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Heterogeneity of Rabbit Aortic Endothelial Cells in Primary Culture[1] (42506)

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Abstract. Factor VIII-related antigen (F8-RAg) and angiotensin-converting enzyme (ACE) are accepted diagnostic markers of endothelial cells in culture. However, when we isolated cells from rabbit thoracic aorta (after collagenase treatment and gentle scraping of the intima) and examined them with immunoperoxidase techniques, we observed two cell types which stained specifically for either F8-RAg or ACE, but not both. Each cell type was morphologically distinguishable in primary culture. F8-RAg-positive cells were recognizable in distinct patches as more elongated, tightly apposed, and firmly adherent cells; they exhibited only faint or no staining for ACE and no accumulation of a fluorescent, acetylated low-density lipoprotein probe (DiI-Ac-LDL), another endothelial cell marker. In contrast, ACE-positive cells were more rounded, less closely apposed, and grew as strict monolayers that exhibited a characteristic cobblestone appearance at confluence; ACE-positive cells were F8-RAg negative, but demonstrated intense labeling with DiI-Ac-LDL. Subcultures of ACE-positive cells were also stained by anti-rabbit thrombomodulin.

Morphological, biochemical, and immunolgical criteria that permit identification of endothelial cells in culture have been established by others: strict monolayer growth and cobblestone appearance at confluence (1), presence of Factor VIII-Related Antigen (F8-RAg) (2), presence of angiotensin-converting enzyme (ACE) (3), and preferential uptake of acetylated low density lipoprotein (4). When we apply these criteria to cells obtained from the intima of rabbit thoracic aorta, we can distinguish two kinds of endothelial cells in primary culture.

Materials and Methods. *Cell culture.* Cells were cultured in Dulbecco's modified Eagles medium (DME) supplemented with Hepes (25 m*M*), ascorbic acid (50 μg/ml), glutamine (2 m*M*), 10% fetal bovine serum (FBS), 10% calf serum, penicillin (100 IU/ml), and streptomycin (100 mg/ml). Powdered medium and supplements were obtained from Sigma or GIBCO, reconstituted with glass-distilled, deionized water, and sterilized by positive pressure filtration through 0.2-μm Sterivex filters (Millipore). Growth medium was changed 24 hr after initial seeding, and every 3–4 days thereafter.

Thoracic aortae, from arch to diaphragm, were removed from young, female New Zealand white rabbits and placed in DME containing antibiotics and 0.5% FBS. After it was trimmed of fat, each aorta was cut lengthwise with scissors to expose the intima, which was irrigated with serum-free DME to wash away blood clots. Aortic segments (2–3 cm long) were placed intima side down in a shallow puddle of sterile collagenase solution (Worthington CLS II, 1 mg/ml DME). After 30 min at 37°C, segments were reinverted in a fresh dish without enzyme and the intimal surface of each segment was gently scraped with a vein stripper. Scrapings were collected after each unidirectional stroke by dipping the vein stripper into pretreated culture dishes containing growth medium.

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Immunocytochemistry. Factor VIII and ACE were detected in aortic-derived cells grown for 16–24 hr on circular glass coverslips (12 mm, Bellco), using an avidin–biotin complex (ABC) adaptation of a standard immunoperoxidase procedure (5). Coverslips were rinsed once in serum-free medium (DME), rinsed three times in Osborn-Weber phosphate-buffered saline (OW-PBS, pH 7.30) (6), and then fixed in 10% neutral-buffered formalin saline (1 min, room temperature), followed by absolute methanol (6 min, −20°C). After rehydration in OW-PBS (15 min, room temperature), coverslips were incubated for 15 min at room temperature with heat-inactivated (56°C, 1 hr) nonimmune rabbit serum (NRS) diluted 1:100 in 0.1% gelatin OW-PBS (NRS:GPBS). Replicate coverslips were then incubated overnight at room temperature in a humid chamber with a primary antiserum (see below) or nonimmune goat serum (NGS). After coverslips were rinsed 3× with OW-PBS, they were incubated for 45 min with biotinyl-rabbit anti-goat IgG (Sigma) diluted 1:400 in NRS:GPBS. Coverslips were rinsed again with OW-PBS, and then incubated 45 min with freshly prepared avidin DH (5 μl/ml) + biotinyl-horseradish peroxidase (5 μl/ml) complex (Vector Labs, Burlingame, CA) in GPBS. Coverslips were rinsed in OW-PBS and incubated (2 to 5 min) in filtered (0.2 μm) diaminobenzidine tetrahydrochloride (DAB, Sigma) substrate (50 mg DAB/100 ml PBS + 25 μl 30% H_2O_2). Coverslips were rinsed and dehydrated through alcohols to xylene and mounted on glass slides (25 × 75 mm) with Krystalon.

