Homing of Hemopoietic Precursor Cells to the Embryonic Thymus: Characterization of an Invasive Mechanism Induced by Chemotactic Peptides

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Abstract. During embryonic development, T cell precursors migrate to the thymus, where immunocompetency is acquired. Our previous studies have shown that avian hemopoietic precursor cells are recruited to the thymus by chemotactic peptides secreted by thymic epithelial cells (Champion, S., B. A. Imhof, P. Savagner, and J. P. Thiery, 1986, *Cell*, 44:781–790). In this study, we have characterized the homing of these precursor cells to the thymus in vivo by electron and light microscopy. Hemopoietic precursors could be seen to extravasate from blood or lymphatic vessels, migrate in the mesenchyme, traverse the perithymic basement membrane, and finally intercalate into the thymic epithelium. Labeled hemopoietic precursors injected into the blood circulation also followed the same pathway. Migrating hemopoietic precursor cells were found to express the fibronectin receptor complex. In the presence of thymic chemotactic peptides, hemopoietic precursors traverse a human amniotic basement membrane. This invasive process was inhibited (*a*) by antibodies to laminin or to fibronectin, two major glycoproteins of the amniotic membrane, (*b*) by monovalent Fab′ fragments of antibodies to the fibronectin receptor, and, finally (*c*) by synthetic peptides that contain the cell-binding sequence Arg-Gly-Asp-Ser of fibronectin. These results indicate that hemopoietic precursors respond to thymic chemotactic peptides by invasive behavior. Direct interactions between basement membrane components and fibronectin receptors appear to be required for this developmentally regulated invasion process.

D URING ontogeny, the thymic rudiment is colonized by hemopoietic precursor cells that subsequently differentiate into T lymphocytes, intra-thymic macrophages, or dendritic cells (55, 47, 64, 70). In birds, T lymphocyte precursor cells are present in the bone marrow, although these precursors may originate from numerous discrete foci dispersed throughout the early embryo (19, 47). There is now ample evidence that the homing of these precursor cells into the thymus is guided by a chemotactic mechanism (5, 14, 44). The thymic epithelium secretes peptides which attract bone marrow–derived hemopoietic precursors (14). The secretion of these peptides is limited to defined periods of embryonic development (5), which are coordinated with the two waves of embryonic thymus colonization (38).

The migration process of hemopoietic precursor cells appears to resemble the behavior of certain other embryonic cells as well as tumor cells (46, 75). Interactions between such cells and their environment during migration can be analyzed in vitro and in vivo using antibodies or other inhibitors to perturb functionally crucial molecules such as fibro-

nectin. The migration of mesenchymal cells during gastrulation, as well as neural crest cells later in development, is inhibited by antibodies to the cell-binding domain of fibronectin. Antibodies to the cellular fibronectin receptor inhibit the attachment and migration of neural crest cells on fibronectin both in vitro and in vivo (10, 21). Synthetic peptides containing the fibronectin cell-binding recognition site prevent the attachment of crest cells and, in vivo, alter migration of amphibian gastrulating cells and of avian neural crest cells (6). Monoclonal antibodies to B16 mouse melanoma cell surface proteins inhibit cell attachment in vitro and block the formation of lung metastases in vivo (83, 84). In mice, lung metastases can be prevented by the preincubation of tumor cells with the cell-binding fragment of laminin (4). Co-injection of fibronectin-derived synthetic peptides together with B16 melanoma cells also considerably reduces the number of lung metastases (36). All of these reports strongly suggest that multiple sequential interactions between invading cells and extracellular molecules are necessary during migration and invasion.

In this study, ultrastructural and immunocytochemical

analyses are performed to define the environment encountered by hemopoietic precursors during thymic colonization. Hemopoietic precursor cells are observed in blood or lymphatic vessels, within the mesenchyme in close interaction with other cell types, and penetrating the thymic epithelium. Hemopoietic precursors injected into the embryonic blood stream can also leave vessels and enter the thymic epithelium. The presence of fibronectin and laminin in the extracellular matrix including the perithymic basement membrane prompted us to analyze the interactions between hemopoietic precursors and these two components in an in vitro assay. The amniotic basement membrane is used to demonstrate that the invasive ability of hemopoietic precursor cells appears to be mediated by the chemotactic peptides produced by the thymus. Crucial components in the invasive process such as laminin or fibronectin are then identified using specific antibodies or synthetic peptide inhibitors.

Materials and Methods

Animals

Embryos of White Leghorn chickens (*Gallus gallus*) and Japanese quails (*Coturnix coturnix japonica*) were used throughout these experiments. Eggs were incubated at 37°C in a humidified atmosphere.

Cells

Hemopoietic precursors. Quail hemopoietic precursor cells were obtained as previously described (5, 14). Briefly, bone marrow from 11.5-d-old quail embryos was transferred to DME (Gibco, Scotland) after aspiration from opened femur and tibia cavities. Cells in DME were layered on a cushion of 30% BSA (Path-O-Cyte 5; Miles Scientific Div., Naperville, IL) in PBS (consisting of 137 mM NaCl, 13 mM KCl, 8 mM $Na_2HPO_4 \cdot 2H_2O$, and 1.5 mM KH_2PO_4), then centrifuged 20 min at 1,800 g. Cells collected at the interface were washed with DME containing 10% FCS (Gibco) before use.

Thymocytes. Thymus from 11-d-old quail embryo was pressed between two glass slides. Mechanically released cells were washed in DME and suspended in DME containing 10% FCS before use.

Cultures

Thymic Epithelial Cell Cultures. Briefly, epithelial cells were prepared from 11.5-d-old quail embryonic thymus by collagenase treatment, followed by sequential plating and recovery of non-adherent cells to remove macrophages, dendritic cells, and fibroblasts. Cells were cultured in DME containing D-valine (Gibco), ascorbic acid (50 μg/ml), reduced glutathione (100 μg/ml), NADH (10 μg/ml), insulin (4 μg/ml), 15% FCS, and 5% chick embryo extract (14).

Embryonic Fibroblast and Kidney Epithelial Cell Cultures. Subcutaneous tissue or kidney rudiment were dissected and dissociated with 0.25% trypsin, 0.2% EDTA solution in PBS (Gibco). The cells were cultured to subconfluency in the medium described for thymic epithelial cells. Fresh medium was conditioned by incubation with these cells for 3 d. Concentrated medium was fractionated on Sephadex G-25 (Pharmacia, Uppsala, Sweden) as previously described (14).

Light Microscopy

Quail embryos were fixed with 3.7% formaldehyde in PBS for 1 h. For Pappenheim panoptic staining, embryos were progressively dehydrated in ethanol and toluene, then embedded in paraffin and sectioned. After progressive rehydration, sections were stained with May-Grünwald solution (11% in H_2O) and in Giemsa solution (1.3% in H_2O) according to Gabe (28). For immunofluorescence, embryos were incubated in a graded series of sucrose solutions (12–18%) in PBS. Sections 8 μm thick were cut on a cryostat (Bright Instrument Co. Ltd, Huntington, England), and deposited on gelatin-coated glass slides before incubation with antibodies. Before immunofluorescence staining, cell suspensions were allowed to adhere by centrifugation on glass slides coated with gelatin. Cells were incubated with antibodies after fixation with formaldehyde (3.7% in PBS). The antibodies

used for staining were rabbit anti-mouse laminin IgG (20 μg/ml), provided by Professor Leo Furcht (University of Minnesota, MN); rabbit anti-chick liver cell adhesion molecule (L-CAM)[1] IgG (50 μg/ml), provided by Professor Gerald Edelman (The Rockefeller University, New York, NY), rabbit anti-chick cellular fibronectin IgG (65), rabbit anti-chick 140-kD fibronectin receptor IgG (15), and mouse monoclonal IgM anti-MB1 (60). This antigen is expressed specifically by quail endothelial cells and all hemopoietic cells aside from mature erythrocytes. The anti-MB1 antibody was directly coupled to FITC on celite (Sigma Chemical Co., St. Louis, MO) as previously described (79); it was also used uncoupled, then localized with a rhodamine-conjugated goat anti-mouse IgG (1:50 in PBS containing 0.5% BSA; Nordic Immunological Laboratories, Tilburg, The Netherlands). The rabbit antibodies were detected with fluorescein-conjugated sheep anti-rabbit IgG (10 μg/ml; Institut Pasteur, Paris, France). The preparations were examined on an Orthoplan epifluorescence microscope (Leitz, Weitzlar, FRG) and photographed with Tri-X Kodak film with a Leitz Orthomat camera.

