Deposition of Fibronectin and Laminin in the Basement Membrane of the Rat Parietal Yolk Sac: Immunohistochemical and Biosynthetic Studies

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ABSTRACT Rat parietal yolk sacs (PYS) at gestational ages 7.5, 9.5, 11.5, 13.5, 14.5, and 16.5 d were reacted with antibodies against laminin or plasma fibronectin. At all times studied, laminin consistently gave a positive reaction with Reichert's membrane and with the cytoplasm of PYS cells. In contrast, fibronectin gave a negative reaction with Reichert's membrane at day 7.5, was weakly positive at day 9.5, and from then on was increasingly positive with maximum reactivity at 14.5 d. By electron microscopic immunohistochemistry, antilaminin reacted strongly with 14.5-d Reichert's membrane and with the contents of the rough endoplasmic reticulum RER cisternae of the PYS cells. Antifibronectin had some spotty reactivity with Reichert's membrane, but the cytoplasm of the PYS cells was negative. The contents of the vitelline vessels and the interface between trophoblast and Reichert's membrane were strongly positive.

Metabolic labeling of PYS cells in organ culture clearly demonstrated the presence of laminin, type IV procollagen, and entactin both in the medium and in tissues, but fibronectin was absent. No component in the medium bound to gelatin-Sepharose columns.

These studies demonstrate that PYS cells, which actively synthesize and secrete basement membrane components, do not synthesize any detectable fibronectin. Furthermore, the antifibronectin staining pattern in the vitelline vessels and trophoblast-Reichert's membrane interface strongly suggests that the fibronectin present in Reichert's membrane is derived from the maternal circulation and is merely "trapped" in the membrane.

The relationship between fibronectin and basement membranes has been controversial. Initial immunofluorescence studies describe the localization of fibronectin in the basement membranes of a variety of tissues (8, 16, 32, 34, 35, 38), but more recent light and electron immunohistochemical studies find that fibronectin and basement membrane antigens have different tissue distributions (1, 21, 37). Because most of the prior studies were performed in tissues with a slow rate of basement membrane synthesis and turnover, they could provide only a relatively static picture. Therefore, we decided to address this question using a more dynamic system characterized by rapid basement membrane synthesis and deposition.

The rodent parietal yolk sac (PYS) in vitro synthesizes, secretes, and deposits onto Reichert's membrane a number of high molecular weight glycoprotein components. These include basement membrane procollagen [pro-α1(IV) and pro-α2(IV)], laminin (PYS A and PYS B), and entactin (PYS C) (2, 4, 7, 11, 12, 31). Biochemical experiments suggest that neither the PYS, the endodermal cells derived from the PYS, nor most PYS carcinoma cells in culture synthesize significant amounts of fibronectin (11–13, 15, 29, 31, 38). In contrast, there are reports that the extracellular matrix associated with such cells and tissues reacts with antibodies directed against fibronectin (13, 34–36, 38, 39).

Here we report that, even at embryonic stages when Reichert's membrane gave a positive reaction with antifibronectin antibody, the rough endoplasmic reticulum (RER) of PYS cells failed to react with this antibody. In addition, biochemical experiments using PYS in culture did not detect the synthesis of fibronectin at a time when other known basement membrane constituents were easily detected. Therefore, we concluded that fibronectin is not produced by the cells synthesizing this base-

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ment membrane, and that in the embryo the origin of fibronectin in Reichert's membrane may be the maternal circulation.

MATERIALS AND METHODS

Tissues: Adult Sprague-Dawley rats were used in this study. Males and females were placed in the same cage at 6:00 p.m. The next morning, females with sperm in the vaginal smear were considered to be fertilized as of the previous midnight. Under ether anesthesia, the uterus was removed at pregnancy dates 7.5, 9.5, 11.5, 12.5, 14.5, and 16.5 d and the uterine segments were placed in PBS. For the biochemical studies, timed-pregnant Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, MA). PYS carcinoma cells were obtained from the ascites form of this tumor (22) grown in CDF rats.

Fixation: For days 7.5, 9.5, and 11.5, entire uterine segments were processed. For older embryos, the PYS was isolated under a dissecting microscope. Tissues were processed as previously described (1, 20–23). Briefly: for light microscopy, tissues or cells embedded in OCT compound were quick-frozen in methylbutane at liquid nitrogen temperature. For electron microscopy, tissues or cells were fixed in 4% formaldehyde in 0.1 M sodium phosphate buffer for 2 h with constant agitation at 4°C and washed in several changes of PBS with 4% sucrose for 16 h. After a last wash in PBS containing 4% sucrose and 7% glycerol, the samples were quick-frozen as above.

Immunohistochemical Staining: Cryostat sections mounted on albumin-coated slides were reacted with the corresponding sera as previously reported (1, 20–23). Ultrathin sections were photographed without any further staining.

Antibodies: All primary antibodies were produced in rabbits. The IgG fraction and Fab fragments were obtained as previously described (20–23). Anti-mouse plasma fibronectin and anti-rat laminin were prepared and characterized by ELISA as described in previous reports (1, 21, 22). Anti-mouse neoplastic basement membrane (NBM) was used as positive control (20, 21, 23). Normal rabbit serum (NRS) was used as a negative control. Goat anti-rabbit Fab and rabbit Fab-peroxidase-anti-peroxidase complex were prepared as previously described (1, 20–22, 30).