Goat anti-human F8-RAg (Atlantic Antibodies) and goat anti-rabbit ACE (Dr. Peter Caldwell, Department of Medicine, Columbia University) were the principal primary antisera employed. Goat anti-rabbit thrombomodulin (TM) (7) and goat anti-human vimentin (VM; Miles Laboratories, Naperville, IL) were used in a limited series of studies. All goat sera were heat inactivated and tested at various dilutions from 1:100 through 1:32000. Reactive cells were unambiguously stained with primary antisera at a working dilution of 1:2000 and showed little or no staining with NGS (at dilutions of 1:500 or less, staining with NGS was quite intense). No staining was observed when biotinyl-rabbit anti-goat IgG or ABC reagent was omitted. Human umbilical vein endothelial cells cultured in 20% FBS:DME (8) were used as a positive control for F8-RAg. Cells from a rabbit tumor (Vx2 papilloma) (9), cultured as described above, were not stained by F8-RAg, ACE, or TM antisera and served as another negative control. No staining of any rabbit cells was observed using anti-VM.

Immunocytochemistry was also performed directly on primary cultures of aortic-derived cells in Falcon dishes (35 or 60 mm). Cells were fixed and rehydrated directly in the culture dishes. For each dish, three Incu-Rings (10 mm, Polysciences) were coated with silicone grease (Dow-Corning) and then pressed onto premarked areas of the culture dish delimiting discrete patches of cells. PBS was aspirated from each ring, and anti-ACE, anti-F8-RAg, or NGS was added. The bottom of each dish was marked to denote where each antiserum or NGS was added.

A sequential, two-color immunoperoxidase procedure (10) was also used to determine if F8-RAg and ACE were present in the same cell. After incubation with biotinylated anti-goat IgG, as described above, replicate coverslips were rinsed in PBS, rinsed in 0.1 *M* acetate buffer (pH 6.0), and incubated 2–5 min in a filtered solution (0.2 μm) of 0.035% DAB, 2.5% (w/v) nickel ammonium sulfate, and 0.1% (v:v) H_2O_2 in 0.1 *M* acetate buffer. Coverslips were rinsed in acetate buffer (3×), OW-PBS (3×), and then reprocessed using the first DAB staining procedure (PBS, pH 7.3) after completion of an overnight incubation with the other primary antiserum (F8 or ACE) or nonimmune goat serum.

Uptake of acetylated low-density lipoprotein fluorescent probe. A fluorescent derivative of acetylated low-density lipoprotein (DiI-Ac-LDL; Biomedical Technologies) was added (20 μg/ml) to endothelial cell cultures growing on coverslips. After 4 hr incubation, coverslips were removed and rinsed two times in probe-free medium, and then immersed in neutral-buffered formalin (5 min, RT). Each coverslip was inverted onto a drop of Aquamount on a 25 × 75-mm glass slide. Cells were first located with Nomarski optics, and then examined un-

der fluorescence illumination, using standard rhodamine filters (Zeiss IM 35).

