Proc. Natl. Acad. Sci. USA
Vol. 75, No. 11, pp. 5548–5552, November 1978
Cell Biology

Fibronectin synthesis by epithelial crypt cells of rat small intestine

(intestinal epithelial cells/extracellular matrix)

ANDREA QUARONI*, KURT J. ISSELBACHER*, AND ERKKI RUOSLAHTI†

* Department of Medicine, Harvard Medical School, and Gastrointestinal Unit, Massachusetts General Hospital, Boston, Massachusetts 02114; and † Division of Immunology, City of Hope National Medical Center, Duarte, California 91010

Contributed by Kurt J. Isselbacher, September 5, 1978

ABSTRACT **Synthesis of fibronectin in an epithelial cell line (IEC-6) established from rat small intestine was demonstrated by using immunofluorescence, radioimmunoassay, and collagen-binding. Internally labeled radioactive fibronectin isolated from the IEC-6 cells gave a single main band in sodium dodecyl sulfate/polyacrylamide gel electrophoresis under reducing conditions. Fibronectin isolated from rat plasma gave two closely spaced bands. The slower one had the same mobility as the epithelial cell fibronectin. The distribution of fibronectin in IEC-6 cells as detected by immunofluorescence was different from that described for fibroblasts and other cell types; fibronectin was present exclusively in regions of cell-to-cell contact. No fluorescence was detected on the surface membrane facing the culture medium or underneath the cells. This suggests that fibronectin may not be involved in the adhesion of the epithelial cells to the growth surface but could mediate cell-to-cell contacts. In microscopic sections of the small intestine, immunofluorescent staining with antifibronectin serum was strong in the basement membrane underlying the epithelial cells in the crypts. The *in vitro* synthesis of fibronectin by the crypt cells and its abundant presence in the basement membrane underlying the same cells *in vivo* suggests that fibronectin is a structural component of the basement membrane, and that it may be, at least in part, synthesized and deposited by the intestinal epithelium.**

Fibronectin (LETS protein) is a large glycoprotein found associated with the extracellular matrix and surface membrane of cultured fibroblasts (1–5). A glycoprotein immunologically and biochemically similar to fibroblast fibronectin is present in human plasma (3, 6, 7) and has been referred to as "cold insoluble globulin." Much of the recent interest in fibronectin has been due to its possible role in malignancy because after viral transformation it largely disappears from the cell surface (1, 2, 4, 5, 8–11). The functional properties of fibronectin *in vitro* have been partially elucidated. It shows a strong and specific affinity to collagens (12–14) and mediates the adhesion of fibroblasts to collagen-coated matrices (15–18) and that of hepatocytes to plastic dishes (19). It may also interact with the cytoskeletal components of cells (20). Addition of fibronectin solubilized from the surface of fibroblasts to cultures of transformed fibroblasts has been found to restore normal morphology and growth properties to such cells (21, 22).

The biological roles of fibronectin *in vivo*, however, are basically still unknown. This glycoprotein has been found in all tissues studied (23, 24); it is especially abundant in basement membranes and in the extracellular connective tissue matrix. Among other roles, fibronectin may, therefore, be involved in cell adhesion and in epithelial/mesenchymal interactions. For further elucidation of its biological role *in vivo*, more information on its cellular origin is needed. In addition to fibroblasts, cultured astroglial cells (8), myoblasts (25–27), and endothelial cells (28, 29) have been found to synthesize fibronectin. Little is known about its synthesis and distribution in cultured epithelial cells.

In this study, we demonstrate that epithelial cell cultures established from rat small intestine, and retaining *in vitro* characteristics of intestinal crypt cells (30), synthesize large amounts of fibronectin. We have isolated and partially characterized this fibronectin and studied its distribution in the cultured intestinal cells and in rat intestine.

MATERIALS AND METHODS

Tissue Culture Conditions. The establishment and characterization of the epithelial intestinal cells (IEC-6) in culture has been described (30). In the present study, the cells were used between the 9th and 20th passage. The cells were grown in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum, 2 mM glutamine, 4 μg of insulin per ml, 50 units of penicillin per ml, and 50 μg of streptomycin per ml, in plastic dishes (Lux Scientific Corp.) or on glass coverslips (for immunofluorescence studies). Other conditions and techniques were as described (30).