Metabolic Labeling: PYS from 14.5-d rat embryos were isolated as described previously and incubated with [^{14}C]proline (4 μCi/ml) in serum-free medium for 5 h at 37°C (5, 6). The incubation medium contained 5 μl/ml aprotinin and 50 μg/ml β-aminopropionitrile. Bovine aortic endothelial cells were isolated and cultured as described by Macarak et al. (17, 18) and metabolic labeling was performed using [^{14}C]proline (10 μCi/ml) in serum-free medium containing 5 μl/ml aprotinin for 24 h at 37°C. The medium from both cultures was harvested, cooled to 4°C, and protease inhibitors were added to give final concentrations of 10 mM N-ethylmaleamide and EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and benzamidine-HCl. The media were subsequently dialyzed against 0.85% NaCl, 0.05 M Tris-HCl (pH 7.2) containing 1 mM PMSF, and the radioactivity was measured by scintillation counting.

After incubation of PYS, tissues were removed from the medium with a forceps, washed thoroughly with cold medium, and frozen in the presence of protease inhibitors as described above. To prepare acellular PYS for extraction, tissues were thawed, washed with distilled water, briefly incubated in an ultrasonic bath, and finally treated with detergent essentially as described by Hogan et al. (12).

Acellular PYS were subsequently extracted at 37°C under reducing and alkylating conditions in 8 M urea, 1 M Tris-HCl (pH 8.5), 2 mM EDTA, 20 mM dithioerythritol (10). After 4 h, the insoluble material was removed by centrifugation (20,000 *g*, 20 min) and reextracted at 4°C in 1% SDS and 5% mercaptoethanol. After 2 h, the insoluble material was again removed by centrifugation. These procedures resulted in the solubilization of essentially all of the radioactivity. The solubilized fractions were subsequently assayed by SDS PAGE.

Affinity Chromatography: The media from both endothelial cell and PYS cultures (≈5 ml) were passed successively through 0.7 × 14-cm columns of Sepharose 4B and gelatin-Sepharose 4B essentially as described by Engvall and Ruoslahti (9). The columns were equilibrated in 0.85% NaCl, 0.05 M Tris-HCl (pH 7.2) containing 1 mM PMSF. The radioactivity that was eluted from the Sepharose 4B column in this buffer was applied to the gelatin-Sepharose 4B column and again eluted with the same buffer. This column was then eluted with this buffer made 1 M in urea and, finally, with this buffer made 4 M in urea. Appropriate fractions were pooled, dialyzed, and lyophilized.

This material was subsequently analyzed by SDS PAGE and fluorography as previously described (3, 4).

RESULTS

Light Microscopy

EMBRYONIC PYS: Antilaminin antibodies gave a consistent pattern regardless of the embryonic stage. All vascular and glandular basement membranes in the endometrium, placenta and embryo were strongly positive (data not shown). In the visceral yolk sac, both basement membranes (3) were positive. In the PYS, Reichert's membrane was always intensely positive, with the reaction product being more intense at both edges of this structure (Fig. 1 *a*). Most importantly, at all times studied the cytoplasm of the PYS cells was also strongly positive with this antibody (Fig. 1 *a*). This cytoplasmic staining had a delicate, reticular appearance clearly delineating the negative nucleus.

By contrast, antifibronectin antibodies gave different results depending upon the embryonic stage. The stroma, vascular lumina, and endothelial cells in the endometrium, placenta, and embryo were consistently positive (data not shown). Reichert's membrane, however, was completely negative at 7.5 d of gestation, was slightly positive at 9.5 d (data not shown), and from then on was increasingly positive, with a maximum at 14.5 d (Fig. 1 *b*). Anti-NBM antibodies were used as a positive control (20, 21, 23). They gave a consistent pattern almost identical to that obtained with antilaminin antibodies (data not shown). NRS reacted neither with Reichert's membrane nor with PYS cells (Fig. 1 *c*).

Note that the most dramatic difference between antilaminin and antifibronectin reactivity was that, at all times studied, even when Reichert's membrane was clearly positive, the cytoplasm of the PYS cells was consistently negative with antifibronectin antibodies (Fig. 1 *a* and *b*).

NEOPLASTIC PYS: The ascites cells and their matrix gave reaction patterns similar to those of their normal embryonic counterparts. Antilaminin and anti-NBM antibodies consistently reacted with the basement membrane matrix and with the cytoplasm of the neoplastic cells (Fig. 2 *a*). Antifibronectin antibodies reacted with the basement membrane matrix, but failed to react with the cytoplasm of the neoplastic cells (Fig. 2 *b*). NRS gave a negative reaction with the cells and their matrix (data not shown).

Electron Microscopy

The ultrastructural and antigenic preservation obtained with formaldehyde fixation compared favorably to that previously reported using periodate-lysine-paraformaldehyde fixation (23, 24).

EMBRYONIC PYS: Only PYS from 14.5 d of gestation (time of maximum staining with antifibronectin antibodies by light microscopy) were examined by electron microscopy.

Antilaminin antibodies consistently reacted with Reichert's membrane. The characteristic granular peroxidase reaction product was distributed throughout the entire thickness of the membrane, with greater intensity at both edges of this structure (Fig. 3 *a*). The intracytoplasmic staining observed by light microscopy corresponded with an intense reaction within the cisternae of the RER (Figs. 3 *a* and 4 *a*).

Antifibronectin antibodies gave more variable results than antilaminin antibodies. Often the interface between the trophoblastic cell surface and Reichert's membrane had the highest accumulation of reaction product (Fig. 3 *b*). This staining pattern suggested a diffusion gradient from the maternal side. However, the cytoplasm of the PYS cells was consistently negative (Figs. 3 *b* and 4 *b*). Occasionally some reaction product could be identified free in the cytosol, but not any more than that seen with NRS. The trophoblastic cells themselves were negative.