Results. The combined collagenase/scraping procedure initially harvested endothelial cells as torn monolayer sheets, which began to attach to the culture dish within an hour. Sheets of attached endothelium gradually "melted down" as attached cells migrated away on the dish. Attachment of endothelial sheets and outgrowth of cells in primary culture required pretreatment of culture dishes with sterile 0.01% Difco gelatin overnight at 37° C (11). Primary cultures were inspected daily with an inverted phase-contrast microscope (Nikon Diaphot). Figure 1 depicts living rabbit aortic cells after 4 and 20 days in primary culture. Two morphologically distinct cell types were consistently observed: one type of cell was more elongated and smaller and appeared to be more tightly affixed to the dish (upper portions of Figs. 1A–C); a second type was larger, more rounded, less tightly apposed, and less firmly attached (lower portions of Figs. 1A–C).

Immunocytochemical identification. Cells on coverslips incubated with normal goat serum in lieu of primary antiserum were invisible or faint brown under brightfield illumination, and were located with Nomarski optics (Olympus BH2). Cells which resembled those in the lower portion of Fig. 1C and incubated with anti-ACE showed diffuse, brown-black staining throughout the cytoplasm (ACE-positive cells; Fig. 2); some ACE-positive cells also showed focal membrane staining. Cells resembling those in upper portion of Fig. 1C were positive for F8-RAg and showed discrete, granular, brown-black, cytoplasmic staining (Fig. 3) surrounding unstained nuclei; this distinctive, perinuclear staining pattern for F8-RAg was also observed in human umbilical vein endothelial cells. Cells passaged from ACE-positive cultures were also stained by anti-TM (not shown).

Using the sequential, two-color staining procedure, more cells on each coverslip were stained than on coverslips incubated with only one primary antiserum. Cells stained using nickel ammonium sulfate showed the same patterns of staining observed with DAB alone, but were bluish-grey instead of brown. However, no additional staining of *individual* cells was discernible, regardless of the order of applying primary antisera. That is, stained cells were positive for either ACE or F8-RAg, but not both. When NGS was used in place of the first antiserum, incubation with the other specific antiserum produced the same staining pattern as the original procedure. Cells in primary culture fixed *in situ* and incubated with specific antiserum within Incu-Rings in the original plastic culture dish displayed the same kind of staining observed in cells subcultured onto glass coverslips.

Accumulation of dil-Ac-LDL. When observed with fluorescent illumination, the majority of cells on coverslips from predominantly ACE-positive cultures incubated with dil-Ac-LDL fluoresced intensely with a bright and persistent red/orange twinkle (Fig. 4). In contrast, cells on coverslips from F8-RAg-positive cultures treated with dil-Ac-LDL were not visible. Cells from replicate cultures not exposed to the probe did not fluoresce.

Discussion. Because our primary goal in isolating and culturing endothelial cells was to obtain cells for use in an *in vitro* bioassay to detect ovarian angiogenesis factors (12), we began with the assumption, suggested by the current literature, that aortic endothelial cells were a homogeneous population. To verify the identity of the cells we isolated, we used accepted criteria for aortic endothelial cells in culture: their derivation from aortic intima; morphology, i.e., epithelioid cells in a characteristic cobblestone monolayer at confluence; and immunoperoxidase staining for F8-RAg and ACE. However, when we applied these criteria, we consistently detected two types of endothelial cells, which were morphologically and immunologically distinguishable, as described above. At first, we suspected that subculture onto glass coverslips may have affected the cells' expression of the immunological markers, but when we performed the immunocytochemical procedures in Incu-Rings on cells in primary culture on plastic dishes we again observed different cells staining for ACE or F8-RAg. In an attempt to clarify the issue, we used another marker of endothelial cells, specific uptake of dil-AC-LDL (4), and again distinguished two types of

