

A MONOCLONAL ANTIBODY (3G5)-DEFINED GANGLIOSIDE ANTIGEN IS EXPRESSED ON THE CELL SURFACE OF MICROVASCULAR PERICYTES

BY RAMESH C. NAYAK, AUDREY B. BERMAN, KATHERINE L. GEORGE,
GEORGE S. EISENBARTH, AND GEORGE L. KING

From the Research Division, Joslin Diabetes Center; the Department of Medicine, Brigham and Women's Hospital; and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215

Studies on isolated pericytes have been hindered by the lack of specific biochemical markers with which to facilitate identification and purification of this cell type. The pericyte is defined by a strictly anatomical criterion *in vivo*, namely its anatomical location within the microvascular basement membrane and surrounding the endothelia (1). In dissociated cultures, pericytes have been identified by morphological and growth characteristics (2–5). A recent study (6) has shown that pericytes can be differentiated from endothelial cells and smooth muscle cells by immunofluorescent staining with antibodies directed against actin isoforms. Endothelial cells were shown to express only nonmuscle actin in stress fibers, whereas smooth muscle cells expressed only muscle actin in stress fibers. In contrast, pericytes were shown to express both muscle and nonmuscle actins in stress fibers. The literature contains additional reports of differential distribution of cytoplasmic and cytoskeletal/contractile proteins in vascular cell types (7–9). However, while the differential distribution of cytoplasmic proteins allows identification of vascular cells *in vitro*, antibodies to these proteins cannot be used as selective agents for purifying cell types as the cells must be fixed and/or permeabilized to make these antigens accessible to antibodies.

In this study we report the characterization of an mAb (3G5) that appears to be a specific marker for pericytes within the microvasculature. The 3G5 mAb has previously been shown to react with brain cells, pancreatic islet cells, adrenal medullary cells, thyroid follicular cells, renal glomerular cells, and peripheral blood T lymphocytes (10, 11). Nonetheless, within the microvasculature, the 3G5 mAb is a unique and useful pericyte marker and a selective agent for enriching these cells.

Materials and Methods

Retinal Cells. Retinal endothelial cells, retinal pigmented epithelial cells, and retinal pericytes were harvested from calf retinal capillaries. Whole retinas were obtained by

This work was supported in part by National Institutes of Health grant EY-05110 (G. L. King), 2R01DK-3208-06 (G. S. Eisenbarth), and by Diabetes Endocrinology Research Center grant DK-36836. R. C. Nayak was supported by fellowship grants from the Juvenile Diabetes Foundation International (386248 and 387261) and currently is supported by NIH grant 1R01 DK-39783-01.

dissection of eyes obtained from a local slaughterhouse and treatment with 0.2% collagenase (CooperBiomedical, Inc., Malvern, PA) in Earle's Balanced salt medium (Irvine Scientific, Santa Ana, CA) for 45 min. They were then homogenized and the homogenate was passed over 210-, 88-, and 53- μ m meshes (Tetko, Elmsford, NY). The material caught by the 53- and 88- μ m meshes was collected and placed in human fibronectin (Collaborative Research, Bedford, MA)-coated tissue culture dishes (Nunc, Roskilde, Denmark) with Dulbecco's modification of Earle's Basal Medium (DMEM)¹ (Irvine Scientific) containing 1% penicillin/streptomycin solution (Irvine Scientific). Initially, the growth of individual cell types was selected using selective growth medium as described below.

Pericytes and Pigmented Epithelial Cells. The growth of retinal capillary pericytes and retinal pigmented epithelial cells from explanted capillary fragments was selected for in medium supplemented with 15% FCS (Gibco Laboratories, Grand Island, NY). Pigmented epithelial cells were identified by electron microscopy and pericytes by morphology using phase-contrast microscopy.

Retinal Endothelial Cells. The growth of retinal endothelial cells from explanted microvessel fragments was selected for in medium supplemented with 10% plasma-derived horse serum (HyClone Laboratories, Logan, UT), 90 μ g/ml heparin (Sigma Chemical Co., St. Louis, MO) and 120 μ g/ml endothelial cell growth factor prepared from bovine brain (12). The endothelial cells were identified by morphology and positive staining with antibody to factor VIII.

Secondary Selection of Cells (Weeding Procedure). After 2–3 d in culture, the attached cells were examined using phase-contrast microscopy and both desired cells and contaminating cells were identified by morphology. Contaminating cells were eliminated by wiping the dish with a sterile cotton swab, followed by washing with calcium- and magnesium-free PBS. This procedure was repeated daily until no contaminating cells were visible and the dishes were confluent.

Brain Cells. Rat brain microvessel pericytes were generously provided by Dr. M. P. Carson (Boston University Medical School) and were isolated as previously described (13).

Endothelial Cells. Aorta were taken from newly slaughtered calves and the intimal layer was treated with 0.25% collagenase for 20 min at 20°C. The released endothelial cells were removed by wiping the intima gently with sterile cotton swabs twice. The swabs were then placed in culture dishes and the endothelial cells were washed out and grown in DMEM supplemented with 5% calf serum and 5% neonatal calf serum. The identity of the endothelial cells was ascertained by cobblestone appearance on morphology. In addition, the cells stained positive for factor VIII antigen and uptake of diacetylated low-density lipoproteins (LDL).

Smooth Muscle Cells. Smooth muscle cells were obtained from the same aorta as the endothelial cells. After removal of the intima, small pieces of the smooth muscle layer (tunica media) were dissected out of the remaining aorta and used to initiate explant cultures for smooth muscle cells. The cells were maintained in DMEM supplemented with 10% FCS. After 2 wk in culture, explanted cells were recovered by trypsinization and maintained in culture.