Anti-NBM antibodies gave a pattern identical to that pre-

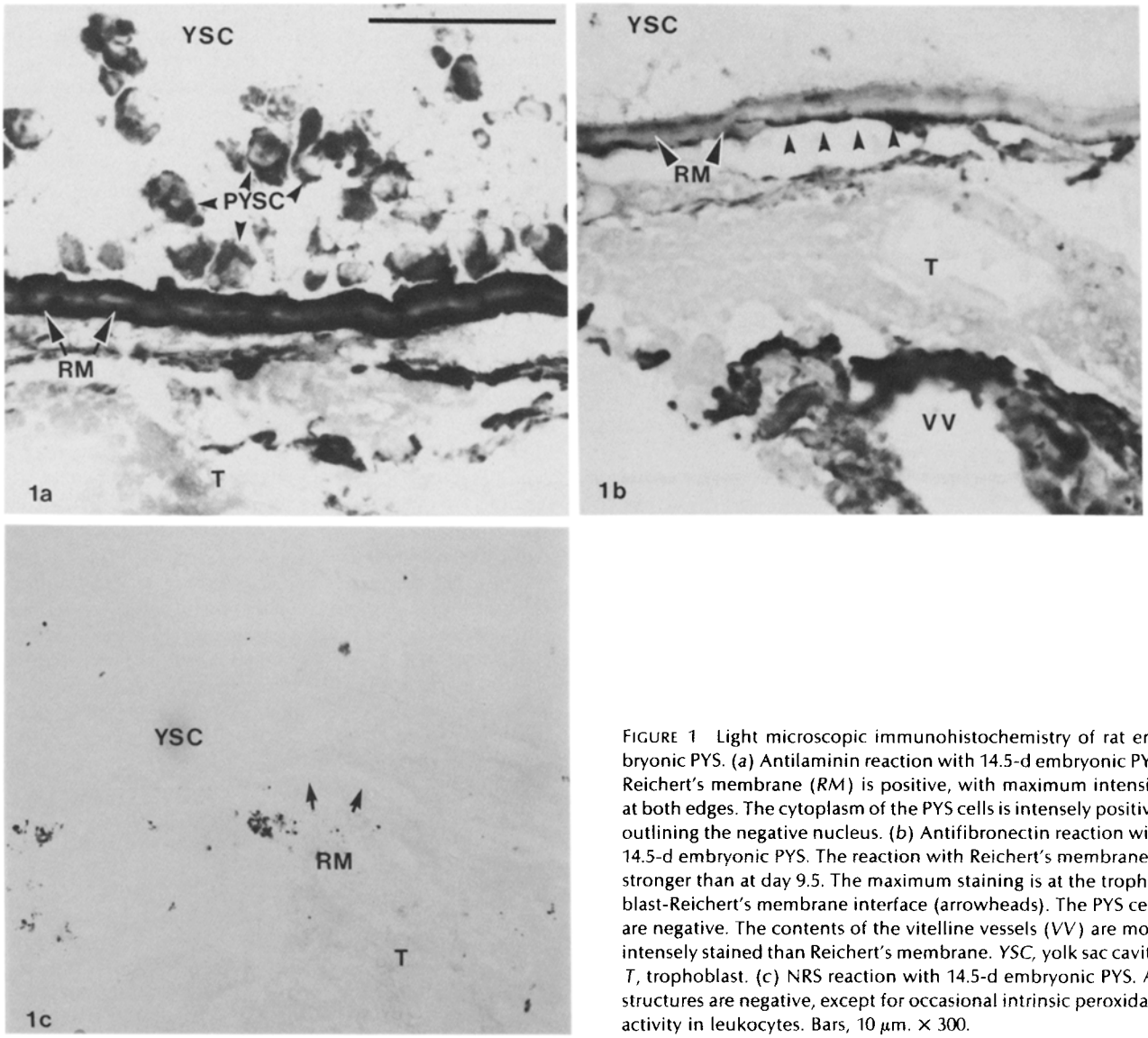

FIGURE 1 Light microscopic immunohistochemistry of rat embryonic PYS. (*a*) Antilaminin reaction with 14.5-d embryonic PYS. Reichert's membrane (*RM*) is positive, with maximum intensity at both edges. The cytoplasm of the PYS cells is intensely positive, outlining the negative nucleus. (*b*) Antifibronectin reaction with 14.5-d embryonic PYS. The reaction with Reichert's membrane is stronger than at day 9.5. The maximum staining is at the trophoblast-Reichert's membrane interface (arrowheads). The PYS cells are negative. The contents of the vitelline vessels (*VV*) are more intensely stained than Reichert's membrane. *YSC*, yolk sac cavity; *T*, trophoblast. (*c*) NRS reaction with 14.5-d embryonic PYS. All structures are negative, except for occasional intrinsic peroxidase activity in leukocytes. Bars, 10 μm. × 300.

viously reported (23) and similar to antilaminin (data not shown). NRS gave only minimal background staining in Reichert's membrane (Fig. 3*c*) and occasional reaction product in the cytosol of the PYS cells.

NEOPLASTIC PYS: Antilaminin antibodies consistently gave a strong, homogeneous reaction throughout the basement membrane matrix (Fig. 5*a*). The contents of the RER of the neoplastic cells, like that of their normal embryonic counterpart, were strongly positive (Fig. 5*a*).