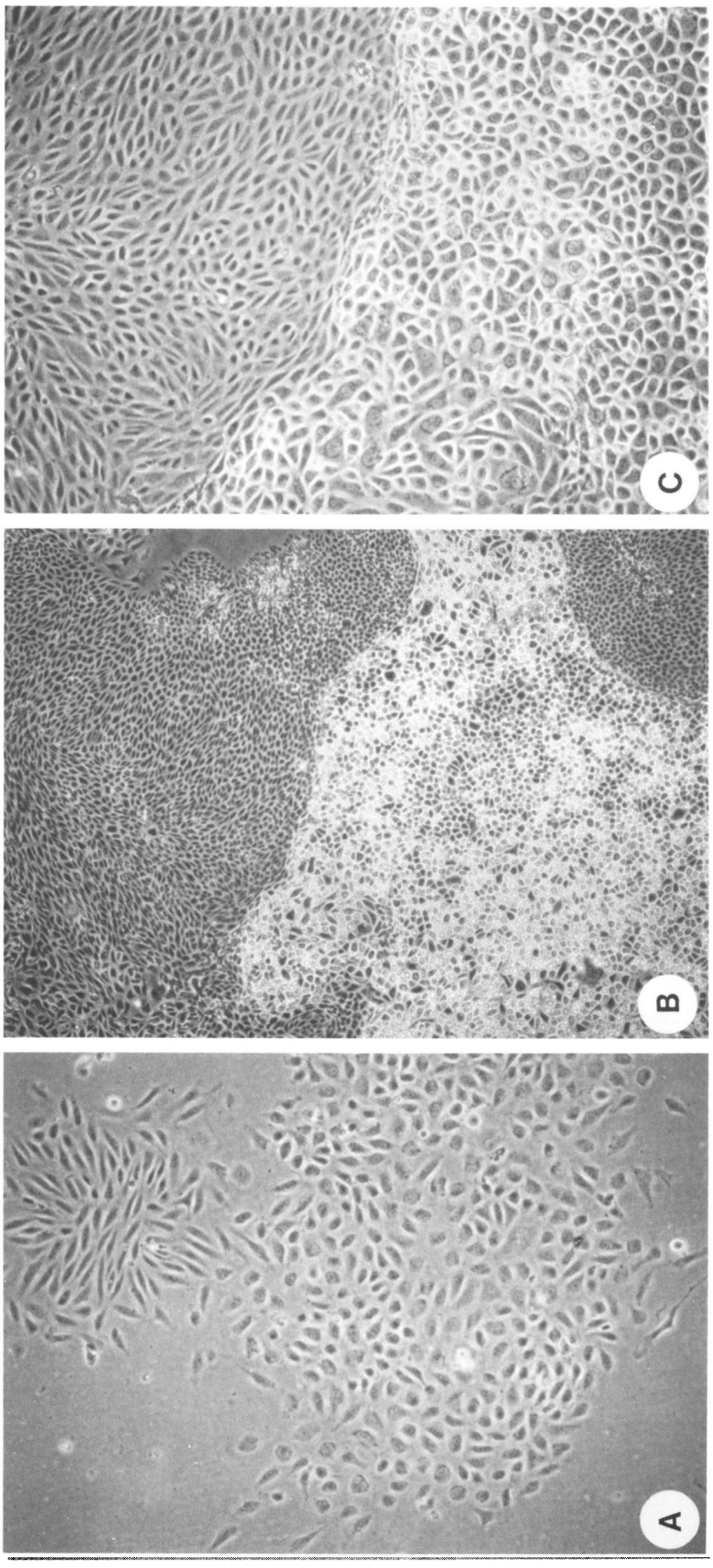

FIG. 1. Rabbit aortic endothelial cells in primary culture exhibit chracteristic cobblestone appearance and strict monolayer growth. Phase contrast. (A) Four days after plating (80×). (B) and (C) depict another primary endothelial cell culture 20 days after plating. (B) (32×); (C) (80×). Factor VIII-positive cells (upper portion) are smaller and more elongated; ACE-positive cells (lower portion) are rounder and less tightly apposed.

