, it is more difficult to identify cell-cell contacts in fibroblasts since these cells tend to overlap in dense cultures, but no staining of any integrin was found with the patterns typical of EC. These data suggest that some well-defined  $\beta_1$  integrins have a specific topography in EC and also in other polarized cells (De Luca et al., 1990), where they contribute to define the typical polarity and the organization of epithelial layered tissues.

Integrin distribution along the outer rim of EC was not homogeneously intense in all areas of the monolayer and was particularly noted in scattered monolayer spots. This could explain why specific antibodies caused discontinuous flaws in the monolayer texture. Molecules specifically located at EC intercellular contacts have recently been identified and denoted as PECAM-1 or CD31 (Newman et al., 1990; Simmons et al., 1990), endoCAM (Albelda et al., 1990) or the  $\text{Ca}^{2+}$ -dependent cell-cell adhesion molecule recognized by the mAb Ec6C10 (Heimark et al., 1990). As reported here, CD31 is also distributed at cell-cell contacts and, similarly to integrins, is not present at free borders of EC, i.e., where they are not reciprocally adjacent. However, the distribution of CD31 is more continuous at contacting EC borders and indeed CD31 antibodies induce a more diffuse monolayer dissociation and change EC morphology more extensively (Albelda et al., 1990). These observations indicate that (a) integrins are not the only molecules regulating the monolayer organization, and (b) the monolayer stability may be controlled by different sets of intercellular molecules, each provided with different morphogenic roles and specific functional properties.

Antibodies to integrins belonging to the  $\beta_1$  subfamily added to confluent EC monolayers induce a significant decrease of endothelial barrier properties at least in vitro assays. Interestingly, this effect was apparent also in conditions where monolayers were virtually intact thus indicating an induced leakage of the cell-cell junctional complex rather than a coarse lesion of the monolayer texture. The possibility that a subtle antibody-induced loosening of cell-matrix interactions could also occur and contribute to increased permeability cannot in principle be excluded. However, no loss of cells was found at antibody concentrations able to cause obvious monolayer flaws, thus suggesting that no significant cell detachment from matrix had occurred.

Integrin localization at intercellular boundaries appeared to be relatively sharper and more intense in EC monolayers that had reached full confluency. Such intercellular enrichment of integrins was accompanied by a relative reduction of integrin localization to cell-matrix adhesions and by a

rearrangement of microfilaments from a predominant stress fiber pattern (Dejana et al., 1988a) to a paramarginal band. This suggests that integrins, once EC have established a compact monolayer organization, tend to relocate from cell-matrix to cell-cell adhesions. This is true for  $\alpha_5\beta_1$  but not for  $\alpha_2\beta_1$ , which is always missing from focal contacts and rather plays its adhesive role by remaining diffuse in the ventral membrane. The preliminary conclusion that may be drawn from this finding is that, in cell types that are programmed to form layers, there is a relative decrease of discrete cell-matrix adhesion sites and associated integrins, which is followed by their relocation to cell-cell adhesions once constituent cells have formed and stabilized reciprocal lateral bonds.

The data reported here have been obtained with cells maintained in the presence of heparin and ECGS. It has been recently found that  $\text{Ca}^{2+}$ -dependent EC contact structure and molecular organization can be abolished by cell interaction with basic fibroblast growth factor or acidic fibroblast growth factor in the presence of heparin (Bavisotto et al., 1990). However, when integrin distribution was studied in cells in primary culture, addition of ECGS and heparin did not reduce the intensity of staining. In addition, cell aggregation, as performed in this study, was not affected by previous exposure of the cells to heparin and ECGS and, in both conditions, it was blocked by  $\alpha_5\beta_1$  integrin antiserum (Lampugnani, M. G., unpublished observations). Altogether, these data suggest that integrin-dependent cell-cell association is a different process than that described by Bavisotto et al. (1990).