Antifibronectin antibodies gave a spotty, uneven reaction with the extracellular basement membrane matrix (Fig. 5*b*) unlike that obtained with antilaminin antibodies. The RER and other intracellular organelles were consistently negative (Fig. 5*b*). Anti-NBM and NRS gave patterns similar to those obtained in the embryonic PYS (data not shown).

Biosynthetic Experiments

In order to determine whether 14.5 d PYS were actually secreting fibronectin, tissues were incubated in vitro with (^{14}C)proline as described in Materials and Methods. The culture medium was subsequently chromatographed on gelatin-Sepharose and analyzed by SDS PAGE. By affinity chromatography, <2% of the applied radioactivity bound to the column and could be eluted with 4 M urea (data not shown). By comparison, 10–15% of the applied radioactivity from similarly treated samples of bovine aortic endothelial cell medium bound to the column and eluted with 4 M urea (data not shown). Analysis of the pertinent chromatographic fractions by SDS PAGE showed that gelatin-Sepharose did not appear to bind any components from PYS medium, whereas fibronectin from endothelial cell medium was specifically bound to the column (Fig. 6*a*). There were insufficient counts of PYS medium bound to gelatin-Sepharose, for analysis. These results demonstrated that there were no newly synthesized components in PYS medium migrating in the same position as fibronectin.

Because fibronectin is often associated with the matrix in in vitro experiments, the labeled PYS tissues were extracted as described in Materials and Methods. Analysis of these extracts by SDS PAGE again did not show any component migrating in the same position as fibronectin (Fig. 6*b*). Note that in both the PYS medium and tissue samples well characterized basement membrane components such as laminin, type IV procollagen, and entactin (2, 4, 7, 11, 12, 22, 31) were detectable.

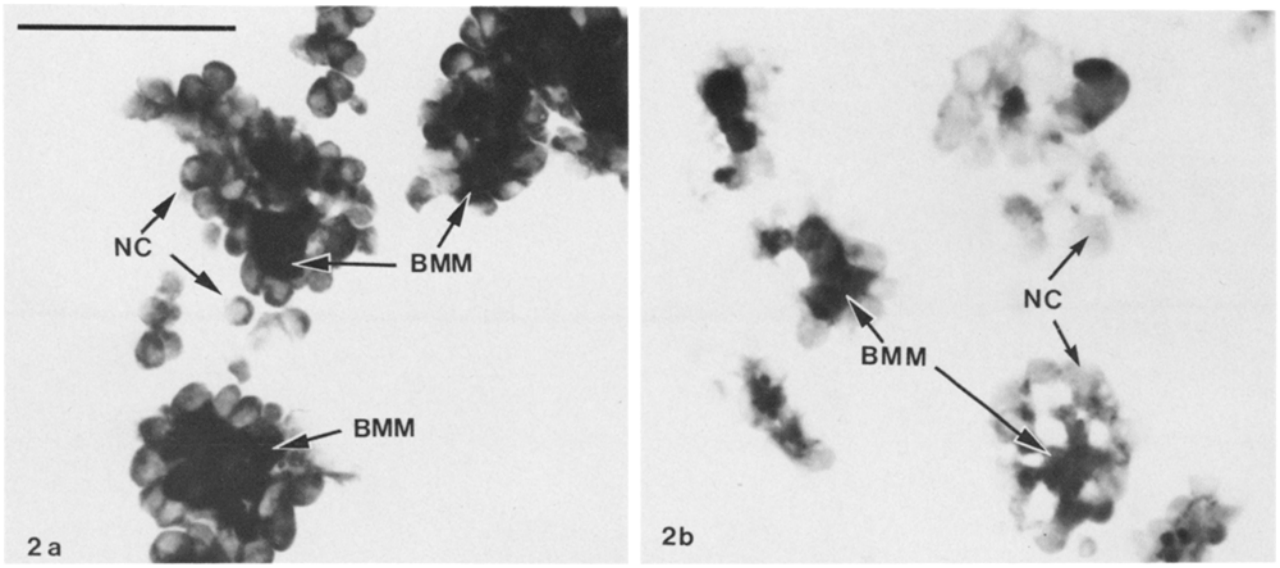

FIGURE 2 Light microscopic immunohistochemistry of rat parietal yolk sac carcinoma, ascites form. Floating in ascites fluid, clusters of neoplastic cells surround a central core of basement membrane matrix. (*a*) Antilaminin reaction with neoplastic PYS. The basement membrane matrix (*BMM*) is strongly positive. The positive cytoplasm of the neoplastic cells (*NC*) outlines the negative nucleus. (*b*) Antifibronectin reaction with neoplastic PYS. The basement membrane matrix is weakly positive. The neoplastic cells have only minimal surface staining. × 300.

DISCUSSION

Fibronectin is a large molecular weight glycoprotein abundant in plasma and tissues (25, 28). Although there are minor differences between plasma and tissue fibronectin, their amino acid composition is similar and antibodies against either of the two recognizes the other (28). Several functions have been ascribed to fibronectin including cell adhesion and collagen-platelet interaction (see references 25, 28 for recent reviews).