[^{14}C]Leucine Labeling of Proteins Synthesized by IEC-6 Cells. The medium of confluent monolayers of IEC-6 cells was removed, and fresh complete medium, lacking L-leucine but containing L-[^{14}C]leucine (New England Nuclear, 56.9 mCi/mmol), was added. After 2 days, the medium was removed, centrifuged (2000 rpm, 15 min), dialyzed against distilled water, and lyophilized. For characterization and isolation of fibronectin, the lyophilized proteins were first dissolved in 0.1 of the original volume of 8 M urea to ensure solubilization and then diluted with phosphate-buffered saline. After harvesting of the medium, the cell layer was washed twice with serum-free medium and dissolved in a solution containing 8 M urea, 1% Triton X-100, 0.02% NaN_3, and 100 mM phenylmethylsulfonyl fluoride (2 ml/dish) (9).

Isolation of Fibronectin. Affinity chromatography on gelatin/Sepharose was used to isolate fibronectin from rat plasma, culture medium, and extracts of IEC-6 cells. The culture medium and cell extract were diluted 1:20 with phosphate-buffered saline for the incubation with gelatin/Sepharose. The details of the purification method have been described (12, 31).

Antiserum to Fibronectin and Radioimmunoassay. Anti-rat fibronectin was prepared (31) by immunizing rabbits with fibronectin isolated from rat plasma by affinity chromatography on gelatin/Sepharose (12, 31). The resulting antiserum reacted with a single line in immunodiffusion against rat plasma and concentrated culture medium from rat fibroblasts. To eliminate possible contaminating antibodies not detectable by this method, we absorbed the serum with rat plasma from which fibronectin had been removed with gelatin/Sepharose. The proteins from such plasma were bound to Sepharose for the absorption. Antibodies crossreactive with bovine fibronectin were removed by absorption with bovine plasma proteins (including fibronectin) bound to Sepharose. Absence of reactivity

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Table 1. Fibronectin content in culture medium of IEC-6 cells

Exp.	Total cell no. $\times 10^{-6}$	Confluency, %	Total fibronectin, μg	Concentration, μg/ml of medium	Total cell protein, mg	Fibronectin synthesized*
1	1.2	80	180	6.0	2.565	35.0
2	30	100	414	6.9	6.414	16.1
3	30	100	390	6.5	6.218	20.3
4	43	85	1140	11.4	9.19	31.0
5	28	55	380	3.8	5.99	31.7
6	20	100	680	17.0	4.28	53.0
7	10	100	390	19.5	2.17	45.6
8	9.6	48	128	3.2	2.05	20.8

Fibronectin was determined in the culture medium of IEC-6 cells by radioimmunoassay. For each experiment, cells were harvested after collection of the medium, and total cell number and total cell protein were determined. The percentage of confluency was estimated by reference to the saturation density of IEC-6 cells.
* μg/mg of cell protein per 24 hr.

to bovine fibronectin was checked by immunodiffusion. The radioimmunoassay was based on inhibition of binding of ^{125}I-labeled rat plasma fibronectin by the antiserum described above. Bovine fibronectin or fresh cell culture medium did not show any inhibition in this assay which detects as little as 5 ng of rat fibronectin per ml. Samples of cell culture medium were typically assayed at a dilution of 1:100; urea extracts of cell layers were diluted 1:50. Fresh extraction solution was not inhibitory when diluted 1:10 or more. The assay has been described in detail (31).

Polyacrylamide Gel Electrophoresis. Electrophoresis was done with 7% polyacrylamide slab gels in the presence of sodium dodecyl sulfate and 2-mercaptoethanol as described by Laemmli (32). After they were stained with Coomassie blue, the gels were dried and subjected to autoradiography.

Immunofluorescence Staining. IEC-6 cells cultured on glass coverslips were washed two or three times with serum-free medium and fixed with ethanol (10 min at −40°C) in some cases followed by acetone (10 min at −40°C). Cryostat sections of rat small intestine were placed on glass slides, allowed to air dry, and used shortly after preparation. Duodenum, jejunum, and ileum were used without detecting significant difference in the distribution of fibronectin. The double antibody technique was used for staining. The first antiserum (anti-rat fibronectin with normal rabbit serum or phosphate-buffered saline serving as controls) at a 1:25 dilution was placed over the slides for 30 min at room temperature, followed by three washes with the buffered saline. Fluorescein-conjugated goat anti-rabbit immunoglobulin (1:25 dilution) was then placed over the slides for 30 min, followed again by three 10-min washes with buffered saline. Finally, the samples were counterstained with Evans blue (0.01% in buffered saline), washed with buffered saline, mounted in a 9:1 mixture of glycerol/buffered saline, and examined under a Zeiss fluorescence microscope (model 260).