Electron Microscopy

Necks from 6-d-old quail embryos were fixed for 1 h with 1% paraformaldehyde (E. Merck AG, Darmstadt, FRG), 2% glutaraldehyde (25% stock, Serva, Heidelberg, FRG) in cacodylate buffer, postfixed with 1% osmium tetroxide (Serva), dehydrated, and embedded in Epon 812 (Serva). Semithin sections were cut in the region of the thymic rudiment and stained with toluidine blue (1%). After localization of the thymic rudiment, ultrathin sections were performed, stained with 2% uranyl acetate (E. Merck AG) followed by 1% lead citrate, and were visualized with a JEM 1200 EX electron microscope (JEOL, Tokyo, Japan).

Human Amnion Basement Membrane

Normal term human placentas were obtained within 3 h after delivery from a local maternity hospital. The amnion was separated from the chorion by blunt dissection and was washed several times in PBS. Epithelial cells were removed by treatment with 0.6 M ammonium hydroxide in PBS for 20 min at 25°C, washed three times with sterile PBS and stored in sterile PBS with penicillin (100 IU/ml; Gibco) and streptomycin (100 μg/ml; Gibco). The amnion membrane was stretched on a nitrocellulose filter (0.45 μm, Millipore Corp., Bedford, MA). Disks of membrane plus filter (13-mm diameter) were punched out with a steel punch and used in Boyden chambers.

Invasion Chambers

Invasion assays were performed in Boyden chambers (8). The "blind well" was filled with 100 μl of the medium to be tested. After centrifugation on a cushion of BSA, 100,000 bone marrow cells were suspended in DME containing 10% FCS, in the upper compartment which was separated from the lower by the membrane and the nitrocellulose filter. Chambers were incubated for 20 h at 37°C (5% CO_2 in a humidified atmosphere). Peptides or Fab' fragments were incubated with the cells in the upper compartment. Fab' fragments were prepared according to Brackenbury et al. (9). The synthetic fibronectin peptides Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro (SP1) and Gly-Arg-Gly-Asp-Ser (GRGDS) were purchased from Peninsula Laboratories, Inc. (Belmont, CA). The control peptides Gly-Arg-Gly-Glu-Ser (GRGES) and Leu-Trp-Met-Arg-Phe-Ala were purchased from Peninsula Laboratories and Serva, respectively. SP1 was >98% pure according to reverse-phase HPLC. GRGDS and GRGES were purified to >98% purity by reverse-phase HPLC on a preparative C18 column (Rainin Instrument Co. Inc., Woburn, MA). Antibodies against laminin (0.1 mg/ml) and fibronectin (0.1 mg/ml) were incubated with the human amnion stretched on Millipore filters for 20 h before washing with DME and setting up of invasive chambers. After incubation, the nitrocellulose filters were washed with PBS, fixed with 3.7% formaldehyde (E. Merck AG), and stained with Giemsa solution (3% in H_2O; E. Merck AG). All of the cells trapped on the nitrocellulose filters were counted.

Chemotaxis Assays

To test chemotaxis, the amniotic membrane and the nitrocellulose filter in the Boyden chamber were replaced by a Nuclepore filter (5-μm pore size; Nuclepore Corp., Pleasanton, CA). The medium to be tested was placed

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1. *Abbreviations used in this paper:* GRGES, Gly-Arg-Gly-Glu-Ser; GRGDS, Gly-Arg-Gly-Asp-Ser; L-CAM, liver cell adhesion molecule; SP1, Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro.
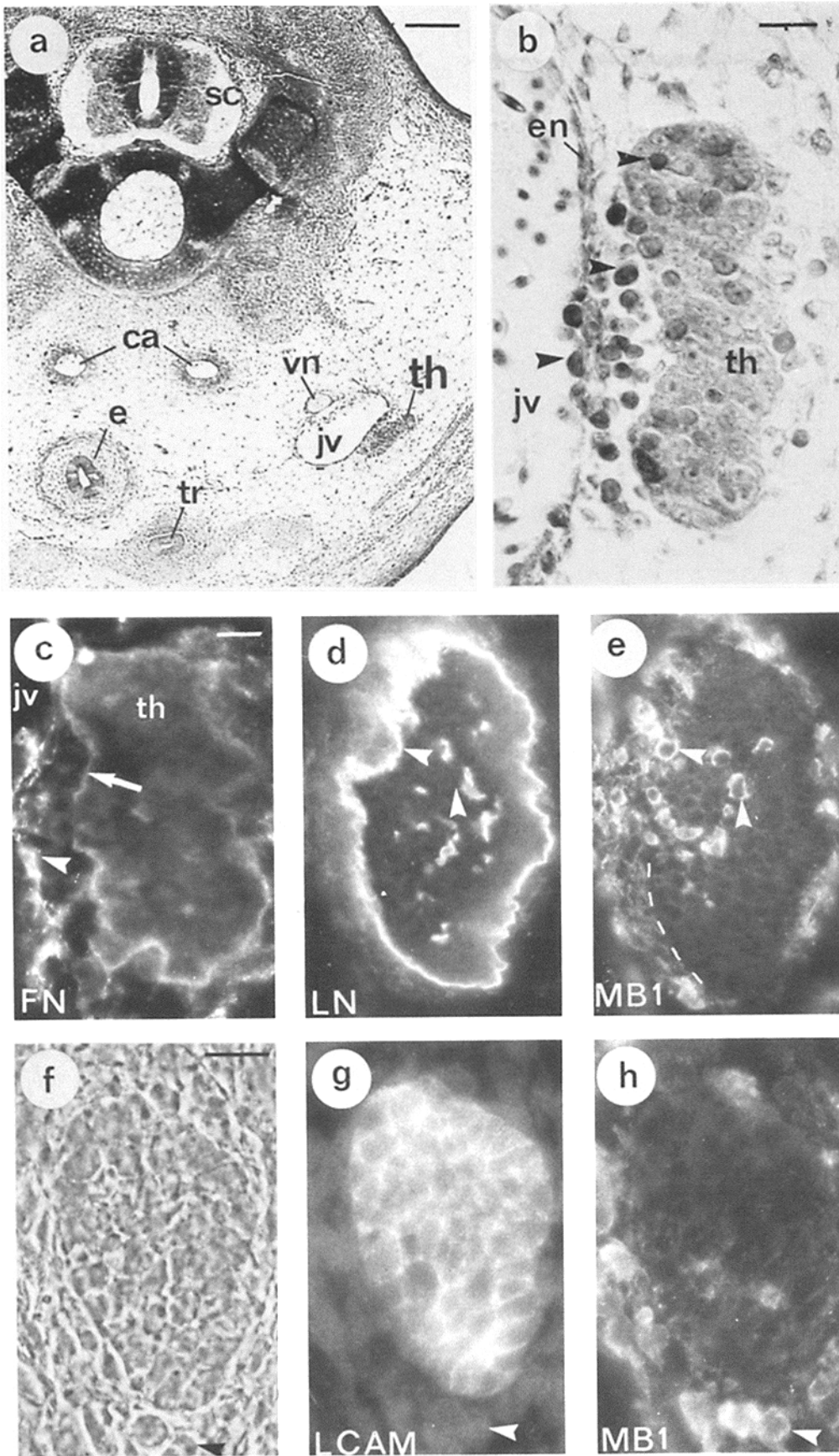
Figure 1. Morphology of the thymus during colonization by hemopoietic precursors: localization of fibronectin, laminin, and L-CAM. (*a* and *b*) 6-d-old quail embryos were fixed and embedded in paraffin. The cervical region containing the thymus was sectioned transversely and prepared for light microscopy by Pappenheim panoptic staining. (*a*) The thymic rudiment (*th*) lies along the jugular vein in the vicinity of the vagus nerve. (*b*) Basophilic hemopoietic precursors, stained densely by the panoptic staining technique, are present in the jugular vein adhering to the endothelium, and can also be found in the mesenchyme and within the thymus (*arrowheads*). (*c–h*) Transverse frozen sections of 6-d-old quail embryos were obtained from the cervical region and immunolabeled. (*c*) Antibodies to fibronectin recognize this molecule around the jugular vein (*arrowhead*) and in the perithymic basement membrane (*arrow*). (*d* and *e*) Double-labeling for laminin and for the hemopoietic cell marker MB1. Laminin (*d*) surrounds the thymus as part of the basement membrane and is also found at local sites within the thymus. Hemopoietic cells entering the thymus labeled by MB1 (*e*) are often located in close proximity to laminin (*arrowheads*). (*f*) Phase-contrast image corresponding to *g* and *h*. (*g* and *h*) Double-labeling for L-CAM and MB1. Only thymic epithelial cells express L-CAM. Arrowhead points to MB1-positive hemopoietic precursor cells outside of the thymus. *ca*, carotid artery; *e*, esophagus; *en*, endothelium; *jv*, jugular vein; *sc*, spinal cord; *th*, thymus; *tr*, trachea. *FN*, fibronectin; *LN*, laminin; *MB1*, hemopoietic cell surface marker antigen. Bars: (*a*) 100 μm; (*b*) 15 μm; (*c–h*) 20 μm.