Coverslips. Plastic coverslips (VWR Scientific, Boston, MA) were sterilized with isopropyl alcohol, rinsed with PBS, and coated with fibronectin before cells were added. All cell types were recovered from culture dishes using 0.25% trypsin in 0.2% EDTA in PBS. They were then grown on coverslips for 2–3 d before staining.

Fluorescence-activated Cell Sorting. Cells to be sorted or analyzed were trypsinized as above and centrifuged for 5 min at 600 g. The cells were washed with DMEM supplemented with 10% FCS and resuspended in 1.0 ml of 3G5 antibody diluted 1:100 in DMEM supplemented with 10% FCS and incubated at 4°C for 30 min before recentrifugation. The pellet was washed twice with PBS and incubated for 30 min at 4°C with 1 ml of fluorescein-conjugated anti-mouse IgG antibody (Tago Inc., Burlingame, CA) diluted 1:20 in DMEM containing 10% FCS. The cells were then washed twice with 1% BSA (Fraction V; Sigma Chemical Co., St. Louis, MO) in PBS, and were resuspended in 1 ml of PBS and sorted or analyzed in an EPICS 752 flow cytometer (Coulter Electronics,

¹ Abbreviation used in this paper: DMEM, Dulbecco's modification of Earle's basal medium.

Hialeah, FL). The 3G5⁺ and 3G5⁻ fractions obtained were plated in 35-mm dishes coated with human fibronectin.

Monoclonal Antibodies. mAbs were produced as previously described (10, 14–16), and prepared for use as an ascites fluid from BALB/c mice. Ascites fluids generally contained 8–14 mg of Ig per ml.

Indirect Immunofluorescence. All reagents were diluted in PBS (pH 7.4) containing 1% BSA. mAbs were diluted 1:100 and fluorescein-conjugated goat anti-mouse IgG (heavy and light chain) (Tago Inc.) was diluted 1:20.

Cells grown on coverslips were washed with PBS–1% BSA three times and incubated with mAb at 20°C for 30 min in a humidified atmosphere. The coverslips were then washed, and incubated with fluoresceinated anti-mouse IgG for 30 min at 20°C. The coverslips were again washed (excess moisture removed by placing the edge of the slip on a paper tissue) and then mounted onto a glass slide with AFT Systems mounting medium (Calbiochem-Behring Corp., La Jolla, CA). The viable cells were then immediately viewed on a Leitz dialux microscope equipped with both phase contrast and fluorescence optics.

Preparation of Rhesus Monkey Retinal Blood Vessels. Trypsinized retinal blood vessels were prepared according to Kuwabara and Cogan (17). Briefly, rhesus monkey retinas were fixed in 4% glutaraldehyde in PBS (pH 7.4) for 30 min at 4°C. The tissue was then transferred to 10% formalin in PBS (pH 7.4) and stored for 1 mo at 4°C. Tissue was cut into 0–5-cm² pieces and washed well with distilled water. The tissue pieces were then placed in 3% trypsin in Tris-buffered saline (pH 7.8) at 37°C for 30–60 min. The digested tissue was then transferred into water and the neuronal cells were shaken out of the tissue, which was then transferred onto glass slides, air dried, and stained by indirect immunofluorescence. Mounted retinal blood vessels prepared as described above were the generous gifts of Mr. T. Schoen and Dr. T. Kuwabara, National Institutes of Health, Bethesda, MD.

Identification of 3G5 Antigen of Capillary Pericytes. 10 plates (100-mm diameter, ~10⁷ cells) of cultured retinal capillary pericytes were harvested by trypsinization, washed in PBS, and homogenized in 3 ml of distilled water. Glycolipids were extracted from the homogenate by the method of Svennerholm and Fredman (18). Briefly, chloroform (C) and methanol (M) were added to the homogenate to give a C/M/H₂O ratio of 4:8:3 and the mixture stirred for 30 min at 20°C. The suspension was centrifuged at 2,000 g for 15 min and the resultant pellet was reextracted (as before). The supernatants were combined and water added to give a C/M/H₂O ratio of 1:2:1.4, which caused a phase partition. The upper methanol/water phase containing the glycolipids was collected and dried by rotary evaporation. The residue was resuspended in C/M 2:1 and one-tenth of the glycolipid extract was applied to each 1-cm lane of an aluminum-backed high-performance thin-layer chromatography (HPTLC) plate (E. M. Science, Cherry Hill, NJ). The TLC plate was developed in an ascending solvent system of C/M/0.25% KCl (aqueous) 5:4:1. When the solvent front reached the top of the plate, the plate was removed and air dried. The lanes of the TLC plate were cut out of the plate, coated with a saturated solution of polyisobutyl methacrylate in hexane (90-s immersion) and air dried. The plates were then immunostained using a modification of the method of Magnani et al. (19). Briefly, the TLC strips were blocked by immersion in PBS containing 3% BSA for 1 h. The lanes were then incubated with either mAb 3G5 or control antibody P3X63 Ag8 at a 1:100 dilution in PBS–3% BSA for 2 h. The strips were washed three times in PBS–3% BSA and then incubated with peroxidase-conjugated rabbit anti-mouse Ig (1:100) (Dako Corp., Santa Barbara, CA). The strips were washed again and then incubated with peroxidase-conjugated swine anti-rabbit Ig (1:100) (Dako Corp.). The color reaction was initiated by addition of 40 mg of 4-chloro-1-naphthol in 50 ml of 50 mM Tris, pH 7.4, containing 0.012% H₂O₂. The mobility of the immunostained band was compared with the mobilities of authentic ganglioside markers purified from bovine brain (Bachem, Inc., Torrance, CA).