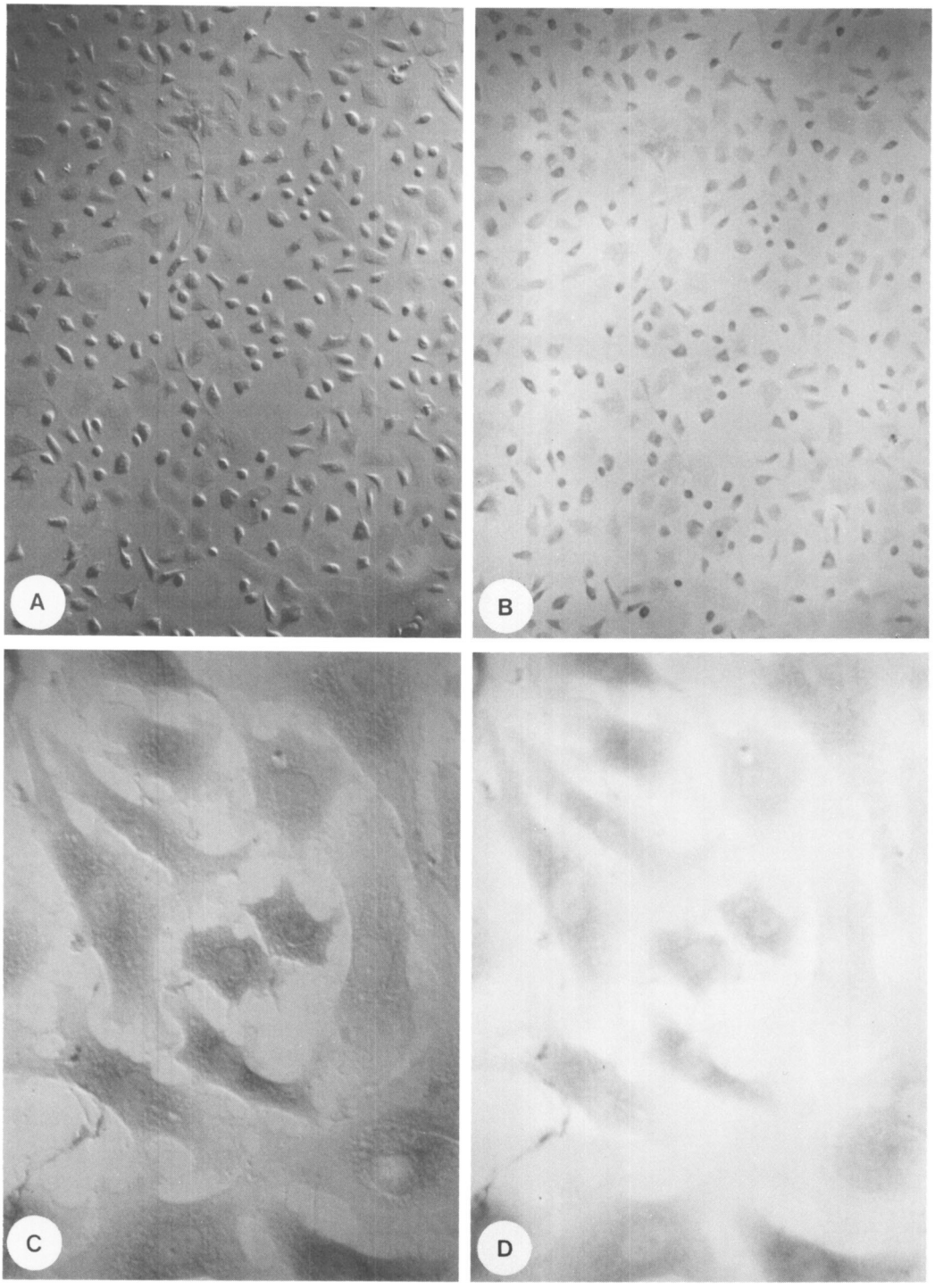

FIG. 2. Rabbit aortic endothelial cells grown on coverslips and immunostained for ACE after formalin: methanol fixation; cells 11 days after isolation. Positive cells displayed diffuse cytoplasmic staining. (A) Nomarski D.I.C. (50×); (B) brightfield (50×); (C) Nomarski (200×); (D) brightfield (200×).