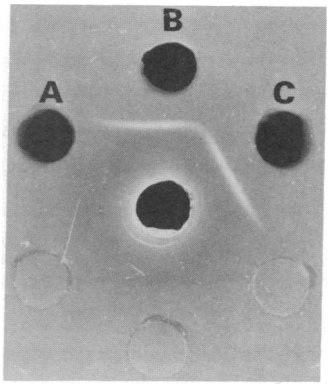

FIG. 1. Immunodiffusion in agarose. Fibronectin purified from bovine plasma (well A), rat plasma (well B), and medium of IEC-6 cells (well C) tested against rabbit antiserum prepared by immunization against rat plasma fibronectin and absorbed with bovine fibronectin.

RESULTS

Fibronectin was detected and quantitated in cultures of IEC-6 cells with a radioimmunoassay specific for rat fibronectin. The amount of fibronectin released into the culture medium by the IEC-6 cells in 24 hr ranged between 16 and 53 μg per mg of cell protein (Table 1). This amount is comparable to that synthesized by cultures of human (33) and rat (E. Hayman and E. Ruoslahti, unpublished results) fibroblasts. No reproducible difference was found between subconfluent and confluent cultures in the amount of fibronectin produced. Fibronectin was found soluble in the medium, and only 5–10% of the total fibronectin synthesized was recovered from the cell layer (Table 2).

Characterization of Fibronectin Produced by IEC-6 Cells. Fibronectin was isolated from the medium and cell extracts of [^{14}C]leucine-labeled IEC-6 cell cultures by using affinity chromatography on gelatin/Sepharose. The same procedure was used to isolate rat plasma fibronectin.

The isolated materials were compared immunochemically and by gel electrophoresis. Fibronectin obtained from plasma and from the IEC-6 culture medium were indistinguishable when tested against an antiserum to plasma fibronectin in immunodiffusion (Fig. 1). Fibronectins produced by fibroblasts and astroglial cells are similarly indistinguishable from plasma fibronectin (3, 8). The fibronectin purified from IEC-6 cells did not react with anti-bovine fibronectin that had been absorbed with rat fibronectin. This was probably due to either a low amount or an absence of fibronectin in the culture medium used for the internal labeling. In other experiments, small amounts of bovine fibronectin were coisolated.

The reaction of immunological identity obtained between

Table 2. Distribution of fibronectin between medium and cell layer

Exp.	Total cell no. $\times 10^{-6}$	Confluency, %	Fibronectin, μg (% of total) Medium	Cell layer
1	20	100	680 (95)	36.0 (5)
2	11	100	390 (93.3)	20.8 (6.7)
3	9.6	48	128 (89)	16.0 (11)

The culture medium of IEC-6 cell cultures was harvested and spun (2000 rpm, 15 min); the cell layer was then washed twice with complete medium, and the cells were scraped into a solution containing 8 M urea, 1% Triton X-100, 0.02% NaN_3, and 100 mM phenylmethylsulfonyl fluoride (2 ml per dish). Fibronectin was determined in the culture medium and solubilized cell layer by radioimmunoassay. Total cell number was measured on parallel cultures, and percent confluency was estimated by reference to the saturation density of IEC-6 cells.

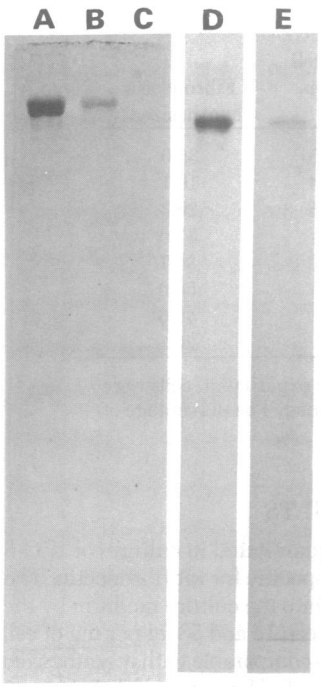

FIG. 2. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. (*Left*) Coomassie blue-stained gel. Lanes: A, reduced samples of fibronectin purified from rat plasma; B, [^{14}C]leucine-labeled culture medium; and C, IEC-6 cell layer. The amount of protein in lane C is too small to give a visible band. (*Right*) Autoradiography of lanes B and C reveals bands in the preparations from IEC-6 cell medium (lane D) and cell layer (lane E).

the IEC-6 product and rat plasma fibronectin and the fact that our antiserum had been depleted of antibodies crossreactive with bovine fibronectin show that the IEC-6 product is of rat origin and is, therefore, produced by the IEC-6 cells. This was also confirmed by demonstrating internal labeling of the isolated IEC-6 fibronectin by means of sodium dodecyl sulfate/gel electrophoresis and autoradiography.