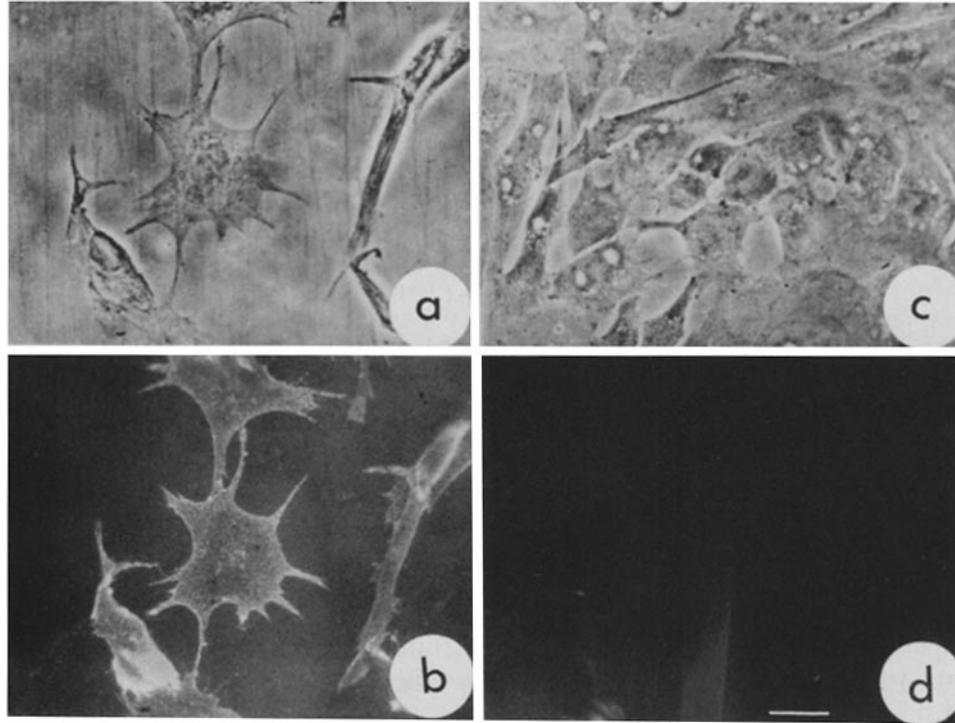


FIGURE 1. Immunofluorescent stain of vascular cells in vitro. (a) Phase-contrast micrograph of bovine retinal pericytes; (b) mAb 3G5 staining of the same field shown in (a); (c) Phase-contrast micrograph of retinal endothelial cells; (d) mAb 3G5 staining of the same field shown in (c). Scale bar = 10 μ m.

Results

Immunostaining of Cells Grown on Coverslips. A panel of mAbs initially collected for their reactivity with pancreatic islet cells, were screened by indirect immunofluorescence for crossreactivity with bovine retinal pericytes. Nine mAbs were screened and only two, 3G5 and HISL-8, were seen to specifically bind to the cell surface of cultured pericytes (in comparison with the control myeloma protein P3X63.Ag8). The mAb 3G5 was selected for further study as the HISL-8 hybridoma line reverted to a nonproducing phenotype. The appearance of 3G5 immunofluorescent staining of bovine retinal capillary pericytes and bovine retinal endothelial cells is shown in Fig. 1. Panel *a* shows the appearance of pericytes grown on coverslips under phase contrast optics and panel *b* is the same field immunofluorescently stained with 3G5. The cells in *a* and *b* showed morphology and growth kinetics typical of pericytes, i.e., they grew very slowly, formed overlapping monolayers at confluence (not shown), and appeared to be large flattened cells which often had dendritic processes (as can be seen in Fig. 1, *a* and *b*). In comparison, mAb 3G5 did not stain bovine retinal endothelial cells (Fig. 1, *c* and *d*). These endothelial cells do stain positively with diacetylated LDL and anti-factor VIII antibodies and do have a typical cobblestone appearance when confluent (not shown). These studies were performed on subconfluent

TABLE I
Summary of mAb 3G5 Binding to Various Vascular Cell Types

| Cell types | 3G5 binding* | P3X63.Ag8 binding |
|-----------------------------------------|--------------|-------------------|
| Bovine retinal capillary pericyte | + | - |
| Rat brain capillary pericyte | + | - |
| Bovine retinal endothelial cells | - | - |
| Bovine retinal pigment epithelial cells | - | - |
| Bovine aortic smooth muscle cells | - | - |
| Bovine aortic endothelial cells | - | - |

* +, Stained cells observed; -, no stained cells observed.

cultures, as confluent cell cultures tended to wash off the coverslips as a sheet (particularly smooth muscle and endothelial cells). The results of immunostaining cells grown on coverslips are shown in Table I. As shown above, bovine retinal capillary pericytes were positively stained with mAb 3G5. Rat brain capillary pericytes were also found to be positively stained with mAb 3G5. In contrast, bovine retinal endothelial cells, aortic endothelial cells, retinal pigment endothelial cells, and aortic smooth muscle cells were all negative for 3G5. All cells were negative for staining with control myeloma protein P3X63.Ag8.