into the lower well, and the chambers were incubated at 37°C with 10^5 cells for 2.5 h as described (14). Migrating cells were collected in the blind (lower) well.

Gel Electrophoresis and Western Blotting

Cells were solubilized in a nonreducing SDS buffer (0.0625 M Tris, pH 7.8, 2% SDS, 10% glycerol). Samples corresponding to 50,000 cells were subjected to electrophoresis in a gradient gel of 4–12% polyacrylamide, electrophoretically transferred to nitrocellulose, and stained with India ink (17 black, Pelikan AG, Hanover, FRG). After three washes in 0.2% Tween in PBS containing 0.5% BSA, the nitrocellulose sheets were incubated for 2 h with antibodies against fibronectin (5 µg/ml) or fibronectin receptor (30 µg/ml), washed three times, and incubated for 30 min with 6 × 10^5 cpm/ml ^{125}I-labeled protein A (specific activity 9 mci/mg; New England Nuclear, Boston, MA). Autoradiography was carried out using Kodak X OMAT RP film with Cronex intensifying screens (DuPont Co., Wilmington, DE).

Intravenous Injections

Hemopoietic precursor cells derived from bone marrow or thymocytes were suspended in DME, and their movements were traced by either of two methods. Cells were either labeled with TRITC (0.01 mg/ml; Research Organics Inc., Cleveland, OH) in PBS for 20 min at 25°C and resuspended in RPMI 1640 medium (Gibco) before injection, or were directly injected and identified by anti–MB1 antibodies, which are specific for quail cells.

After opening the shell, the vitelline membrane was pulled out by forceps. 3–4 × 10^5 cells were injected carefully with a micropipette (30-µm diameter; Drummond Scientific Co., Broomall, PA) into the omphalomesenteric vein of 7-d-old embryos. After further incubation (15 h), the embryos were fixed in 3.7% formaldehyde, dehydrated, embedded in paraffin, and serially sectioned. Injected cells were identified by epifluorescence.

Results

Histological Studies of Thymic Colonization

The thymus is located in the cervical region of quail embryos (Fig. 1 a). At 6 d of embryogenesis, it consists of dense continuous rows of epithelial cells arrayed parallel to the jugular vein. Hemopoietic precursors invading this epithelial rudiment were basophilic cells of the blast type, and were densely stained by Pappenheim panoptic staining (Fig. 1 b). Some precursors adhered to endothelial cells of perithymic blood or lymphatic vessels including the jugular vein. A large number of precursors were found lodged in the perithymic mesenchyme, and others were present within the thymic tissue in direct contact with epithelial cells (Fig. 1 b).

The possible association of precursor cells with fibronectin or laminin was explored. In the 6-d-old quail embryonic thymus, fibronectin surrounded the jugular vein (arrowhead, Fig. 1 c) and was present in the perithymic basement membrane (arrow, Fig. 1 c). Low levels of fibronectin could also be detected within the thymic epithelium. Anti–laminin antibodies strongly decorated the corrugated perithymic basement membrane (Fig. 1 d). Laminin was also found in a more dispersed state within the thymic epithelium. The hemopoietic precursor cells could be specifically localized by labeling with anti–MB1 antibodies. These cells, corresponding to the basophilic cells described in Fig. 1 b were detected both outside and inside of the basement membrane (Fig. 1, d and e). The cells appeared to enter the thymus in restricted regions located close to the jugular vein (Fig. 1 b). Inside the thymus, these cells often clustered in regions containing laminin (Fig. 1, d and e). The epithelium of the embryonic thymus was characterized by the presence of substantial amounts of L-CAM (29, 76) (Fig. 1, f and g). Again,
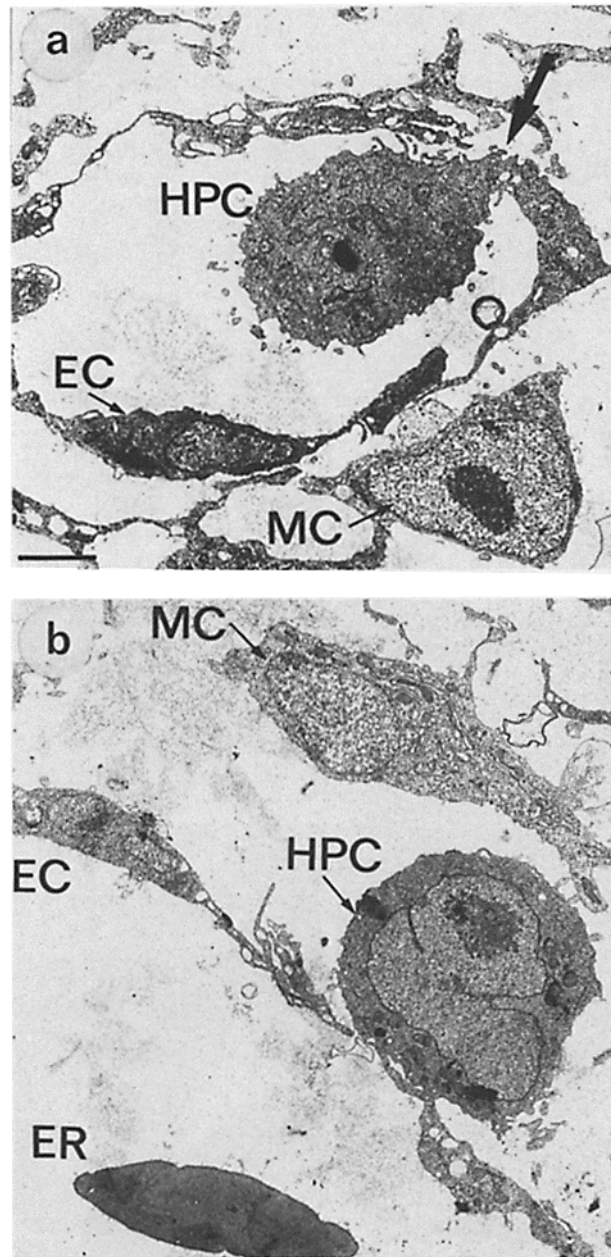

Figure 2. Electron micrographs of hemopoietic precursors leaving vessels in the perithymic area. Sections were obtained from 6-d-old quail embryos embedded in Epon. Basophilic hemopoietic precursor cells were identified by toluidine blue staining of semi-thin sections. Subsequent serial ultrathin sections of the same cells were examined by transmission electron microscopy. Hemopoietic precursor cells can be recognized in vessels close to the thymus. Two stages of traversal through the endothelial wall can be seen. (a) The hemopoietic precursor cell is sending a pseudopod into an opening of the endothelium (arrow). (b) The hemopoietic precursor cell is leaving the vessel and entering a region containing mesenchymal cells. EC, endothelial cell; ER, erythrocyte; HPC, hemopoietic precursor cell; MC, mesenchymal cell. Bar, 2 µm.