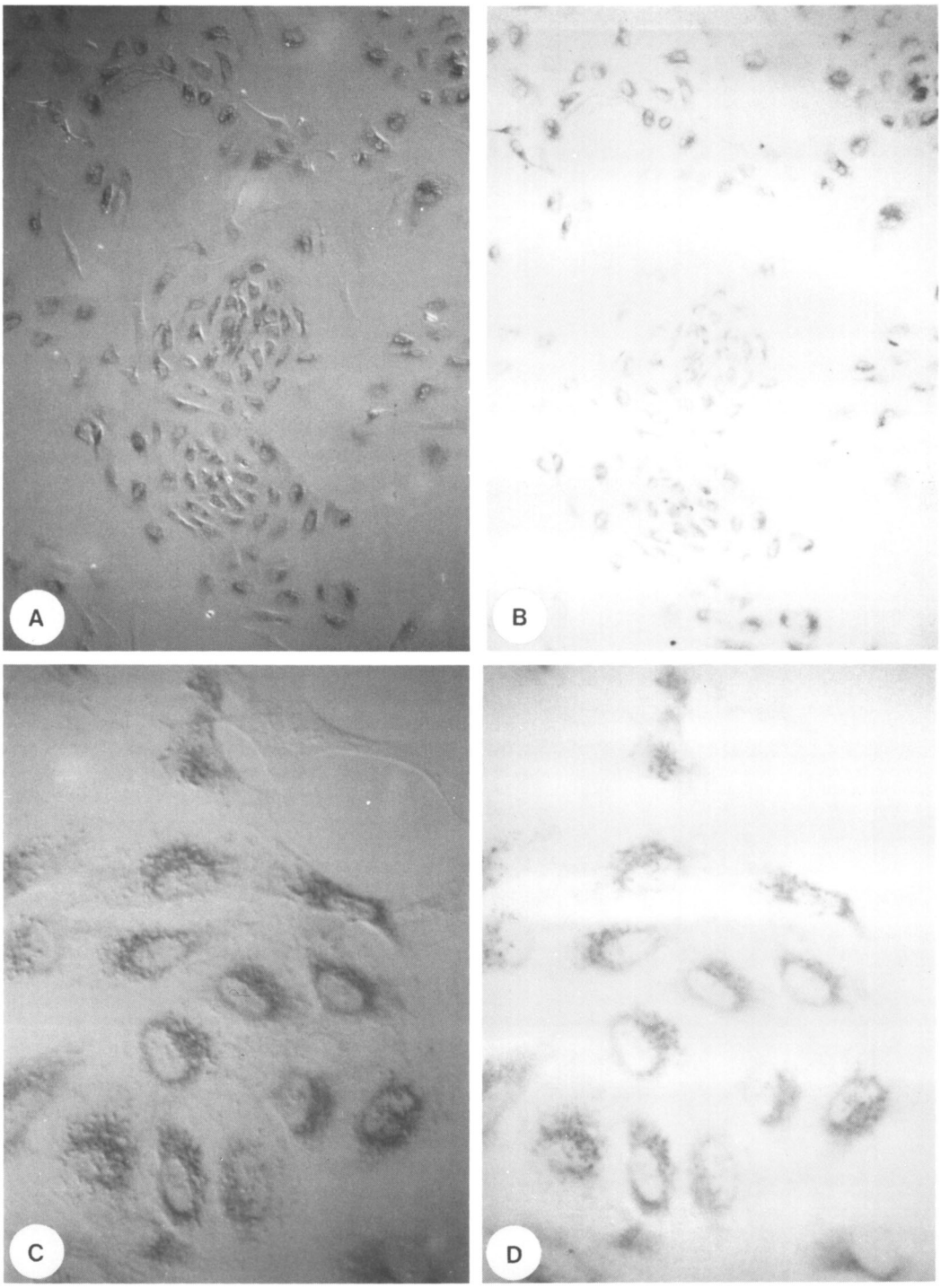

FIG. 3. Rabbit aortic endothelial cells grown on coverslips and immunostained for F8-RAg after formalin: methanol fixation; cells 14 days after isolation. Positive cells displayed distinctive, granular, perinuclear cytoplasmic staining. (A) Nomarski (50×); (B) brightfield (50×); (C and D) as in (A) and (B); (C) Nomarski (200×); (D) brightfield (200×).