Fluorescence-activated Cell Sorting and Analysis. Trypsinized cells in suspension were stained by indirect immunofluorescence using mAb 3G5 and control myeloma protein P3X63.Ag8. The stained cell suspensions were analyzed by FACS (Fig. 2). The 3G5⁺ cells of pericyte cultures are seen as a broad peak encompassing a large range of fluorescence intensity with a sharp initial peak of negative cells (Fig. 2a). Fig. 2, b, c, and d, show the staining profile of mAb 3G5 on bovine retinal pigmented endothelial cells, bovine retinal endothelial cells, and bovine aortic smooth muscle cells, respectively. Each of these three cell types gave a sharp peak of little or no fluorescence intensity with mAb 3G5, which in each case represented negative staining as the 3G5 stain was identical to the P3X63.Ag8 control staining (not shown). In some cultures of smooth muscle cells a small proportion (~5%) of the cells stained positively with 3G5, probably reflecting contamination with aortic intimal cells which we have found to be 3G5⁺ (our unpublished observation). These results obtained by FACS confirm the fluorescence microscopy results showing specific 3G5 staining of pericytes. These results also suggest that selection of intensely 3G5⁺ cells from retinal microvascular cell cultures will effectively purify pericytes. To evaluate the utility of mAb 3G5 as a means of purifying pericytes, retinal pigmented endothelial cells and retinal capillary pericytes were mixed in a ratio of 9:1, respectively, and cocultured. The mixed culture was then trypsinized, fluorescently labeled with mAb 3G5, and sorted with a FACS. Cells were collected in two fractions; one of high fluorescence intensity and one representing all the remaining cells. The two

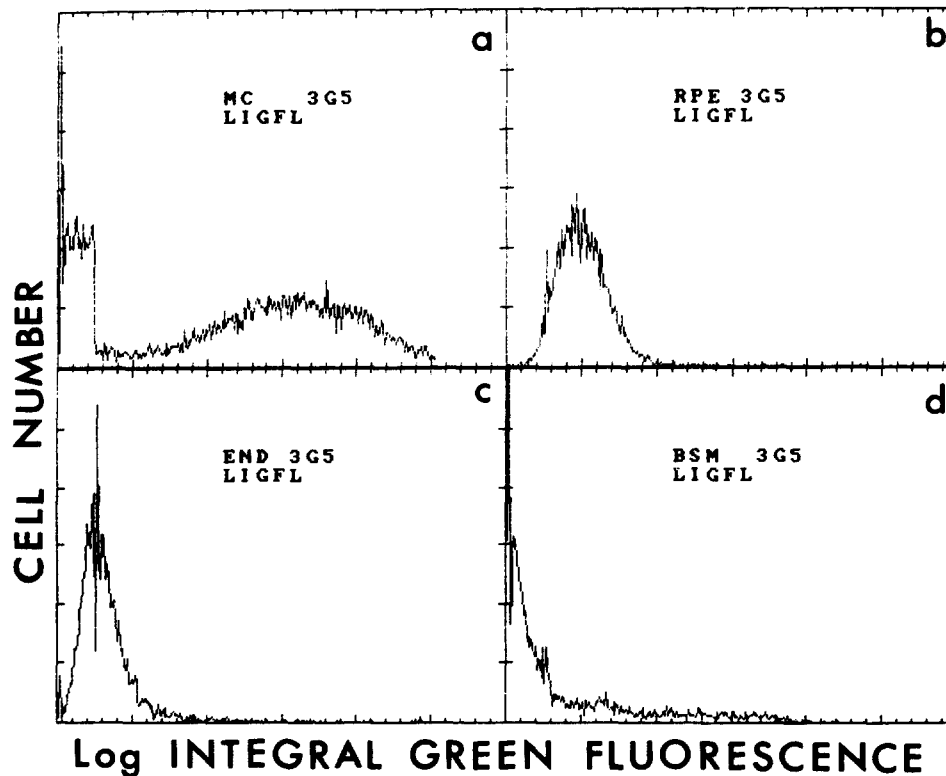


FIGURE 2. Fluorescence-activated flow cytometry of mAb 3G5-stained vascular cells. (a) Bovine retinal pericytes; (b) Bovine retinal pigmented epithelial cells; (c) Bovine retinal endothelial cells; (d) Bovine smooth muscle cells.

fractions were plated and cultured for 1 wk, and the high fluorescence intensity culture was reanalyzed for 3G5 staining by FACS. The large majority of cells in the mixed culture were negative, with only a small tail of 3G5⁺ cells evident (representing ~8% positive cells, Fig. 3a). The reanalysis of cells selected for by 3G5 staining and sorting is shown in Fig. 3b. The shape of the peak is very similar to that seen previously with 3G5-stained pericyte cultures; furthermore, quantitation indicates that >70% of cells expressed the 3G5 antigen as compared with ~8% indicating a greater than nine-fold enrichment over the level in the mixed coculture.

mAb 3G5 Staining of Retinal Capillaries In Vivo. The 3G5 staining pattern on retinal capillaries prepared from the rhesus monkeys is shown in Fig. 4a. The fluorescence was limited to the external margins of the capillary walls and numerous protrusions of the capillary external margins were seen to be intensely stained. This intense staining of protrusions of the capillary external margin is consistent with staining of capillary pericytes *in vivo*. Furthermore, the staining of the capillary external margin was continuous, as would be expected from the known 1:1 distribution of pericytes to endothelial cells in retinal capillaries (20). Control staining with the P3X63.Ag8 myeloma protein is shown in Fig. 4b. Weak, diffuse fluorescence is associated with the microvessels after control