double staining for hemopoietic precursor cells demonstrated an accumulation of precursor cells readily distinguishable at the periphery of the thymic epithelium (Fig. 1 h). These cells are clearly distinct from the thymic epithelial cells which form a well-delineated structure.
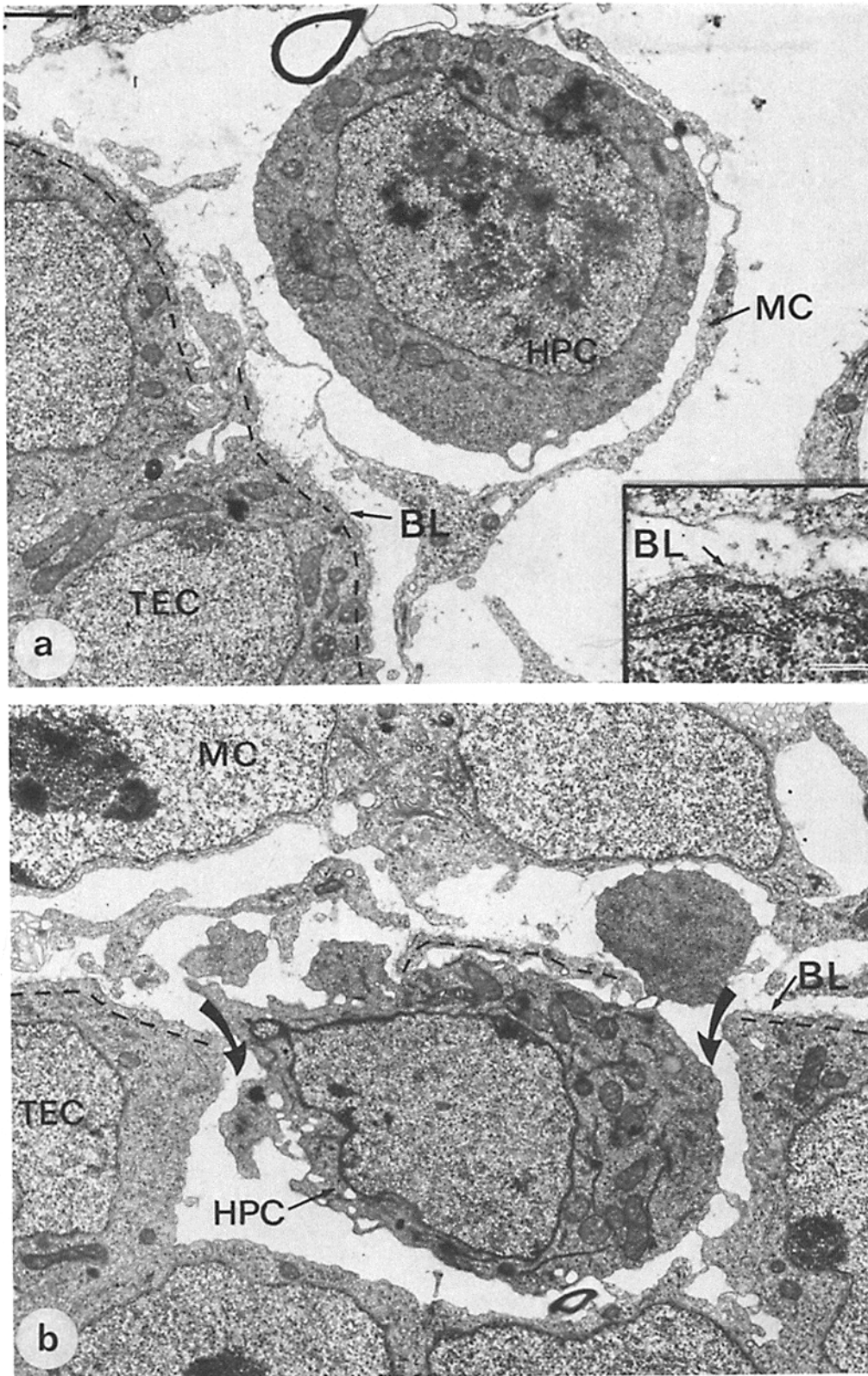
Figure 3. Electron micrographs corresponding to the early phases of hemopoietic precursor homing. Ultrathin sections of 6-d-old quail embryo thymic rudiments. At this stage of development, the thymus consists of an epithelium surrounded by a basal lamina. (*a*) A hemopoietic precursor almost completely enveloped by a mesenchymal cell process is in close proximity to the perithymic basal lamina. The basal lamina is shown more clearly in the inset. (*b*) A hemopoietic precursor penetrates the basal lamina and perturbs cell–cell contacts in the thymic epithelium (*large arrows*). Contacts between precursors and epithelial cells can be observed. Epithelial cell surfaces covered by the wispy basal lamina are indicated by the dotted lines. *BL*, basal lamina; *HPC*, hemopoietic precursor cell; *MC*, mesenchymal cell; *TEC*, thymic epithelial cell. Bar, 1 μm. (*Inset*: bar, 2.8 μm.)

On semi-thin sections (not shown), hemopoietic precursors were identified by their basophilic staining and their correspondence to the MB1⁺ cells previously described. Subsequent ultra-thin sections were examined by electron microscopy. Hemopoietic precursor cells could be found in large numbers in the perithymic area of the 6-d-old embryonic thymus, particularly in vessels located within 40 μm of the thymic epithelium (Fig. 2 *a*). The cells displayed a rounded morphology with a diameter of ≈8 μm, and the cell surface was heavily convoluted. The cytoplasm was rela-

tively electron dense, with numerous mitochondria and free ribosomes; but, no differentiated organelles such as secretory granules. These cells often appeared to be departing from blood or lymphatic vessels (Fig. 2 b). In most cases, hemopoietic precursor cells could be seen in the mesenchyme surrounding the thymus. They appeared to be in close contact with mesenchymal cells, and in many cases, they were partially covered by thin sheets or processes extending from adjacent mesenchymal cells (Fig. 3 a).

The basal lamina lining the thymic epithelial cells appeared in sections as a continuous line that was interrupted locally, especially when hemopoietic precursors were adjacent (Fig. 3 a) or penetrating it (Fig. 3 b). The hemopoietic cells penetrating into the epithelium at this stage were often characterized by a large cytoplasmic extension still left protruding outside of the basal lamina. Hemopoietic precursors then established close relationships and contacts with the epithelial cells (Fig. 3 b). After completion of this stage of thymic colonization, the basal lamina was once again found by electron microscopy to be intact, completely surrounding the thymus (not shown).

Injection of Hemopoietic Precursor Cells into Blood Vessels of Living Embryos

The path of homing to the thymus could also be demonstrated using exogenously injected precursor cells in interspecies transfers. About 3–4 × 10^5 living quail hemopoietic precursor cells were injected into extraembryonic veins of 7-d-old chicken embryos. This embryonic stage corresponds to the beginning of the first wave of colonization of the thymus in vivo. After 24 h, the quail hemopoietic precursors were found adhering to, and penetrating, the endothelium of numerous embryonic vessels or were detected migrating in

the mesenchyme (Fig. 4, a–d). In several embryos, injected cells were found colonizing the thymus, which was as yet nonvascularized (Fig. 4, e–h). Control cells (i.e., thymocytes from 11-d-old quail embryos), were found to leave vessels in various other regions of chick embryos, but could never be found within the thymus (not shown).

Analysis of Fibronectin Receptor Molecules on Hemopoietic Precursor Cells before and after the First Colonization Period of the Thymus

Blood cells of 5-d-old quail embryos were double immunolabeled for fibronectin receptors and for MB1 molecules. All MB1-positive hemopoietic precursors expressed immunofluorescence for the fibronectin receptor even before the embryonic thymus was seeded by these cells (arrows in Fig. 5, a–c). Mature embryonic erythrocytes showed no reaction with either antibody. In the colonized thymus of 6-d-old embryos, hemopoietic precursors present both in the thymus (arrowhead) and in its immediate environment (arrow) also expressed a low level of fibronectin receptor molecules on their plasma membranes as shown by similar double labeling for fibronectin receptor and MB1 molecules (Fig. 5, d–f). Such double staining performed after the completion of thymus colonization in 10-d-old embryos yielded comparable results (Fig. 5, g–i). Thymic epithelial cells were found to express large amounts of fibronectin receptors both at cell–cell and at cell–substrate (basement membrane) contacts (Fig. 5 f).