Fibronectin isolated from the IEC-6 culture medium produced a single band in gels stained for protein. This band correlated with the more slowly migrating of the two closely spaced bands obtained with plasma fibronectin (Fig. 2). The amount of fibronectin isolated from IEC-6 cell extracts was insufficient to be detected by protein staining (Fig. 2, lane C) but was detectable by autoradiography as a band that comigrated with the band obtained from the medium (Fig. 2, lanes D and E).

Distribution of Fibronectin in Intestinal Epithelial Cells *In Vitro*. Immunoreactive fibronectin was identified in cultured IEC-6 cells by the double-antibody immunofluorescent technique. An antiserum prepared by immunization against rat plasma fibronectin was used. The antiserum was depleted of antibodies crossreacting with bovine fibronectin of fetal calf serum.

Fibronectin in IEC-6 cells was exclusively associated with an extracellular matrix which often appeared to be composed of bundles of fibrillar structures linking adjacent cells (Fig. 3 *a* and *b*). Fixation of the cells with acetone (Fig. 3*b*) failed to uncover any fibronectin present on the growth substratum beneath the cell monolayer. Likewise, when the cells were treated with EDTA with resultant rounding and partial detachment of the cells from the substratum (Fig. 3 *c* and *d*), fibronectin became organized in a meshwork of large fibrillar structures still linked to the cells; no fluorescence was detected, however, on the surface of the coverslip after cell removal. Brief treatment of the cell monolayer with trypsin completely abolished the fluorescence (data not shown).

Distribution of Fibronectin in Rat Small Intestine. Cryostat sections of rat small intestine were used to study the tissue distribution of fibronectin. The highest concentration of fibronectin (Fig. 4 *a* and *d*) was detected in the region of the crypts of Lieberkühn; the fluorescence was particularly strong in the basement membrane underlying the epithelial cells (Fig. 4 *c* and *e*), and more patchy and weaker in the stroma. Fluorescence associated with the base of the epithelial cells progressively decreased in intensity along the villus axis (Fig. 4*a*) becoming weak and discontinuous at the villus tips (Fig. 4*b*). Some staining was detected in the lamina propria of the villus (Fig. 4 *a* and *b*), possibly associated with blood vessels. Fluorescence in the muscularis mucosa was strong and rather diffuse (Fig. 4*d*), but other relevant portions of the small intestine appeared totally negative (Fig. 4 *a* and *d*). In particular, no fibronectin was detected in the cytoplasm or at the lateral and luminal sides of the epithelial cells.