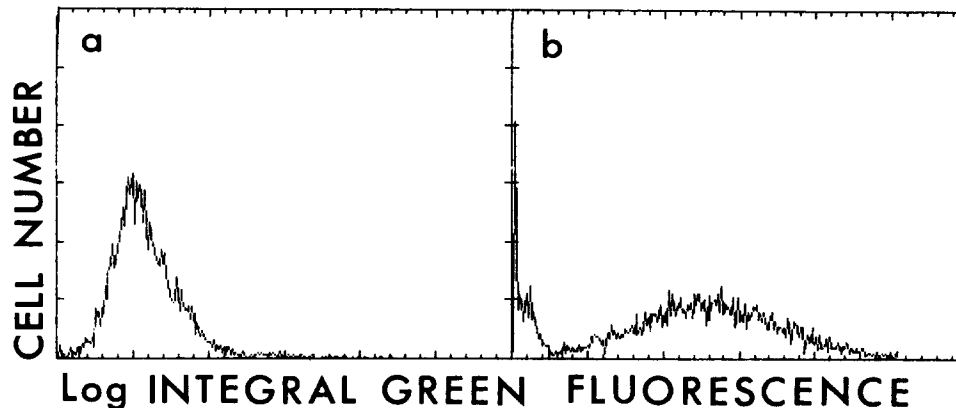


FIGURE 3. Enrichment of 3G5⁺ cells from mixed retinal cell culture by FACS. (a) Cell sorter analysis of 9:1 mixture of retinal pigmented epithelial cells/retinal pericytes; immunofluorescently stained with mAb 3G5. (b) Cell sorter analysis of 3G5⁺ cells selected from a; grown in tissue culture, and restained with mAb 3G5 before reanalysis.

staining. Control antibody-stained preparations showed more intense, but diffuse staining of erythrocytes within the microvessels. Attempts to stain the endothelium with antibodies to factor VIII gave identical staining to that seen with P3X63.Ag8 antibodies, presumably due to denaturation of factor VIII antigen by glutaraldehyde fixation and/or trypsinization.

Identification of the 3G5 Antigen of Retinal Pericytes. The 3G5 antigen of cultured pericytes migrated on TLC as a single band below the GM2 ganglioside marker and above the GM1 ganglioside marker (Fig. 5, lane 2). In contrast, no band was seen in the lane incubated with the P3X63.Ag8 myeloma protein (Fig. 5, lane 1). The pericytes 3G5 antigen has the same migration on TLC as the antigen found in pancreas and T lymphocytes (11, 21) and comigrates with one of several 3G5 antigens found in brain extracts (21).

Discussion

An mAb (3G5) to pancreatic islet cells, which crossreacts with bovine retinal capillary pericytes, has been identified by staining the cell surface of viable pericytes using indirect immunofluorescence. The staining is specific for pericytes, within the context of the microvasculature, as the antibody did not detectably bind to endothelial cells, retinal pigmented epithelial cells, or aortic medial smooth muscle cells. Furthermore, mAb 3G5 stained retinal capillaries *in vivo* with a fluorescence distribution consistent with pericyte staining. The prominent staining of protrusions of the capillary wall is similar to the description of pericyte staining in periodic acid Schiff (PAS)-stained capillary preparations of the retina (22), and the apparently continuous staining of the external capillary wall may be expected in view of the recent report of extensive pericyte coverage of the endothelial cell tube in retinal capillaries (23). The pattern of staining observed with mAb 3G5 is due to specific binding of the antibody, as similar staining could not be obtained using the control antibody P3X63.Ag8. Attempts to stain these preparations with antibodies to factor VIII (not shown) gave the same diffuse nonspecific staining pattern observed with P3X63.Ag8 myeloma

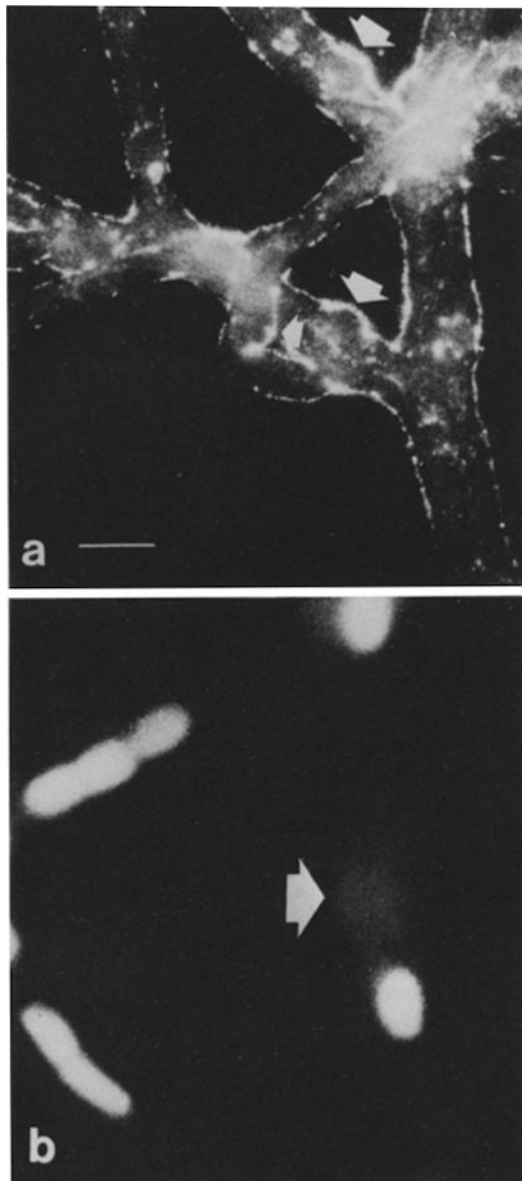


FIGURE 4. mAb 3G5 staining of retinal microvessels in vivo. (a) Distribution of mAb 3G5 stain on trypsin-digested preparations of rhesus monkey retinal microvessels. Arrows indicate stained protrusions consistent with binding to pericytes. Scale bar = 10 μ m. (b) Control (P3X63.Ag8 myeloma protein) staining on trypsin-digested preparations of rhesus monkey retinal microvessels arrow indicates putative pericyte.