Analysis of Fibronectin Receptors on Hemopoietic Precursors before and after Chemotactic Migration

Hemopoietic precursor cells derived from bone marrow of 11.5-d-old quail embryos can migrate along a chemotactic
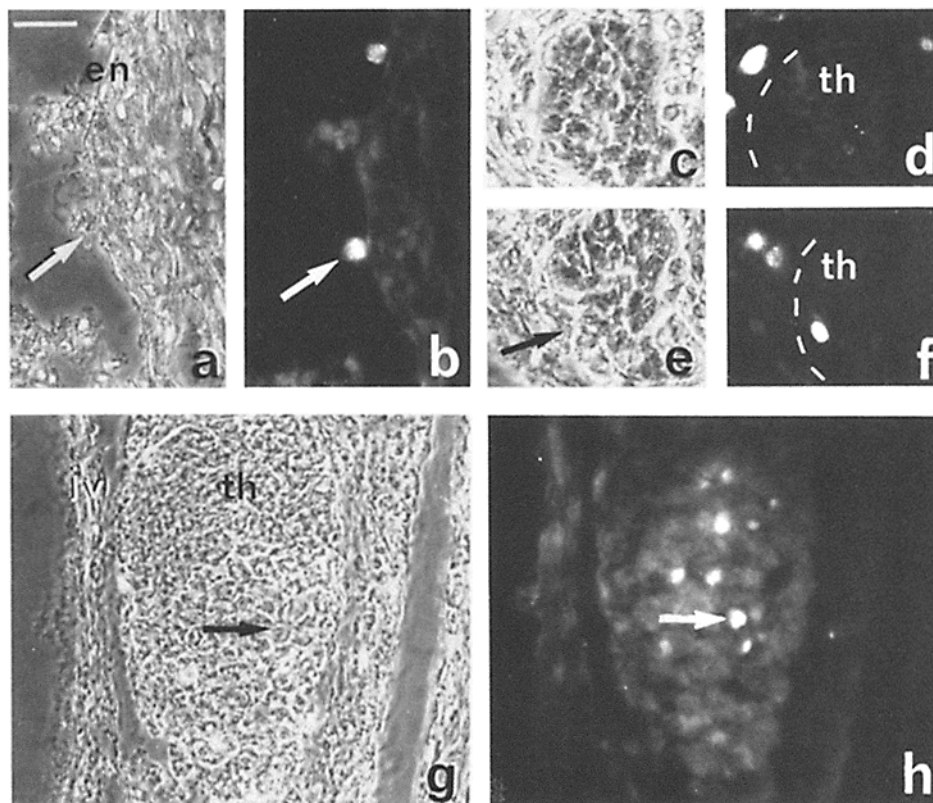

Figure 4. Localization of injected quail hemopoietic precursor cells in chick embryos. 1 d after intravenous injection of quail bone marrow cells into chick embryos as described under Materials and Methods, histological sections of 8-d-old chick embryos were screened for quail hemopoietic precursor cells by fluorescence microscopy. (a–b) Quail hemopoietic precursor cells detected by species-specific anti–MB1 antibodies are found adhering to the endothelium of vessels. (c–f) In the perithymic area, stained cells are found in the mesenchyme as well as within the thymus. (g–h) Using a different labeling approach, hemopoietic precursors covalently labeled before injection by TRITC are also found in the thymus. Arrows point to quail hemopoietic precursor cells. *en*, endothelium; *jv*, jugular vein; *th*, thymus. Bar, 20 μm.
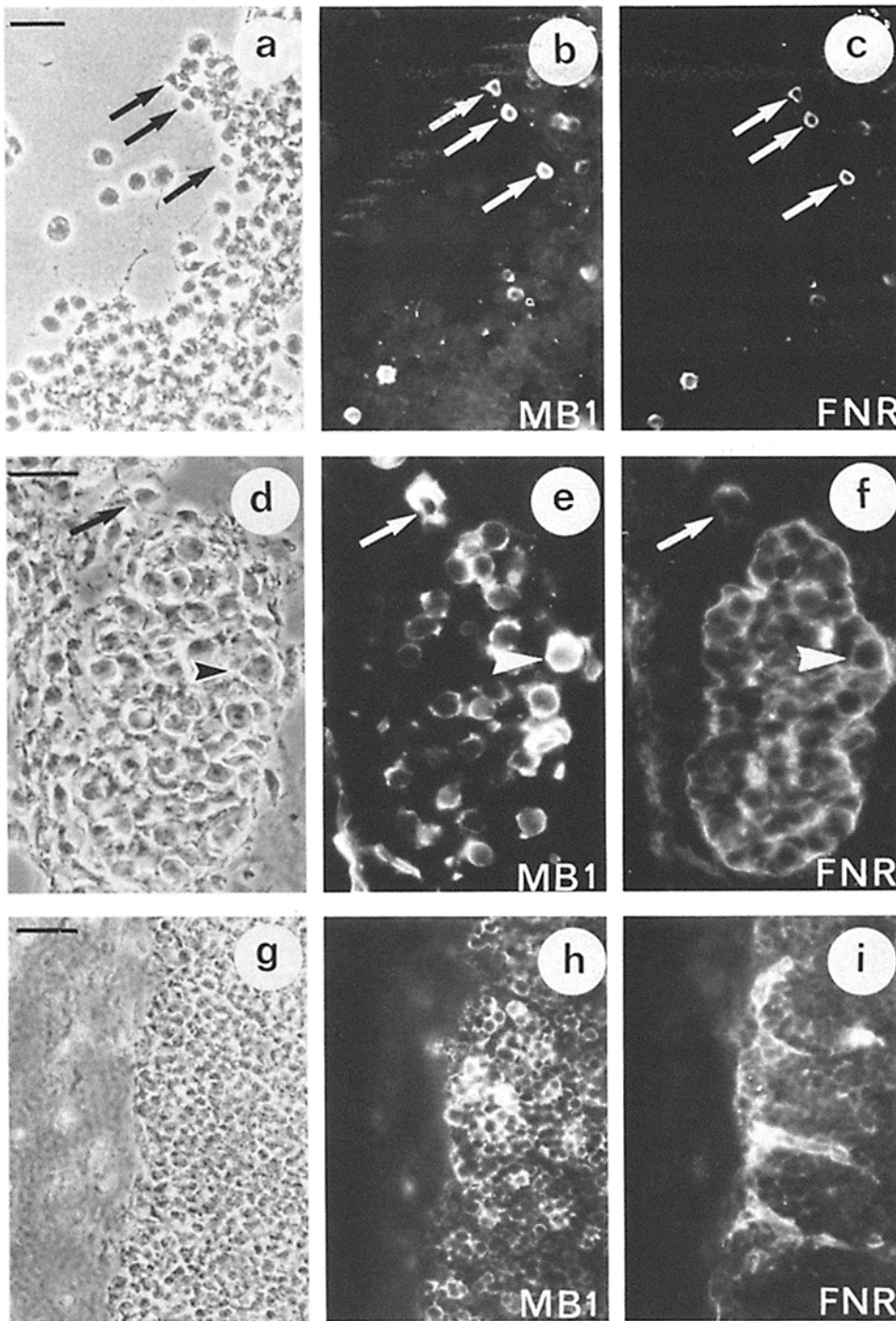
Figure 5. Localization of fibronectin receptor on hemopoietic precursor cells before and after their homing into the thymus. Frozen sections were examined by double-staining immunofluorescence using anti–MB1 and anti–fibronectin receptor antibodies. (*a–c*) Transverse section through a large blood vessel of a 5-d-old quail embryo; small blood cells are stained for both MB1 and fibronectin receptor (*arrows*). Erythrocytes are all negative. (*d–f*) Sections of a 6-d-old quail thymus. Most MB1-positive hemopoietic precursor cells also show immunofluorescence for the fibronectin receptor either outside (*arrow*) or inside (*arrowhead*) the thymus. (*g–i*) Sections of an 11-d-old quail thymus. T lymphocytes express high amounts of MB1 antigen, and they also contain fibronectin receptor. The heavily stained stacks of cells in *i* correspond to mesenchymal infiltrations including blood vessels. *MB1*, hemopoietic cell surface marker; *FNR*, fibronectin receptor. Bars: (*a–f*) 20 µm; (*g–i*) 35 µm.