On the basis of immunofluorescence and immunohistochemical studies, several authors (8, 16, 26, 32, 34) have suggested that fibronectin is a component of basement membranes. However, recent studies from our laboratories using antibodies against basement membrane matrix, type I collagen, and fibronectin in a concerted fashion have demonstrated that fibronectin is not present in most basement membranes (1, 21). Our previous studies used two different rabbit polyclonal antibodies—one directed against mouse plasma fibronectin (also used in the present study) and the other directed against bovine plasma fibronectin. Both antibodies failed to react with most basement membranes (21). In addition, three monoclonal antibodies directed against different domains of human plasma fibronectin (supplied by Drs. M. Pierschbacher and E. Ruoslahti) also failed to react with human colonic, endometrial, and cervical basement membrane (data not shown).

In the current study we used the embryonic and neoplastic rat PYS, systems characterized by rapid basement membrane synthesis and deposition. In both systems, and at all times studied, antilaminin reacted intensely with the basement membrane matrix *and* the contents of the RER cisternae of the cells responsible for basement membrane synthesis (Figs. 1 *a*, 2 *a*, 3 *a*, 4 *a*, and 5 *a*). This is in contrast to the results obtained with antifibronectin which had no reaction at 7.5 d of gestation and only a weak reaction with the basement membrane at later stages of gestation (Figs. 1 *b*, and 3 *b*). This reaction of antifibronectin with Reichert's membrane was consistently weaker than the reaction on the same sections with either plasma or placental stromal structures. Most significantly, antifibronectin reacted neither with the embryonic cells nor with their neoplastic counterpart (Figs. 1 *b*, 2 *b*, 3 *b*, 4 *b*, and 5 *b*). The immunohistochemical results are summarized in Table I.

The biosynthetic and immunohistochemical findings are in agreement. At day 14.5 of gestation (the time of maximum staining of Reichert's membrane with antifibronectin), neither the culture medium nor the tissue contained any detectable fibronectin (Fig. 6). On the other hand, medium from bovine aortic endothelial cells (cells known to synthesize fibronectin) (17, 18) provided a distinct band in the molecular weight region of 220,000 (Fig. 6 *a*). Similarly, cultured neoplastic PYS cells failed to demonstrate any synthesis of fibronectin (data not shown). These results indicated that PYS cells that are actively synthesizing basement membrane components do not synthesize or secrete any detectable amounts of fibronectin.

The following considerations suggest an origin for the fibronectin found in Reichert's membrane. Because the embryonic PYS acts as a selective filter through which all metabolites in maternal plasma must pass to reach the embryo (14, 27), Reichert's membrane is continuously exposed to maternal plasma fibronectin. The pattern of staining observed in these studies—intense staining within vitelline vessels and at the interface between the trophoblast and Reichert's membrane, lack of staining early in development (7.5 d) and only weak staining at later times—suggests a diffusion and trapping of plasma fibronectin from the maternal circulation. A similar suggestion has been made by others (13, 15, 31). The same situation applies to the tumor. The aggregates of neoplastic cells are floating in ascites fluid (a fluid in equilibrium with plasma). Thus, the fibronectin present in the basement membrane matrix could well be of plasma origin. In contrast, the PYS carcinoma grown in the subcutaneous form (not in direct contact with plasma) has a basement membrane matrix negative for fibronectin (1). This interpretation is also in agreement with our previous findings in a variety of basement membranes (1, 21).

In summary, the current studies demonstrated that PYS cells that actively synthesize and secrete basement membrane com-