protein. As these retinal microvessel preparations are fixed with glutaraldehyde and trypsinized to release the neurons that obscure the vessels, the factor VIII antigen and other glutaraldehyde/trypsin-sensitive protein antigens will have been destroyed, thus explaining the absence of specific anti-factor VIII staining. The 3G5 antigen, however, is not a protein and is therefore glutaraldehyde/trypsin-resistant and consequently maintained in these preparations. The pericyte 3G5 antigen was demonstrated to be a glycolipid antigen that migrates between GM2 and GM1 gangliosides on TLC. This antigen is insensitive to the action of trypsin and, therefore, the mAb was used to stain viable trypsinized

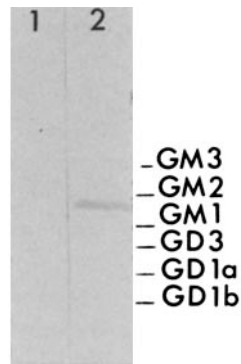


FIGURE 5. Identification of the 3G5 antigen of tissue-cultured bovine retinal pericytes. (Lane 1) Pericyte glycolipid extract chromatographed by TLC and stained with P3X63.Ag8 myeloma protein; (lane 2) pericyte glycolipid extract chromatographed by TLC and stained with mAb3G5. The position of authentic ganglioside markers is shown to the right of lane 2. The ganglioside nomenclature is that of Svennerholm (39).

cells. The mAb 3G5 was seen to stain pericyte cultures with a broad range of fluorescence intensity, which at its lowest overlaps the profile of negative cells. These 3G5⁻ pericytes are, as yet, otherwise unidentified but are most probably contaminating negative cells, as it would be expected to take more than one cycle of cell sorting to purify a cell type to homogeneity. Another possible factor that may explain the presence of 3G5⁻ pericytes is the possible downregulation of antigen expression during particular stages of the cell cycle. We are currently investigating the possible cell cycle regulation of 3G5 antigen in microvascular pericytes. These results indicate that mAb 3G5 may be a useful marker of microvascular pericytes. However, we have only, thus far, studied pericytes from central nervous system tissue. Consequently it remains to be ascertained whether this antibody will specifically stain microvascular pericytes of extraneural tissues. Nonetheless, using immunofluorescently stained trypsinized cells, we have shown that mAb 3G5 can be effectively used to selectively enrich pericytes from a mixed population of retinal cells using a FACS. We have not as yet attempted to enrich pericytes from mixtures with endothelial cells; however, as endothelial cells may be expected to be the major contaminant of pericyte cultures, cell sorting with mAb 3G5 may be of greatest use in this context. This antibody may also be of use in depleting pericytes from vascular cell cultures by complement-mediated cytotoxicity, as it is an IgM class antibody and consequently fixes complement, or alternatively, by the production of mAb 3G5-conjugated toxins.

The embryonic origin of pericytes remains essentially unknown, although it has been suggested that mesenchyme-derived cells form the pericytes of capillaries in amphibian larvae (24). This conclusion was based on continuous observation of cellular migration in the translucent tails of tadpoles. Investigations using biochemical markers in studies of development of capillary pericytes have not been reported. The availability of mAbs will enable immunohistochemical studies to further define the embryonic origin of pericytes and their differentiation program. It has been suggested that the pericyte is a relatively undifferentiated cell type that has pluripotential properties. Pericyte pluripotentiality has been suggested on the basis of expression of both muscle and nonmuscle actins (6) and the observation that a small population of infantile hemangiopericytoma cells appear to transform into cell types other than pericytes (25). The tissue distribution of the 3G5 antigen outside of the vascular system suggests that mAb

3G5 may be considered as a neuroendocrine cell marker (10), although it does react with nonneuroendocrine cell types as well (11). The 3G5-reactive cells include cells of the brain, pancreatic islet cells, adrenal medullary cells, thyroid follicular cells, renal glomerular cells, and T-lymphocytes. Clearly the 3G5 antigen is expressed on terminally differentiated cell types and is probably a differentiation antigen of the "jumping type" as defined by Springer (26). The relationship of pericytes to smooth muscle cells is also brought into question, as pericytes have been shown to express muscle type contractile proteins but the 3G5 antigen is not found on either smooth or skeletal muscle (10). Further studies pursuing our observation (unpublished) that aortic intimal cells, but not medial cells, express the 3G5 antigen may enable the question of the relationship of smooth muscle cells to pericytes to be addressed more fully, as the pericyte has been considered by some authors to be similar to the so called "myointimal cell" (27) and the intermediate cell of Robertson (28) and Page et al. (29). These cells were considered to be dedifferentiated smooth muscle cells or to be derived by growth from smooth muscle cells of the tunica media (1). These studies may be of some functional significance, as the pericyte has been reported to have contractile capabilities and thus a role in capillary contraction (30). This, however, remains a controversial conclusion.

The 3G5 antigen of pericytes was found to be a glycolipid of mobility intermediate between the ganglioside markers GM2 and GM1. This mobility is identical to that observed of both the T lymphocyte (11) and pancreatic islet cell antigen (21). In the brain, however, mAb 3G5 binds to at least five glycolipid bands on TLC including the high-mobility band found in pancreatic islets, pericytes, and T lymphocytes (21). The available evidence indicates that multiple forms of the 3G5 antigen exist only in the brain and that the high-mobility 3G5 antigen of brain is expressed in extraneural tissue. Clearly then, the expression of the 3G5 epitope is differentially regulated, suggesting that a more complex pattern of expression of enzymes involved in the biosynthesis of the 3G5 glycolipid antigen exists in the brain than in extraneural tissues. The function of the 3G5 antigen is unknown, but gangliosides have been shown to have roles in cellular recognition and as onco-developmental antigens (31), as modulators of receptor phosphorylation (32), and as targets of autoimmunity (21, 33, 34). Furthermore, a ganglioside of similar mobility has been shown to be distributed in a dorso-ventral gradient in the developing rat retina, suggesting a role in the establishment of visual system topography (35).