gradient in vitro (14). This stage of development corresponds to the second colonization period of the thymus during embryogenesis. Hemopoietic precursor cells of such embryos were analyzed by double immunofluorescence using anti–fibronectin receptor and anti–MB1 antibodies. Before chemotactic migration, only 61% of the cells expressed both fibronectin receptors and MB1 molecules (Fig. 6 *A*). In contrast, cells that migrated represented a uniform population (99.5%) of double-positive cells (FNR$^+$, MB1$^+$; Fig. 6 *B*). At least some of the nonmigrating 26% subpopulation of cells that displayed the fibronectin receptor but lacked MB1 (FNR$^+$, MB1$^-$) may be contaminating fibroblasts from the bone marrow stroma. In the colonized 11-d-old embryonic

thymus, >80% of double-positive cells can be obtained after mechanical extrusion (Fig. 6 *C*). At this stage of development, the lymphoid cells form the majority of the cells of the thymus (Fig. 5 *h*). In addition, cells from the stroma (MB1$^-$) are not efficiently dissociated by mechanical disruption and are therefore not included in this analysis.

Analysis of Fibronectin Receptor on Hemopoietic Precursors by Immunoblotting before and after Chemotactic Migration

The expression of fibronectin receptors on bone marrow–derived hemopoietic precursors was analyzed by immunoblot-
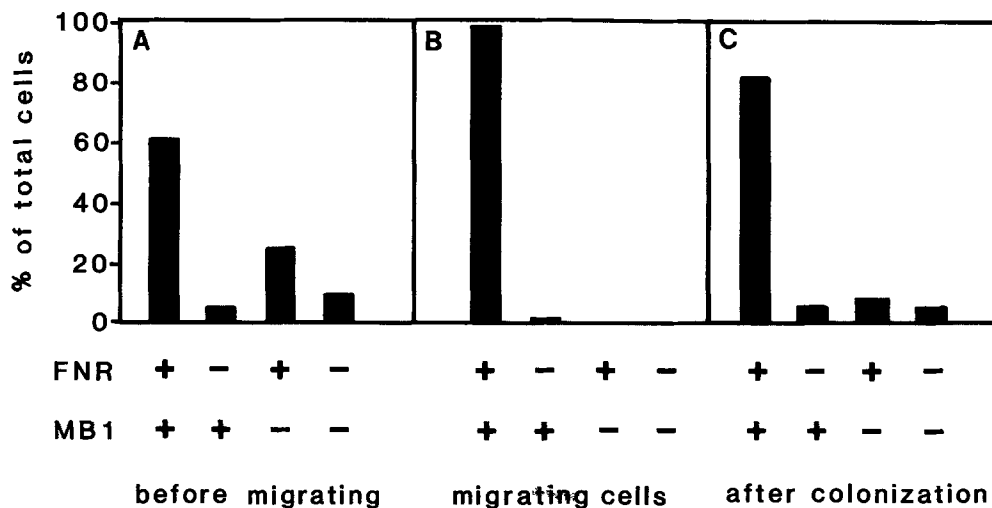
Figure 6. Analysis of hemopoietic precursor cells and thymocytes for fibronectin receptor molecules before and after chemotactic migration in vitro, and within the thymus of 11-d-old quail embryo after colonization. (A) 11.5-d-old embryonic bone marrow cells were separated by centrifugation on a cushion of BSA, and analyzed for FNR and MB1 by immunofluorescence and phase-contrast microscopy. (B) These cells were loaded in the upper compartment of chemotactic chambers. After chemotactic migration, the bone marrow cells collected from the lower chamber were analyzed for MB1 and FNR by immunofluorescence and phase-contrast microscopy. (C) 11-d-old embryonic thymuses were mechanically dissociated. The cell suspension contains mostly lymphoid cells which still display the FNR.

ting. The suspension of bone marrow cells before chemotactic migration contained significant amounts of fibronectin receptors (Fig. 7 a), a part of which should correspond to the contaminating fibroblasts. After migration, fibronectin receptors are still detectable on the surface of hemopoietic precursors (FNR+, MB1+) that responded to the chemotactic peptide (Fig. 7 b). It should be stressed that the amount was substantially lower than that obtained from an equivalent number of quail cultured fibroblasts (Fig. 7 c). Fibronectin was detected in bone marrow cells extracts (Fig. 7 a'), also at a lower level as compared to cultured fibroblasts (Fig. 7 c'). In contrast, the hemopoietic cells after migration did not contain any fibronectin (Fig. 7 b'). These results indicate that the hemopoietic precursor cells express fibronectin receptors, although at a lower level than fibroblasts both from the bone marrow stroma or from quail embryos.

In Vitro Induction of Invasive Properties by Thymic Chemotactic Peptides

The effect of chemotactic peptides from thymic epithelial cells on the invasiveness of hemopoietic precursor cells was studied in Boyden chambers containing human amniotic basement membrane. After treatment with ammonium hydroxide, the amnion loses its epithelial cell layer, leaving a thick basement membrane containing laminin and fibronectin (2, 67, and data not shown). Hemopoietic precursors were incubated on such basement membranes for 20 h. Cells that had successfully traversed the amnion and had accumulated on the underlying nitrocellulose filters were stained and counted.

Both thymic epithelial cell–conditioned medium and Sephadex G-25–purified chemotactic peptides produced a four- to fivefold increase in the number of hemopoietic precursors migrating through the amnion (Table I). In contrast, conditioned media derived from embryonic fibroblasts and kidney epithelial cells did not contain the chemotactic activity. When the attracting medium was placed in the upper compartment together with the cells, the migration index was slightly lower than in controls, indicating that the conditioned medium does not provide factors inducing chemokinesis in this system (Table II). The migration index was

reduced when monovalent Fab' fragments against the fibronectin receptor complex were incubated with the cells in the upper compartment. This effect was dose dependent, and as much as 80% inhibition was obtained when 1 mg/ml Fab' was used (Fig. 8).

In a complementary approach, amniotic membranes were precoated in Boyden chambers for 20 h with antibodies against fibronectin or laminin before incubation with hemopoietic precursors. The resulting inhibition of hemopoietic precursor cell invasion exceeded 50%, and a mixture of both antibodies resulted in >70% inhibition of invasiveness (Fig. 9).

A similar inhibitory effect was obtained with the synthetic peptides GRGDS and with the decapeptide SP1 (also containing the cell-binding adhesive recognition sequence of fibronectin, Arg-Gly-Asp-Ser). A considerable inhibition was achieved at 2 mg/ml (Fig. 10). The effect was dose dependent (not shown), and control peptides had minimal effects on migration.

Discussion

Our major findings can be summarized as follows. In vivo, ultrastructural and immunocytochemical analyses suggested that hemopoietic precursor cells interact with fibronectin and laminin during an invasive type of migration towards the thymus.