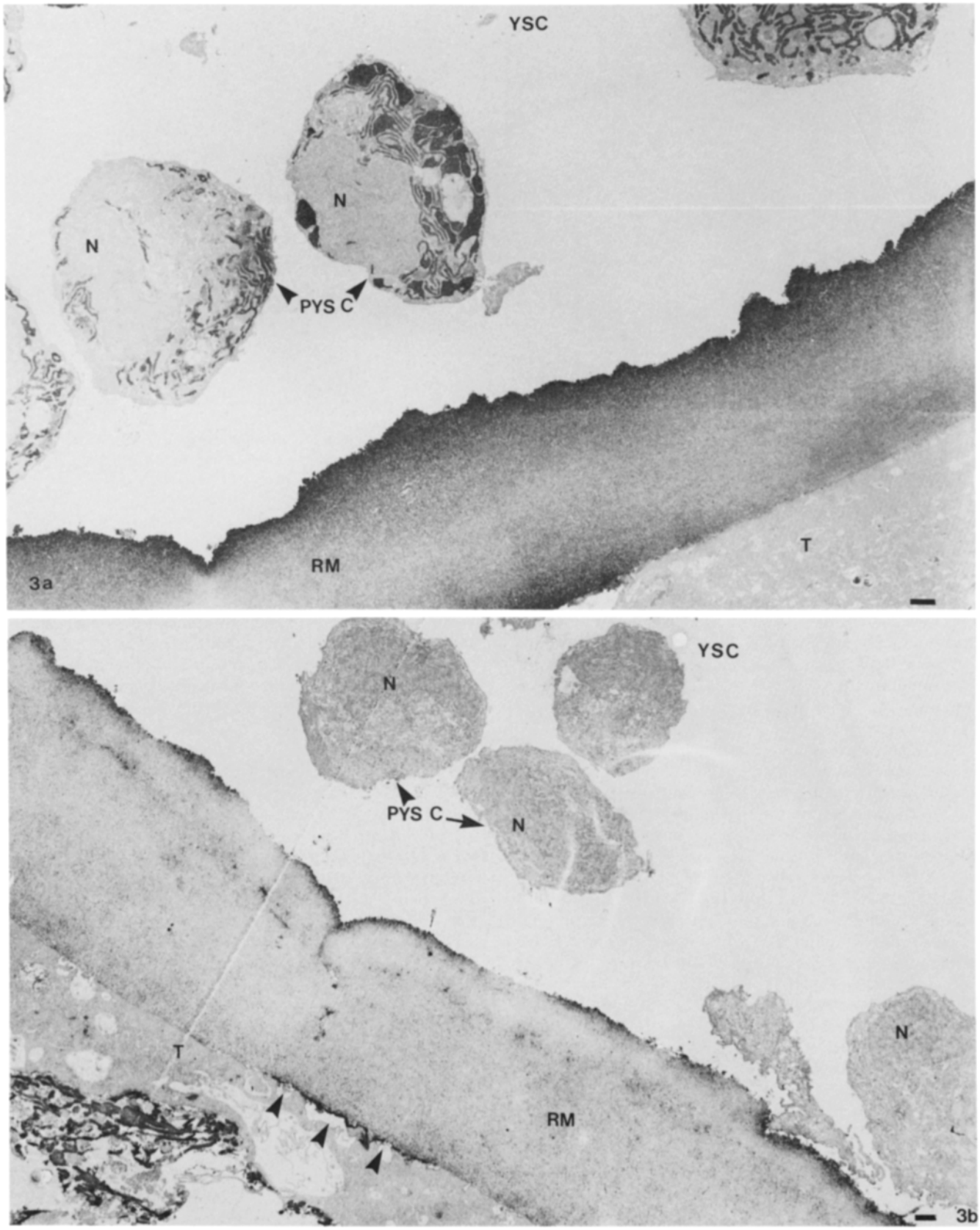

FIGURE 3 Electron microscopic immunohistochemistry of 14.5-d rat embryonic PYS. (*a*) Antilaminin reaction. Reichert's membrane (*RM*) is positive throughout, with the maximum staining occurring at its edges. The contents of the RER of the PYS cells (*PYSC*) are also positive. The trophoblast-Reichert's membrane interface is negative. × 4,200. (*b*) Antifibronectin reaction. Reichert's membrane is weakly positive, with the greatest intensity at the trophoblast-Reichert's membrane interface (arrowheads). The RER of the PYS cells is negative. The contents of the vitelline vessels are positive. × 3,800. (*c*) NRS reaction. No reaction product is present in any structure. Bars, 1 μm. × 4,400.