The 3G5 mAb may also be of use in facilitating the unambiguous demonstration and quantitation of pericyte loss reported to occur in both human and canine diabetes (20, 36–38) through development of double-staining procedures using mAb 3G5 and anti-endothelial cell antibodies. This approach may also enable accurate quantitation of endothelial cell/pericyte ratios in various capillary beds.

In conclusion, mAb 3G5 is potentially an excellent tool for studying both the differentiation and function of microvascular pericytes, and the functional role of the 3G5 cell surface ganglioside.

Summary

The identification of microvascular pericytes *in vitro* relies principally on morphological characteristics and growth dynamics, as there is a paucity of immunochemical markers for these cells. Consequently, an attempt was made to identify mAb reagents that would aid in both the rapid identification and enrichment of retinal capillary pericytes in vascular cell cultures.

A panel of mAbs raised by xenogeneic immunization of mice with various tissues was screened for immunoreactivity with dissociated cultures of bovine retinal capillary pericytes. Two antibodies from the panel (3G5 and HISL-8) were seen to react with pericytes by indirect immunofluorescence. The mAb 3G5 was selected for further study. mAb 3G5 did not react with dissociated cultures of smooth muscle cells, endothelial cells, or retinal pigmented endothelial cells. The pericyte 3G5 antigen was insensitive to the action of trypsin; therefore, mAb 3G5 was used to selectively purify pericytes from trypsinized mixed retinal cell cultures by flow cytometry. 3G5⁺ pericytes (representing 8% of cells in a mixed retinal cell culture) were enriched at least nine-fold to represent >70% of cells. The mAb 3G5 stained retinal capillaries *in vivo* with a fluorescence distribution consistent with pericyte staining. The 3G5 antigen of cultured pericytes was found to be a glycolipid of mobility intermediate between ganglioside markers GM1 and GM2.

We thank Mr. Daniel Rosenberg and Ms. Terri-Lyn Bellman for excellent secretarial assistance. We are indebted to Dr. T. Kuwabara and Mr. T. Schoen (National Eye Institute) and Dr. M. P. Carson (Boston University Medical School) for generously providing retinal capillary preparations and brain microvessel pericyte cultures, respectively.

Received for publication 6 October 1987 and in revised form 23 November 1987.

References

1. Rhodin, J. A. G. 1968. Ultrastructure of mammalian venous capillaries, venules and small collecting veins. *J. Ultrastruct. Res.* 25:452.
2. Buzney, S. M., S. J. Massicotte, N. Hetu, and B. R. Zetter. 1983. Retinal vascular endothelial cells and pericytes. Differential growth characteristic *in vitro*. *Invest. Ophthalmol. & Visual Sci.* 24:470.
3. Cohen, M. P., R. N. Frank, and A. A. Khalifa. 1980. Collagen production by cultured retinal capillary pericytes. *Invest. Ophthalmol. & Visual Sci.* 19:90.
4. Frank, R. N., V. E. Kinsey, K. W. Frank, K. P. Mikus, and A. Randolph. 1979. *In vitro* proliferation of endothelial cells from kitten retinal capillaries. *Invest. Ophthalmol. & Visual Sci.* 18:1195.
5. Gitlin, J. D., and P. A. D'Amore. 1983. Culture of retinal capillary cells using selective growth media. *Microvasc. Res.* 26:74.
6. Herman, I. M., and P. A. D'Amore. 1985. Microvascular pericytes contain muscle and non-muscle actins. *J. Cell Biol.* 101:43.
7. Joyce, N. C., P. Decamilli, and J. Boyles. 1984. Pericytes, like vascular smooth muscle, contain high levels of cyclic GMP-dependent protein kinase. *Microvasc. Res.* 28:206.
8. Joyce, N. C., M. F. Haire, and G. F. Palade. 1985. Contractile proteins in pericytes. I. Immunoperoxidase localization of Tropomyosin. *J. Cell Biol.* 100:1379.
9. Joyce, N. C., M. F. Haire, and G. F. Palade. 1985. Contractile proteins of pericytes.