In vitro, hemopoietic precursor cells transiently expressed substantially augmented invasive properties when exposed to chemotactic peptides produced by the thymic epithelium. Such stimulated hemopoietic precursors readily traversed a thick human amniotic basement membrane. Antibodies directed against fibronectin and laminin, two components of the amniotic membrane, inhibited this invasion by hemopoietic precursors. The fibronectin receptor was also found to be present on these hemopoietic precursor cells. Fab' fragments against the fibronectin receptor, or synthetic peptides containing the fibronectin-binding recognition site for this receptor, also blocked the invasive process. Thus, the developmental process of invasive migration by hemopoietic precursors appears to require interactions with laminin and
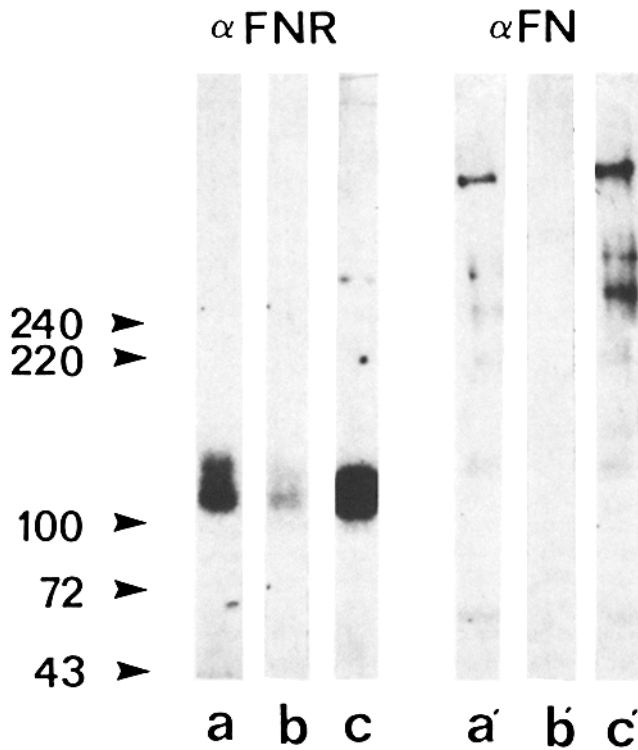
αFNR   αFN

240 ▶
220 ▶

100 ▶

72 ▶

43 ▶

a b c   a´ b´ c´

Figure 7. Western blot analysis of bone marrow–derived hemopoietic precursor cells for fibronectin receptor and for fibronectin. Western blot of a polyacrylamide 4–12% SDS gel after electrophoresis under nonreducing conditions. (a and a′) Original total suspension of bone marrow cells. (b and b′) Hemopoietic precursors after chemotactic migration to the lower compartment of Boyden chambers. (c and c′) Cultured quail embryonic fibroblasts. (a–c) Autoradiograms were labeled for the fibronectin receptor using a monospecific rabbit polyclonal antibody. A 140-kD band characteristic of the fibronectin receptor (band 3) is found in all three cell populations. (a′–c′) Autoradiograms were labeled for fibronectin using a monospecific rabbit polyclonal antibody. The 440-kD band corresponding to nonreduced fibronectin is only found with cultured fibroblasts and with the total suspension of bone marrow cells. It is absent from the migrating cells. Aliquots were normalized to equal cell number; molecular mass markers were human red cell membrane proteins after chemical reduction.

fibronectin, and the fibronectin receptor appears to play an important role in these interactions.

Sequence of Thymic Colonization by Hemopoietic Precursors

The morphological data from sections of 6-d-old quail embryos suggested that hemopoietic precursors cross the endothelial wall of the jugular vein or of other perithymic blood or lymphatic vessels. This pathway would imply a passive mechanism of transport of these cells from their original sites of emergence via the blood circulation to thymic vessels, followed by active invasiveness during and after extravasation. Consistent with these observations, quail bone marrow–derived hemopoietic precursor cells, when injected intravascularly into a chick embryo, were also found to home to the thymic rudiment. In fact, this mode of migration using the vascular route has also been described in adult mice after injecting hemopoietic bone marrow precursors into irradiated animals (39, 49, 71). The experiments described here,

Table I. Invasiveness of Hemopoietic Precursors in the Presence of Thymic Chemotactic Peptides

Medium tested	Migration index
Thymic epithelial cell–conditioned medium	4.5 ± 1.1
G-25 fraction	3.7 ± 0.9
Kidney epithelial cell–conditioned medium	0.85 ± 0.18
Fibroblast-conditioned medium	0.90 ± 0.22

Amniotic basement membranes were mounted in Boyden chambers, the lower wells of which contained the medium to be tested. The upper wells were loaded with 1.5×10^5 hemopoietic cells and incubated for 20 h at 37°C.
The migration index was calculated as the ratio between the number of cells attracted by the conditioned medium and the number of cells found in the presence of control culture medium (DME for G-25 fraction). Values represent the mean of quadruplicate samples ± standard deviation.

however, did not require preceding damage to the host, since they were performed during a period permissive for thymic colonization.

After leaving the vascular system, hemopoietic precursor cells were found transiently passing through the mesenchyme towards the thymic rudiment. This location in the perithymic mesenchyme has also been described for precursors in human and mouse embryos (35, 52). In the avian embryo, grafting of hemopoietic foci results in a wide dispersal of precursor cells in the perigraft area of the host (47). Our results agreed with these observations, but they suggested some additional role for mesenchymal cells. These mesenchymal cells, whose unusual cytoplasmic protrusions were found to closely surround hemopoietic precursors, might facilitate their translocation. Nevertheless, activated hemopoietic precursors migrated through a basement membrane devoid of accessory cells.

As shown by immunofluorescence and by electron microscopy, the thymic rudiment was originally completely surrounded by a basement membrane. Before entering the thymus, hemopoietic precursors disrupted and penetrated this membrane separating this organ from the mesenchyme. Such invasive migration may involve proteolytic activities. In fact, the production of plasminogen activator by hemopoietic cells has been described during ontogeny of the bursa of Fabricius (81). In addition, the invasive properties of metastatic cells seem to be correlated with the secretion of specific proteases that degrade the extracellular matrix, including basement membranes (42, 51, 57, 68).

After disrupting and crossing the basement membrane, the migrating hemopoietic cells are confronted by the cells of the thymic epithelium. The epithelium was remodeled during and after colonization with the formation of adhesive interactions between epithelial cells and hemopoietic precursors.

Table II. Discrimination between Chemotactic and Chemokinetic Activities in the Invasive Process

Lower well	Upper well	Migration index
Control medium	Conditioned medium	0.4 ± 0.3
Conditioned medium	Control medium	5.0 ± 3.5

Checkerboard analysis was performed by testing thymic epithelial cell–conditioned medium alternatively in the upper well or in the lower well of Boyden chambers containing amniotic basement membranes. The cells were deposited in the upper wells in all cases. Migration index was calculated as described in Table I. Values represent the mean of triplicate samples ± standard deviation.
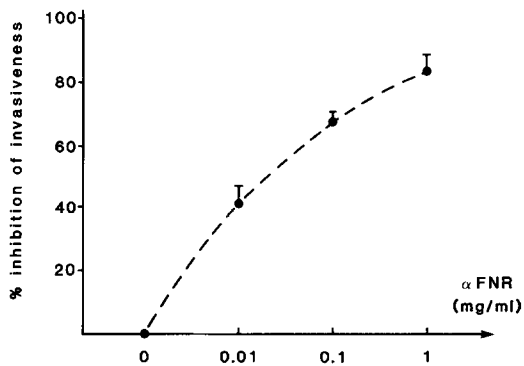
Figure 8. Dose–response inhibition of invasiveness across basement membranes by antibodies against fibronectin receptors. The compartments of Boyden chambers were separated by the human amnion membrane. During migration assays, cells were incubated with monovalent antibodies to the fibronectin receptor at increasing concentrations. Fixation and counting were performed after 20 h incubation at 37°C. Inhibition is calculated in all cases as % = 100× (1 - the ratio between cell number found in the presence of Fab' fragments and cell number found in the absence of treatment). Each value indicates mean of quadruplicate samples ± standard deviation.

Contacts between epithelial cells and hemopoietic precursors were found to continue for as long as 2 d after the colonization period in quail thymus (Savagner, P., unpublished results). Such direct contacts have been shown to be involved in T lymphocyte differentiation (23, 40), with a contribution from peptides secreted by the thymic epithelium (3, 16, 17, 32).

Involvement of Extracellular Matrix in Migration of Hemopoietic Precursor Cells

Fibronectin is well documented to promote cell migration, in particular during embryonic development (recent studies include 7, 10, 20, 65, 85). In birds and in mammals, a surface glycoprotein complex of 120–160 kD acts as a fibronectin receptor mediating low affinity interactions (1, 11, 34, 59). Recently, fibronectin receptors have been reported to be present on bone marrow cells, reticulocytes, and a subset of thymocytes (13, 31, 58). In all of these cases, the receptor appears to be involved in static adhesive events. However, fibronectin receptors have now been demonstrated on hemopoietic precursor cells, which migrate to the thymus in vivo and penetrate a basement membrane in vitro. Their unpolarized distribution and low expression might be in accordance with the high motility of hemopoietic precursor cells. Motile neural crest cells also show a similar diffuse distribution. In contrast, stationary cells accumulate the fibronectin receptor in prominent streaks at cell–substrate contact sites (21).