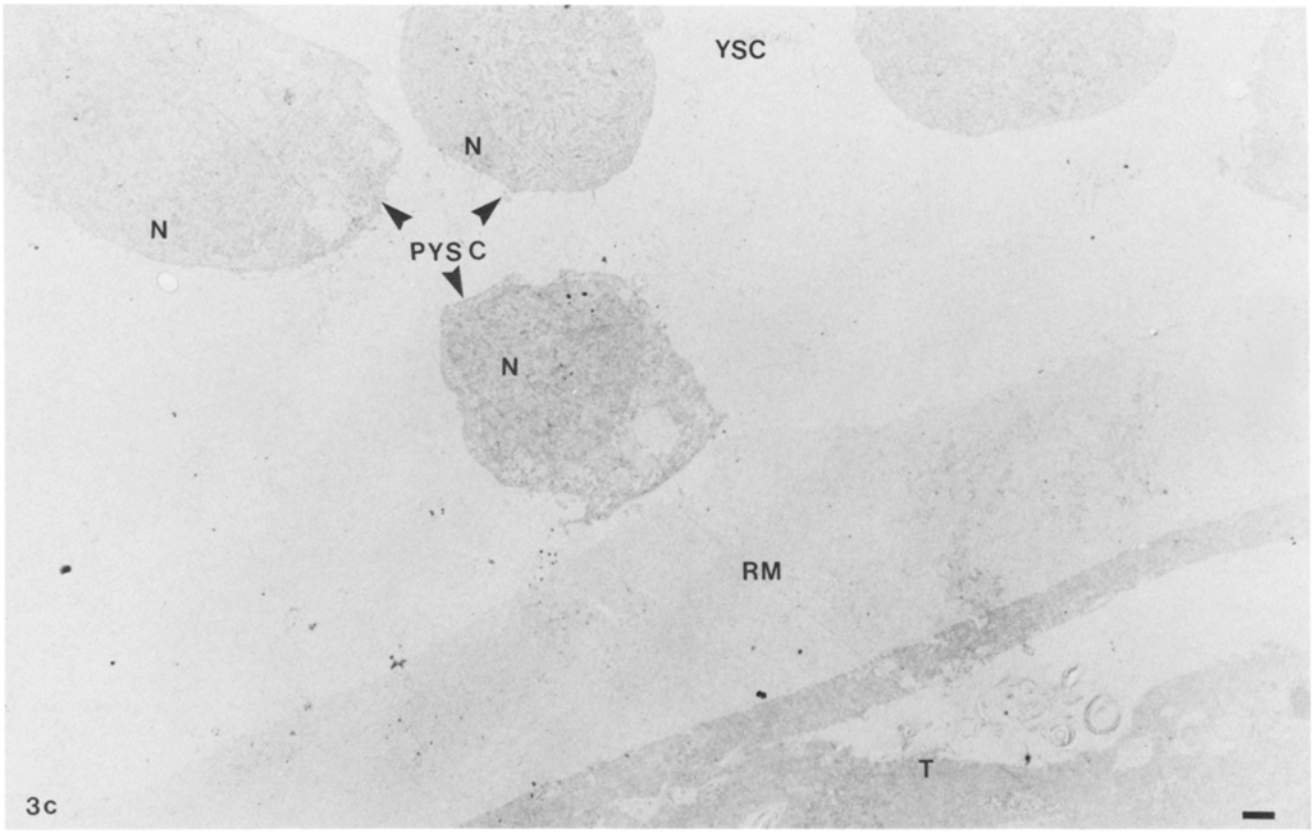

FIGURE 3C

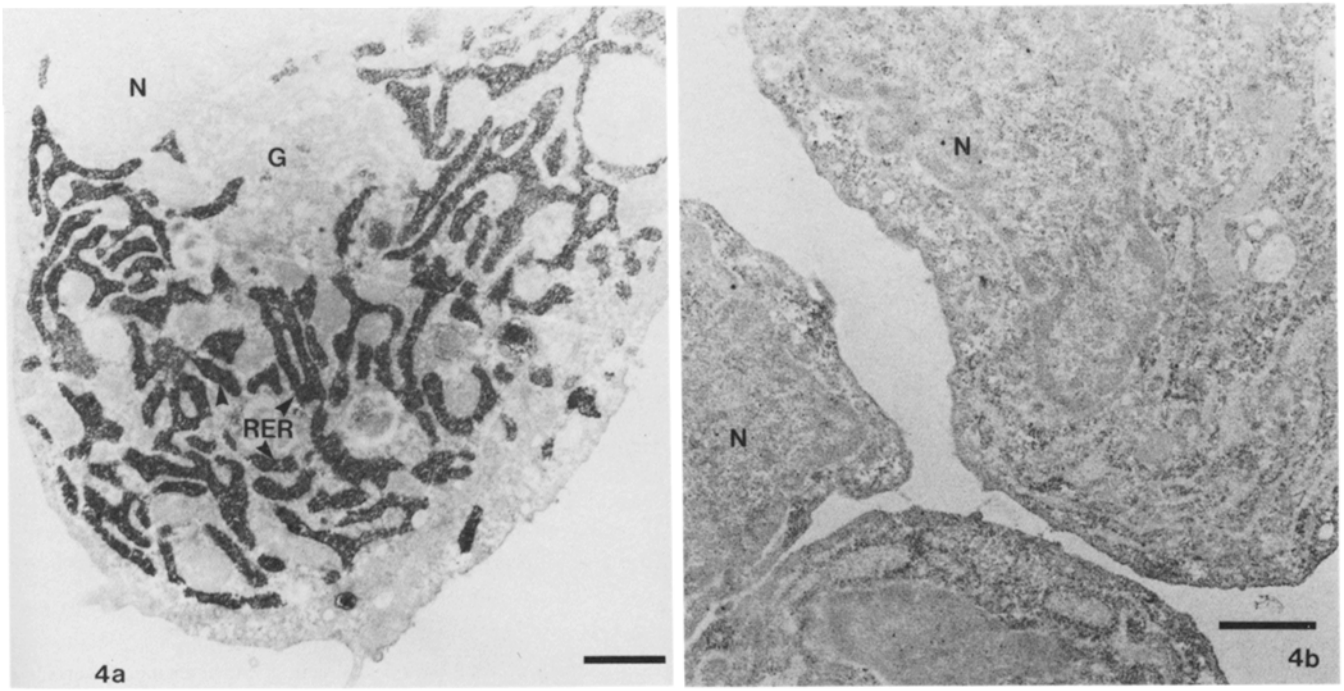

FIGURE 4 Electron microscopic immunohistochemistry of 14.5-d rat embryonic PYS cells. (*a*) Antilaminin reaction. Portion of a PYS cell. The characteristic granular peroxidase reaction product is localized exclusively within the RER. *N*, nucleus; *G*, Golgi complex. × 10,500. (*b*) Antifibronectin reaction. Portions of three PYS cells. The RER and all other cytoplasmic organelles are negative. × 13,000.