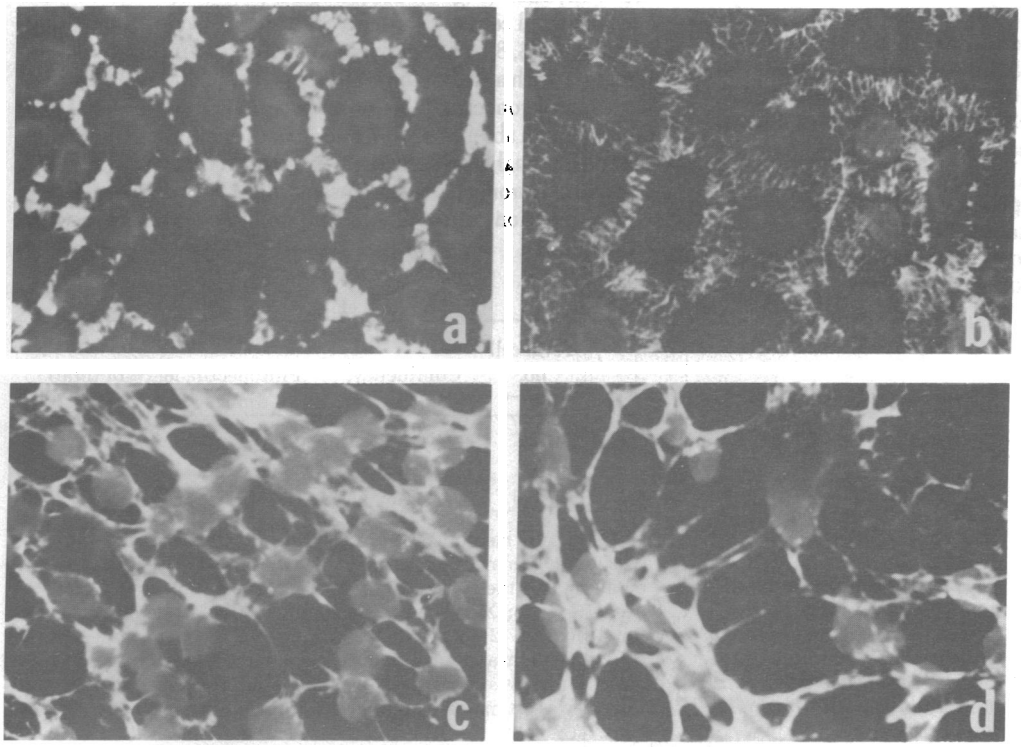

FIG. 3. Distribution of fibronectin in cultured intestinal epithelial (IEC-6) cells. Confluent coverslip cultures of IEC-6 cells were rinsed with serum-free medium and fixed with ethanol (*a* and *c*) or ethanol and acetone (*b* and *d*) at −40°C. Fibronectin was detected by indirect immunofluorescence (here appearing white against a dark background) with an antiserum specific for rat fibronectin and counterstained with Evans blue. (×800.)

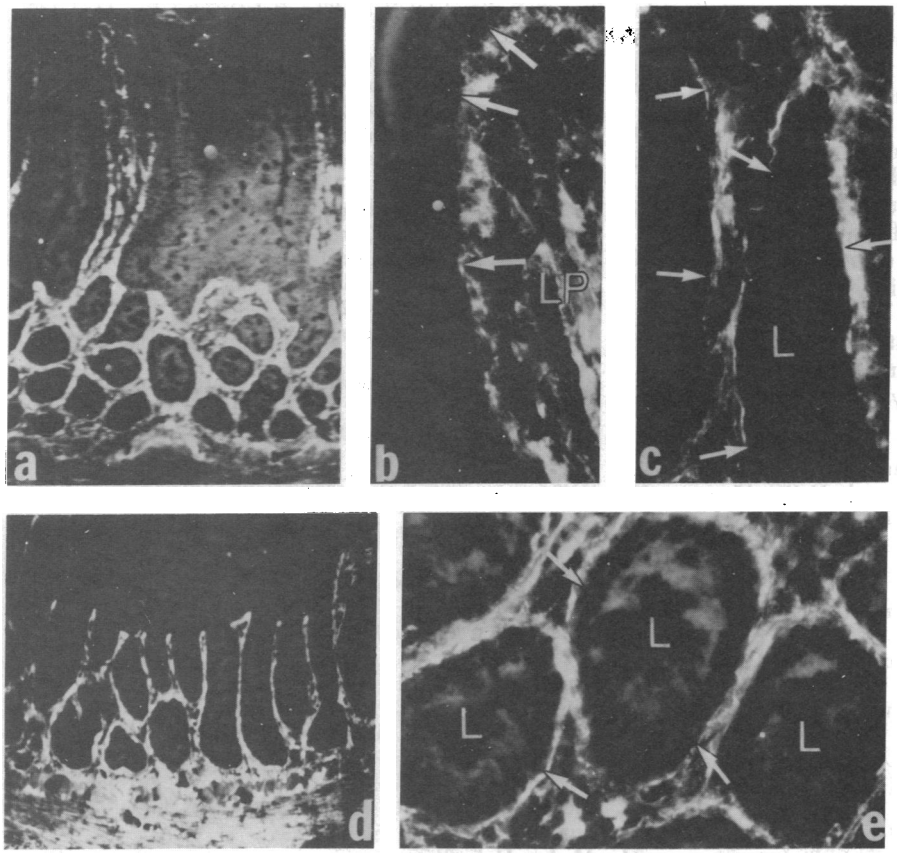

FIG. 4. Distribution of fibronectin in rat small intestine. Cryostat sections (2–4 μm thick) of rat small intestine were stained by indirect immunofluorescence, using an antiserum specific for rat fibronectin, and counterstained with Evans blue. (*a* and *d*) Longitudinal sections of small intestine. (*b*) Villus tips. Arrows indicate the base of the epithelial cells; LP = lamina propria. (*c* and *e*) Higher-magnification view of intestinal crypts showing the localization of fibronectin at the base of the epithelial cells (arrows); L = lumen. (*a* and *d*, ×200; *b*, *c*, and *e*, ×800.)