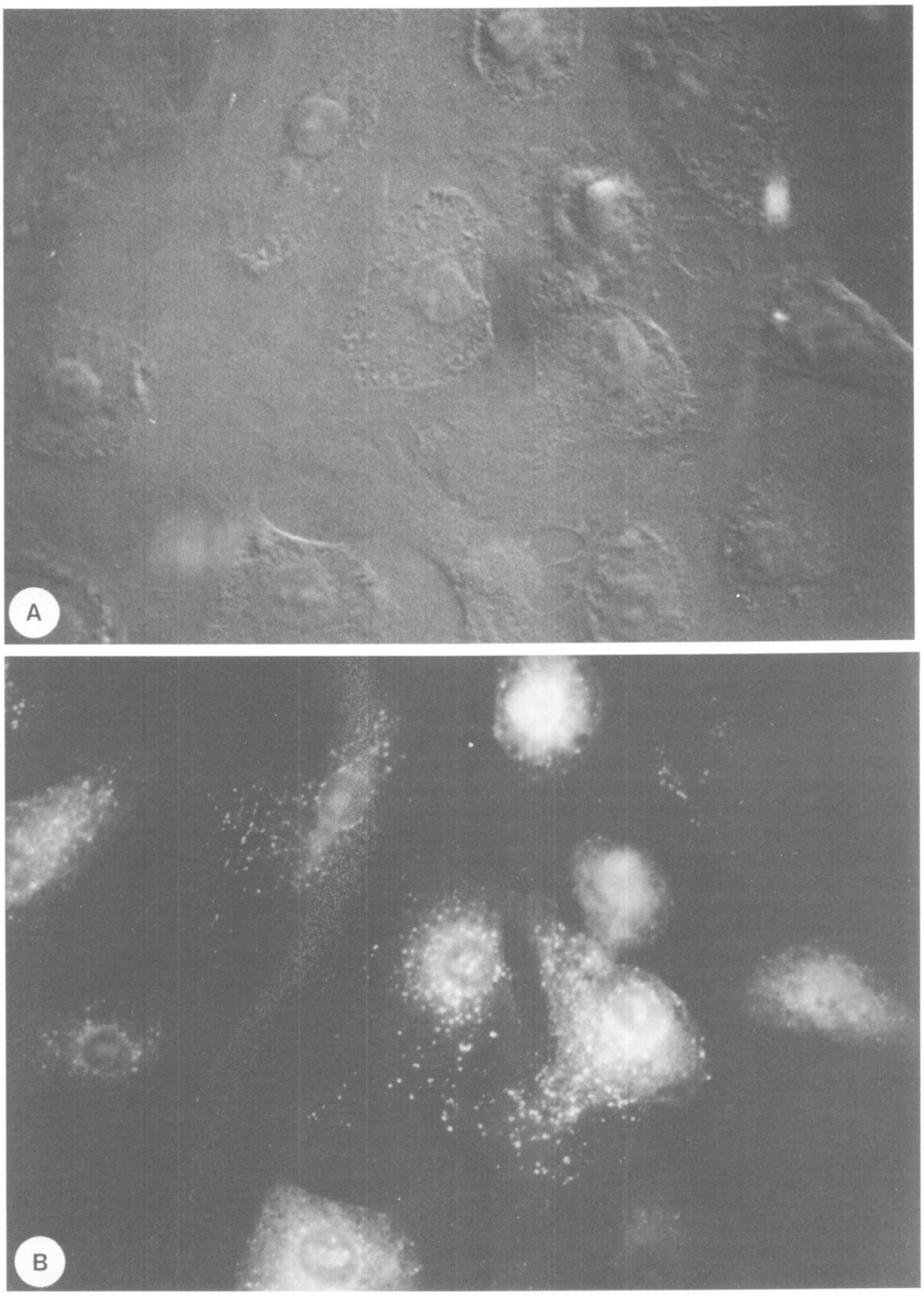

FIG. 4. Rabbit aortic endothelial cells grown on coverslips and incubated with fluorescent Ac-LDL probe (20 μg/ml) for 4 hr at 37°C, and then fixed in formalin. (A) Nomarski; (B) mercury lamp with rhodamine filters (360×). Fluorescence indicates accumulation of DiI-Ac-LDL. Cells in the center of (B) are decorated with the fluorescent marker, whereas cells in each corner visible with Nomarski optics (A) are not decorated (B). These cells are from the same culture depicted in Fig. 1A and Figs. 2A and B.

cells. Although it was apparent that two cell types were distinguishable—one F8-RAg positive but ACE and dil-AC-LDL negative, the other F8-RAg negative but ACE and dil-AC-LDL positive—we performed a final test using a sequential staining procedure for F8-RAg and ACE and discerned no additional staining of individual cells.

Although our isolation and culture procedures may have depleted F8-RAg from ACE-dil-Ac-LDL-positive cells, that other, morphologically distinguishable cells retained the F8-RAg marker but were negative for ACE and dil-AC-LDL vitiates the assumption that aortic endothelial cells are homogeneous. In addition, although each primary antiserum may not have been entirely monospecific, the presence of other antibodies cannot explain the differentiable staining of aortic cells (neither antiserum stained rabbit Vx2 tumor cells, or, in a limited series, cells isolated from rabbit oviductal mucosa or rabbit uterine endometrium).

We have routinely subcultured individual batches of ACE-positive rabbit aortic endothelial cells for 6–8 months; these cells maintain their characteristic cobblestone monolayer appearance at confluence and are positive for ACE, TM, and dil-Ac-LDL throughout this period. In