- II. Immunocytochemical evidence for the presence of two isomyosins in graded concentrations. *J. Cell Biol.* 100:1387.
10. Powers, A. C., A. Rabizadeh, R. Akesson, and G. S. Eisenbarth. 1984. Characterization of monoclonal antibody 3G5 and utilization of this antibody to immobilize pancreatic islet cell gangliosides in a solid phase radioassay. *Endocrinology.* 114:1338.
 11. Rabinowe, S. L., R. C. Nayak, K. Krisch, K. L. George, and G. S. Eisenbarth. 1987. Aging in man: linear increase of a novel T-cell subset defined by antiganglioside monoclonal antibody 3G5. *J. Exp. Med.* 165:1436.
 12. Maciag, T., J. Cerundolo, S. Ilsley, P. R. Kelley, and R. Forand. 1979. An endothelial cell growth factor from bovine hypothalamus: identification and partial characterization. *Proc. Natl. Acad. Sci. USA.* 76:5674.
 13. Carson, M. P., and C. C. Handenschild. 1986. Microvascular endothelium and pericytes: high yield, low passage cultures. *In Vitro Cell Dev. Biol.* 22:344.
 14. Eisenbarth, G. S., H. Oia, A. F. Gazdar, W. L. Chick, J. A. Schultz, and R. M. Searce. 1981. Production of monoclonal antibodies reacting with rat islet cell membrane antigens. *Diabetes.* 30:226.
 15. Srikanta, S., and G. S. Eisenbarth. 1986. Islet cell antigens: initial studies of their biology and function. *Mol. Biol. & Med.* 3:113.
 16. Srikanta, S., M. Telen, J. T. Posillico, R. Dolinar, K. Krisch, B. F. Haynes, and G. S. Eisenbarth. 1987. Monoclonal antibodies to a human islet cell surface glycoprotein: 4F2 and LC7-2. *Endocrinology.* 120:2240.
 17. Kuwabara, T., and D. G. Cogan. 1960. Studies of retinal vascular patterns. Part 1. Normal Architecture. *Arch. Ophthalmol.* 64:904.
 18. Svennerholm, L., and P. Fredman. 1980. A procedure for the quantitative isolation of brain gangliosides. *Biochim. Biophys. Acta.* 617:97.
 19. Magnani, J. L., M. Brockhaus, D. F. Smith, and V. Ginsburg. 1982. Detection of glycolipid ligands by direct binding of carbohydrate binding proteins to thin layer chromatograms. *Methods Enzymol.* 83:235.
 20. Cogan, D. G., and T. Kuwabara. 1967. The mural cell in perspective. *Arch. Ophthalmol.* 78:133.
 21. Nayak, R. C., P. G. Colman, and G. S. Eisenbarth. 1987. How are monoclonal antibodies related to autoimmune serology. *In Endocrine and Other Organ-oriented Autoimmune Disorders.* Bailliere's Clinical Immunology and Allergy. Vol. 1. D. Doniach and G. F. Battazzo, editors. 81-99.
 22. Kuwabara, T., and D. G. Cogan. 1963. Retinal vascular patterns. VI. Mural cells of the retinal capillaries. *Arch. Ophthalmol.* 69:492.
 23. Frank, R. N., S. Dutta, and M. A. Mancini. 1987. Pericyte coverage is greater in the retinal than in the cerebral capillaries of the rat. *Invest. Ophthalmol. & Visual Sci.* 28:1086.
 24. Clark, E. R., and E. L. Clark. 1925. The development of adventitial (Rouget) cells on the blood capillaries of amphibian larvae. *Am. J. Anat.* 35:239.
 25. Eimoto, T. 1977. Ultrastructure of an infantile hemangiopericytoma. *Cancer (Phila.).* 40:2161.
 26. Springer, T. A. 1980. Cell surface differentiation in the mouse: characterization of "jumping" and "lineage" antigens using xenogeneic rat monoclonal antibodies. *In Monoclonal Antibodies. Hybridomas: A New Dimension in Biological Analyses.* R. H. Kennet, T. J. McKearn, and K. B. Bechtol, editor. Plenum Press, New York. 185-217.
 27. Buck, R. C. 1961. Intimal thickening after ligation of arteries: an electron microscopic study. *Circulation Res.* 9:418.

28. Robertson, A. L. 1968. Oxygen requirement of the human arterial intima in atherogenesis. *Prog. Biochem. Pharmacol.* 4:305.
29. Page, I. H., J. G. Green, and A. L. Robertson. 1966. The physician's incomplete guide to atherosclerosis. *Ann. Intern. Med.* 64:189.
30. Tilton, R. G., C. Kilo, J. R. Williamson, and D. W. Murch. 1979. Differences in pericyte contractile function in rat cardiac and skeletal muscle microvasculatures. *Microvasc. Res.* 18:336.
31. Hakomori, S.-I. 1981. Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. *Annu. Rev. Biochem.* 50:733.
32. Hanai, N., G. Nores, and S.-I. Hakomori. 1987. Presence of lysoglycosphingolipids in A431 cells: their inhibition of tyrosine phosphorylation of epidermal growth factor (EGF) receptor. *Fed. Proc.* 46:1926. (Abstr.)
33. Nayak, R. C., M. A. K. Omar, A. Rabizadeh, S. Srikanta, and G. S. Eisenbarth. 1985. "Cytoplasmic" islet cell antibodies: evidence that the target antigen is a sialoglyconjugate. *Diabetes.* 34:617.
34. Nayak, R. C., P. G. Colman, P. A. Halban, and G. S. Eisenbarth. 1987. Identification and purification of islet ganglioside antigen 3G5 and islet cell autoantigen from human pancreas. *Diabetes.* 36(Suppl. 1):64. (Abstr.)
35. Constantine-Paton, M., A. S. Blum, R. Mendez-Otero, and C. J. Barnstable. 1987. A cell surface molecule distributed in a dorsoventral gradient in the perinatal rat retina. *Nature (Lond.)*. 324:458; and *Nature (Lond.)*. 325:284 (Erratum).
36. Cogan, D. G., D. Toussaint, and T. Kuwabara. 1961. Retinal vascular patterns. IV. Diabetic retinopathy. *Arch. Ophthalmol.* 66:366.
37. Engerman, R., J. M. B. Bloodworth, and S. Nelson. 1977. Relationship of microvascular disease in diabetes to metabolic control. *Diabetes.* 26:760.
38. Speiser, P., A. M. Gittelsohn, and A. Patz. 1968. Studies on diabetic retinopathy. III. Influence of diabetes on intramural pericytes. *Arch. Ophthalmol.* 80:332.
39. Svennerholm, L. 1963. Chromatographic separation of human brain gangliosides. *J. Neurochem.* 10:613.