Recently, it has been shown that the 120–160-kD protein complex can also be involved in laminin binding (34). The role of laminin in adhesion and migration of different cell types is well documented (54, 62, 73, 78). In fact, we found laminin located in the perithymic basement membrane and even within the epithelium. The migration of hemopoietic precursors could be potentiated by the combined interaction of the 120–160-kD complex with laminin and fibronectin. However, a laminin receptor shown to be clearly distinct
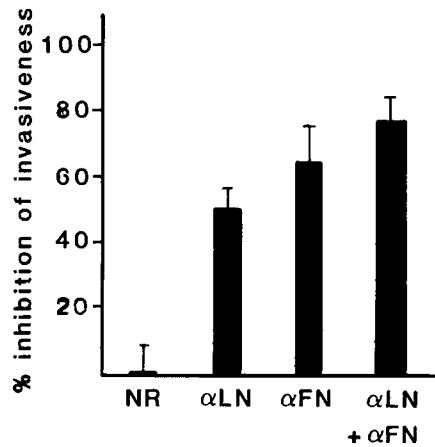

Figure 9. Inhibition of invasiveness by antibodies against fibronectin and laminin. Monospecific polyclonal antibodies against laminin (0.1 mg/ml) or fibronectin (0.1 mg/ml), and control rabbit antibodies (0.1 mg/ml) were preincubated with amniotic basement membranes alone for 20 h before loading cells into the Boyden chambers. In both experiments, cell migration was allowed to proceed in the presence of bound antibodies for 20 h more in invasion chambers. Cells which traversed the amniotic basement membrane were fixed and counted. Inhibition is calculated in all cases as % = 100× (1 - the ratio between cell number found in the presence of antibodies and cell number found in the absence of antibodies). Each value indicates the mean of triplicate samples. Differences between specific antibodies treatment and controls are significant at $P < 0.05$ level in all cases, according to Student's t test.

from the 120–160 kD complex could also be involved in such interactions (12, 48, 50, 61).

Invasiveness of Hemopoietic Precursor Cells In Vitro

Cell invasion assays have been developed using cultured cell monolayers (41, 69, 82), organ cultures (53, 63), or extracellular matrices (30, 56, 66). Each model system has specialized applications, and no "universal" in vitro assay system seems superior for quantitating cell invasiveness. We used as an assay the human amnion in Boyden chambers, because it allowed us to establish a concentration gradient of low molecular weight–diffusible molecules through an intact basement membrane from both sides.

The invasive migration of hemopoietic precursors through the amniotic basement membrane could be inhibited by antibodies to fibronectin and to laminin, by Fab' fragments against the fibronectin receptor, and by synthetic peptides containing the cell-binding adhesive recognition sequence (Gly-Arg-Gly-Asp-Ser [GRGDS]) of fibronectin. These results suggest that laminin, fibronectin, and their receptors participate in some steps of the invasive process of hemopoietic precursors. In vivo, several experiments also suggest a role for cell–laminin and cell–fibronectin interactions in the invasive process of metastasizing cells (4, 74) and during embryo implantation (26). Recently, intravascular injection of the peptide Gly-Arg-Gly-Asp-Ser (GRGDS) along with B16 melanoma cells was shown to prevent experimental lung metastasis (36). However, in our experiments, the inhibition of invasive migration was never larger than 80%, suggesting the possible involvement of other molecules than the receptors for fibronectin and laminin (37). It is also important to
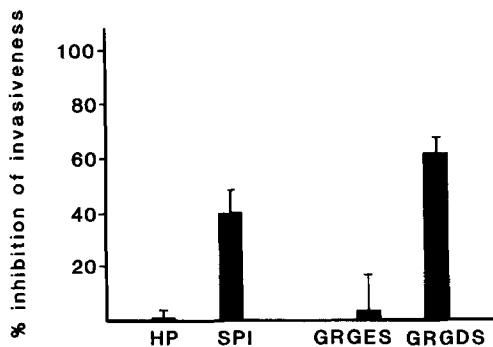
Figure 10. Inhibition of invasiveness by synthetic peptides containing the Arg-Gly-Asp-Ser cell-binding recognition site of fibronectin. Cells were incubated with 2 mg/ml of each peptide (SP1 and GRGDS) for 20 h in invasion chambers. After migration, the cells were fixed and counted. As controls, the hexapeptide HP (Leu-Trp-Met-Arg-Phe-Ala), with an amino acid sequence unrelated to fibronectin and the peptide GRGES (with only a single amino acid exchange of a Glu for an Asp residue) were used. Percent inhibition of invasiveness was calculated as described for Fig. 9. Each value represents the mean of four samples $\pm$ standard deviation. Inhibition value differences are significant at $P < 0.05$, according to Student's t test. It should be noted that the equal concentration by weight of the decapeptide SP1 used for comparison with the pentapeptide is equivalent to only about half of the latter's molar concentration.

emphasize that our assays do not differentiate between inhibition of initial attachment and of later steps in the invasive migration process. Nevertheless, interaction with laminin or fibronectin appears to constitute a necessary step in the many events involved in this developmentally programmed invasion of basement membrane.

Chemotaxis and Hemopoietic Cell Invasiveness

Penetration of human amniotic basement membranes by hemopoietic precursors in vitro was provoked by chemoattractant peptides produced by thymic epithelial cells. This activation includes a change of shape from round to polarized cells, directed migration, and possibly secretion of different proteases. Our chemotactic peptide could only activate hemopoietic precursors before the cells colonized the thymus. After residing in the thymus, thymocytes could no longer respond to the chemotactic peptide in vitro. On these cells, the cell surface receptors for the chemotactic peptide seem to be down regulated (14). In conclusion, the chemotactic and invasive behavior appears to be temporary and stage dependent during thymic morphogenesis. This is in contrast with similar but ongoing events described for polymorphonuclear leukocytes in adults during inflammation. However, the presence of formyl-Met-Leu-Phe, an efficient synthetic chemoattractant, does induce polymorphonuclear cells to migrate, to penetrate through human amnion (67), and to secrete proteases present in intracytoplasmic granules (25, 33).

In leukocytes, and as now shown in hemopoietic cells, signal molecules induced the invasive behavior of cell populations via specific receptors. In another series of experiments, a low-metastatic lymphoma was fused with nontransformed macrophages or with T cells. Some hybrids developed a capacity for constitutive invasion independent of signal molecules (18, 43). However, on hemopoietic precursor cells, we show that this behavior can be induced by thymic peptides

in a transient event that is precisely programmed during a specific stage of embryonic development.

To What Extent Are Embryonic Cells Invasive?

Embryogenesis involves different migration patterns for numerous cell populations. Most migratory cells move actively in mesenchymal cell-free spaces, or passively in blood vessels. However, passing through basement membranes constitutes a specific property of highly invasive cell populations like hemopoietic precursors. It is important to note that such invasiveness is not required for highly migratory cell populations like most neural crest cells (77). For instance, after grafting to ectopic sites, trunk neural crest cells show active migration, but are never seen passing through basement membranes (22; and C. A. Erickson, personal communication). During peripheral gangliogenesis in the gut, subpopulations of neural crest cells can be found mingling with smooth muscle precursors before the formation of muscular layers and basement membranes (80). In vivo, melanocytes seem to be the only well-described neural crest–derived cells to invade an epithelium (the epidermis), during migration (45, 72). However, when confronted in vivo by a human amnion, these cells are unable to pass through this thick membrane (30).

In the hemangioblastic lineage, in common with the hemopoietic precursors, endothelial cells constitute a potentially highly invasive cell population. During embryonic vascularization and neovascularization towards normal tissue or tumors, endothelial cells can migrate and invade epithelia through basement membranes. Their migration is enhanced by well-described substances like heparin or heparin fragments secreted by mast cells or angiogenin secreted by tumor cell lines (24, 27).

However, to our knowledge, the migration of hemopoietic precursors to the thymic rudiment constitutes the first documented example of an invasive migration induced by chemotactic peptides during embryogenesis. Since the behavior of activated hemopoietic precursors appears comparable to that of invasive, metastasizing cells, it would be interesting to test the possible involvement of oncogenes in its regulation.

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