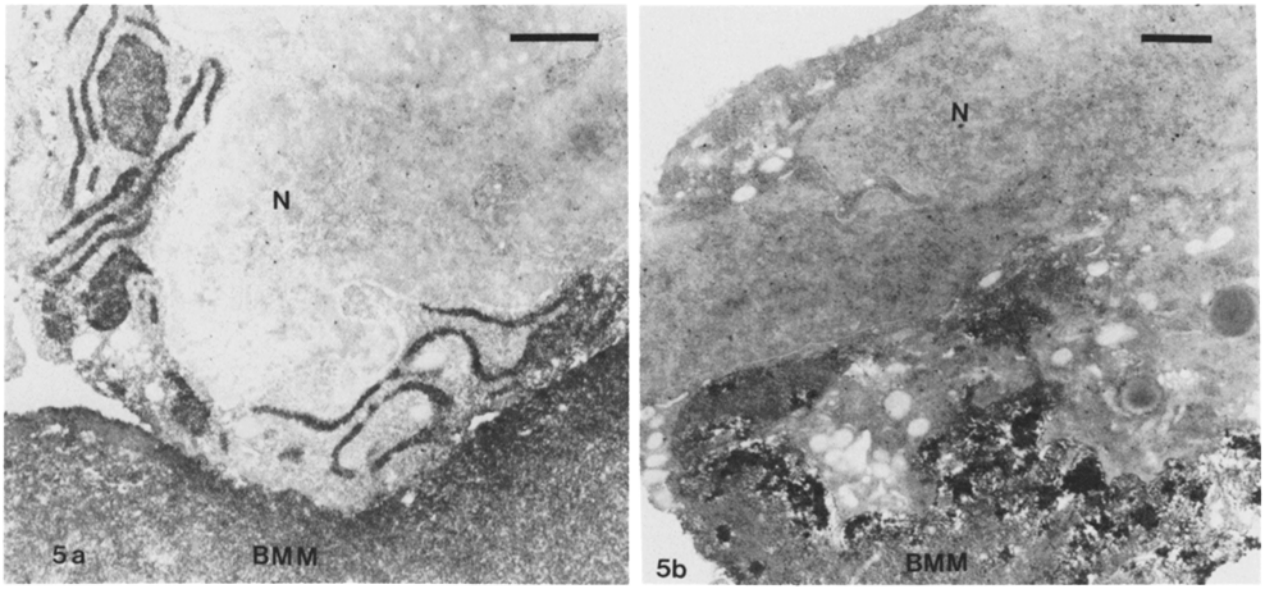

FIGURE 5 Electron microscopic immunohistochemistry of PYS carcinoma, ascites form. Portions of neoplastic cells and basement membrane matrix. (*a*) Antilaminin reaction. The basement membrane matrix (*BMM*) is homogeneously positive. The contents of the RER of the neoplastic cell are positive. All other cell organelles are negative. × 11,800. (*b*) Antifibronectin reaction. The BMM is positive in a spotty, uneven fashion. The reaction product is particularly intense in the BMM adjacent to the cell membrane. All cellular organelles are negative. × 10,000.

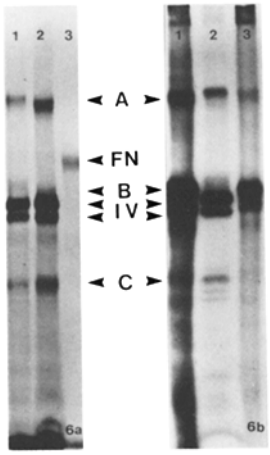

FIGURE 6 SDS PAGE analysis of [^{14}C]proline-labeled PYS samples. (*a*) Fluorogram of gelatin-Sepharose fractions. Lane *1*, PYS medium; lane *2*, PYS medium not bound to gelatin-Sepharose column; lane *3*, bovine aortic endothelial cell medium, fraction bound to column and eluted with 4 M urea. (*b*) Fluorogram of [^{14}C]proline-labeled PYS tissue solubilized by reduction and alkylation (lane *1*) and subsequently by SDS-mercaptoethanol (lane *3*). For comparison, a PYS medium sample is shown (lane *2*). The components indicated are: laminin subunits (*A* and *B*), fibronectin (*FN*), basement membrane procollagen chains pro-α1 and pro-α2 (*IV*), and entactin (*C*).

ponents such as type IV procollagen, laminin, and entactin (2, 4, 6, 7, 12, 22 and 31) do not synthesize any detectable fibronectin. Furthermore, the immunohistochemical staining pattern strongly suggested that the fibronectin present in the basement membrane is "trapped" plasma fibronectin.

Whether or not fibronectin represents a component of basement membranes remains somewhat controversial. Based on light microscopic studies, several authors (16, 32, 34) conclude that fibronectin is found in basement membranes. Electron microscopic studies, however, demonstrate that in most cases fibronectin is actually located in the adjacent connective tissue matrix (21). This distinction between basement membrane and adjacent matrix is beyond the resolving power of the light microscope (19, 21). Nevertheless, several electron microscopic studies find some fibronectin antigen within a particular basement membrane—the glomerular basement membrane (8, 19, 21, 26). This finding is interpreted by some (8, 26) to indicate that fibronectin is part of basement membranes, whereas we

TABLE I

Summary of Immunohistochemical Results

	Laminin		Fibronectin	
	7.5 d	14.5 d	7.5 d	14.5 d
PYS cells	+++	+++	–	–
Reichert's membrane	+++	+++	–	+
Reichert's membrane-trophoblast interface	–	–	++	+++
Vitelline vessels	–	–	+++	+++

suggest that this is plasma fibronectin trapped in the glomerular filter (21).

For the purpose of this discussion, perhaps basement membranes can be divided into two classes: those whose major function is filtration (glomerular and Reichert's), and those whose major function is attachment and support (epidermal and lens capsule). The studies to date indicate that both classes of basement membranes contain type IV procollagen, laminin, and heparan sulfate. Only those basement membranes with a major filtering function (i.e., continuously exposed to circulating plasma) contain some fibronectin. Therefore, we would like to propose that type IV procollagen, laminin, and heparan sulfate, for example, represent "intrinsic" components of basement membranes, whereas fibronectin that is deposited from plasma and is present only in those basement membranes with a major filtering function represents one (of perhaps several) "extrinsic" component.

We are grateful to Dr. B. R. Olsen for his helpful discussion of the concept of "intrinsic" and "extrinsic" basement membrane components, to Dr. E. Macarak for providing bovine aortic endothelial cell culture medium, to Ms. Louise Price, Ms. Vicki L. Sabo, and Ms. Janine Crossland for their excellent technical assistance, and to Mrs. Shawn D. Turner for typing this manuscript. C. C. Clark is grateful to Dr. N. A. Kefalides for continuous support and encouragement.

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Reprint requests should be addressed to Dr. Martinez.

Note Added in Proof: While this manuscript was being reviewed, it came to our attention that similar conclusions about the distribution of laminin and fibronectin antibodies in murine Reichert's membrane have been obtained by S. Semhoff, B. L. M. Hogan, and C. R. Hopkins (EMBO Journal, in press). We thank Dr. Hogan for making this information available to us prior to publication.

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