We still ignore whether the endothelial localization of integrins to cell-cell borders corresponds to their organization in specific junctional structures. It has to be noticed, though, that vinculin, a cytoskeletal protein present in adhesion junctions of epithelial cells (Geiger et al., 1985), codistributed with integrins at EC borders while talin, that is absent from such junctions (Geiger et al., 1985; Nuckolls et al., 1990) did not.

A major issue was whether the presence of integrins at EC borders was induced by culture conditions or could be also detected in freshly isolated endothelium. To address this issue, islets of umbilical vein endothelium were obtained by mild dissociation, let adhere very shortly and studied by immunocytochemistry. Since the cellular islets represented bits of the native endothelium, we believe they may be considered as fragments of the original inner lining of the vascular wall. Since  $\beta_1$  integrins and also  $\alpha_v$  were detected at intercellular borders in the islets, we feel confident in assuming that integrins might play a role also in the physiology and in the morphogenesis of the vascular lining in vivo.

This paper reports a novel pattern of integrin distribution in vascular endothelia. A similar distribution for  $\alpha_2\beta_1$  and  $\alpha_3\beta_1$  has been reported in keratinocytes (Carter et al., 1990; De Luca et al., 1990; Larjava et al., 1990) and  $\alpha_3\beta_1$  in many different normal and transformed cell types (Kaufmann et al., 1989) and suggested to be responsible for the onset and maintenance of cell association. It could be that this type of distribution and functional role is specific for polarized epithelial cells and for functions related to epithelial physiology but the type of junction(s) that integrins can form remains undefined. Integrins can be linked in a type of homotypic or heterotypic interaction or require the presence

of plasma or matrix protein. Integrin heterotypic interaction has already been described for LFA-1 interaction with ICAM-1 and ICAM-2 (Marlin and Springer, 1987; Stauton et al., 1989),  $\alpha_4\beta_1$  with V-CAM (Elices et al., 1990) or in leukocyte adhesion (Campanero et al., 1990). Integrin-mediated cell adhesion with an intermediate plasma protein has also been described in GpIIb-IIIa ( $\alpha_{GP}\beta_3$ )-mediated platelet aggregation in the presence of fibrinogen (Marguerie et al., 1979). Conversely, homotypic interaction between integrin molecules has not yet been described and remains a possibility to be explored.

Finally, we would like to point out that at least one integrin heterodimer, namely the prototype FN receptor  $\alpha_5\beta_1$  (Pytela et al., 1985a) expressed by human EC, may modulate its cellular topography and can be relocated also to peripheral cell-cell contacts. This event is prompted by a signal generated by the reciprocal contact of cells. Since the  $\beta_1$  chain of the FN receptor is considered as the chief linker for the microfilamentous cytoskeleton via a cascade of connecting proteins at cell-matrix junctions (for review see Burridge et al., 1988), it would be interesting to understand how its biochemical properties change in the new position also in view of the fact that talin is missing from cell-cell junctions (Geiger et al., 1985). In a somewhat different way,  $\alpha_2\beta_1$ , a collagen and LM receptor of EC (Languino et al., 1989), concentrates at cell-cell junctions without having been previously assembled in discrete cell-matrix junctions. All these data suggest that integrins, more than any other adhesion molecules, are endowed with a considerable biochemical and functional adaptability that highlights their key role in the control of cellular associations.

We are greatly indebted to K. Burridge, W. Carter, D. Cheresch, B. S. Collier, C. Damsky, J. W. Fenton II, J. Gordon, M. Hemler, W. Knapp, J. A. McDonald, J. I. Old, A. Sonnenberg, G. Tarabozetti, G. Tarone, J. van Mourik, V. L. Woods, and L. Zardi for supplying reagents used in this study.

This investigation was supported by Centro Nazionale delle Ricerche Special Projects "Biotecnologie e Biostrumentazione" and "Tecnologie Biomediche e Sanitarie", NATO Research Grant International Collaboration, and Associazione Italiana per la Ricerca sul Cancro (AIRC).

Received for publication 30 July 1990 and in revised form 19 October 1990.

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