DISCUSSION

In the present study we have demonstrated the synthesis of fibronectin by cultured rat small intestine epithelial cells (IEC-6 cells), which retain *in vitro* characteristics of small intestinal crypt cells (30). Identity to plasma fibronectin was established on the basis of: (*i*) indistinguishable immunological properties (Fig. 1), (*ii*) similar mobility on sodium dodecyl sulfate gels (Fig. 2), and (*iii*) similar affinity for denatured collagen. The specificity of the antiserum used in these studies (31) and the comigration of protein and radioactivity on sodium dodecyl sulfate gels for both medium and cell layer fibronectin (Fig. 2) demonstrate that the isolated fibronectin is a product of the IEC-6 cells and not a contamination from the fetal calf serum present in the culture medium.

In this study, we have compared isolated fibronectin from plasma, spent culture medium (soluble), and tissue culture cells (insoluble) by gel electrophoresis. Reports on the electrophoretic mobilities of plasma- and cell culture-derived fibronectins are not in agreement (8, 14, 34). Our results suggest that small but reproducible differences exist. Thus, gel electrophoresis of fibronectin from rat plasma revealed two closely spaced bands in approximately equal amounts (Fig. 1), similar to what has been found with mouse plasma fibronectin (31); in contrast, fibronectin from IEC-6 medium and the cell layer resulted in a single band, with the same mobility as the more slowly migrating of the two bands from plasma fibronectin. At present, one can only speculate on the molecular basis for this difference, which might reflect a difference in primary structure, glycosylation, or proteolytic degradation.

The soluble and insoluble forms of fibronectin isolated from IEC-6 cell cultures showed no difference in mobility on sodium dodecyl sulfate gels, similar to what has been found with fibroblast fibronectin (33, 35). These results suggest that no major modification of fibronectin is required for its incorporation in the extracellular matrix.

The IEC-6 cells appeared active in the synthesis of fibronectin, releasing an average of 30 μg of cell protein per ml per day into the medium, a value very similar to that found for fibronectin synthesis by fibroblasts (33). Most of the fibronectin was present in soluble form in the culture medium (Table 2) and no reproducible difference was found between confluent and subconfluent cultures (Table 1).

Immunofluorescence staining of monolayers of IEC-6 cells with a specific antiserum showed that fibronectin is a component of the extracellular matrix. A striking finding was its presence exclusively in regions of cell-cell contact. Fibronectin has also been found in this location in cultures of fibroblasts (11, 36, 37), but in those cases most of the fibronectin was present in the extracellular material beneath the cells. In the endothelial cells (28, 29) and in epithelial cells from hamster kidney and rat liver (38), fibronectin has been found almost exclusively beneath the cells.

On the basis of the above findings and the selective affinity of fibronectin for collagen (12–14), fibronectin is generally considered an important factor in cell adhesion (15–19). Although the IEC-6 cells appear to be tenacious in their adhesion to plastic and glass surfaces (30), our results seem to exclude a role for fibronectin in their attachment to the growth surface, suggesting the involvement of attachment factors other than fibronectin.

In the rat small intestine *in vivo*, the distribution of fibronectin was found to be similar to what has been observed in other tissues (23, 24), the basement membrane underlying the epithelial cells being the region in which this glycoprotein was most concentrated. It is noteworthy that the amount of fibronectin detected by immunofluorescence in the basement membrane was highest in the crypts and decreased towards the tip of the villi.

If the crypt cells were as active fibronectin producers *in vivo*

as they are *in vitro*, at least part of the fibronectin present in the basement membrane underlying the epithelium could be synthesized by these cells and deposited in the basement membrane. A layer of fibroblasts, however, is present beneath the basement membrane (39) and is likely to participate in the synthesis of basement membrane components. If, indeed, fibronectin mediates cell adhesion *in vivo*, the decreasing amount of fibronectin in the basement membrane along the villus could provide the basis for the greater tendency of the villus cells to detach (40). Such a role for fibronectin *in vivo* would be in contrast to its apparent lack of involvement in the attachment of the IEC-6 cells to the growth substratum. However, the geometry of the cells *in vitro* may be different from their organization *in vivo*. Furthermore, the basement membrane *in vivo* and the glass or plastic surfaces *in vitro* may impose different requirements for cell adhesion.

We thank Aulikki Pekkala for technical assistance. This work was supported by Grants AM 01392 and AM 03014 from the National Institutes of Health, by U.S. Public Health Service Training Grant AM 07191, and by Grants CA 22108 and CA 16434 from the National Cancer Institute.

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