contrast, expression of F8-RAg by the other cell type observed in primary culture was generally not detectable after the second week. It is unlikely that F8-RAg+ cells are smooth muscle cells, because they were clearly distinguishable from a third type occasionally observed, which grew in multilaminar foci in a "hill and valley" pattern typical of smooth muscle cells in culture, as described by others (13), and which were F8-RAg and ACE negative. F8-RAg also disappears within days from porcine aortic endothelial cells in primary culture (2). After examining rabbit endothelial cells isolated from aorta, brain, and fat, DeBault and co-workers (7) concluded that F8-RAg is not a reliable marker for cultured endothelial cells in this species and proposed that TM be used instead. Using the lectin *Dolichos biflorus* agglutinin as a strain-specific marker, Schmidt and co-workers (14) demonstrated mosaicism in the aortic endothelium of mouse aggregation chimeras. The presumptive heterogeneity of rabbit aortic endothelial cells we observed in culture may, in turn, be a reflection of diverse clonal histories of aortic endothelium during fetal angiogenesis.

In summary, we have used several accepted criteria to identify rabbit endothelial cells in culture: their derivation from aortic intima; morphology in culture, i.e., cobblestone monolayer at confluence; immunoperoxidase staining of F8-RAg or ACE; and specific uptake of a fluorescent acetylated low-density lipoprotein probe. Applying these criteria to cells isolated from monolayer fragments of intima from rabbit thoracic aorta, we detected two endothelial cell types which are morphologically, immunologically, and biochemically distinguishable in primary culture.

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