

## CULTURED ENDOTHELIAL CELLS FROM LYMPHATICS OF NUDE MICE PARASITIZED BY *BRUGIA MALAYI*

B.H. Kwa, R. Buck, J.K. Nayar, A.C. Vickery

University of South Florida College of Public Health (BHK, RB, ACV), Tampa, Florida and IFAS, Florida Entomology Laboratory (JKN), University of Florida, Vero Beach, Florida, USA

### ABSTRACT

*Endothelial cells from dilated inguinal lymphatics of congenitally athymic nude mice, parasitized by adult Brugia malayi, were placed in culture. Cells formed a loose monolayer and exhibited a typical cobblestone appearance. When microfilariae were present in cultures, they frequently appeared to be attached to the monolayer by one end. Approximately 75% of the primary explant cells were positive for Factor VIII-associated antigen, comparable to bovine artery endothelial cells used as a control. With few exceptions, cultures were uncontaminated with fibroblasts or other non-endothelial cell types. Large granular cells with characteristics of mononuclear/macrophage cells appeared in long term and unpassaged cultures. Cells remained viable in culture for an average of 60 days, 5 to 6 passages, before becoming highly vacuolated and assuming a rounded configuration. Viability of the cells was dependent upon heparin, serum and endothelial cell growth factor.*

*The propensity of the lymphatics of nude mice to become greatly dilated in the presence of viable adult worms of B. malayi will prove to be important not only for the study of the effects of the parasite and its products upon endothelial cells, but also because a source of murine lymphatic endothelial cells can be readily available for functional studies.*

Despite extensive study, the patho-

genesis of filarial lymphedema remains poorly understood. Previously we demonstrated that congenitally athymic nude mice can be parasitized by *Brugia* larvae with later maturation of adult worms in the peripheral lymphatic system (1). Because vascular endothelium is closely linked to both cellular immunity and inflammation, processes associated with lymphatic dysfunction, we have attempted to culture endothelial cells obtained from limb lymphatics of male nude mice infected 6-7 months earlier with *Brugia malayi* larvae (L3).

### MATERIALS AND METHODS

Male nude C3H/HEN mice, inoculated approximately 200 days previously (1) with 50 L3 of *B. malayi* subcutaneously into the inguinal region, and which exhibited dilated lymphatics afferent to the superficial inguinal nodes, were sacrificed by cervical dislocation. Nodes and associated lymphatics were removed aseptically into PBS for removal of fat, connective tissue, microfilariae (Mf) and worms. Lymphatics were placed in HEPES-buffered Medium 199 (Gibco) containing 5% fetal bovine serum, antibiotics (10000 units/ml penicillin; 10mg/ml streptomycin) and nonessential amino acids, then teased apart and dispersed with collagenase (230 units/ml) for 10 min at room temperature. These primary explant cells were washed into conical centrifuge tubes and washed 3x (10 min

at 620g). Pelleted cells were suspended in Medium 199 as above, with 20% fetal bovine serum and supplemented with heparin (156 units/ml; Gibco) and endothelial cell growth factor (3mg/ml, Sigma) then placed in one well of a 24-well plate for incubation at 37°C in 5% CO<sub>2</sub> in air. The medium was changed at two day intervals to remove Mf if present, from culture wells. CCL 209 Bovine pulmonary artery endothelial cells (AtCC, USA) were similarly cultured. To split cultures for seeding additional wells or chamber slides, cells were treated with trypsin-EDTA for 10 min.

For examination of Factor VIII positivity (2), lymphatic and 209 cells were transferred to and grown for 10 days in chamber slides. Cultures were washed with PBS, fixed for 5 min with cold 70/30% acetone/methanol, and washed 4x in PBS. Rabbit anti-human Factor VIII antibody (Behring diagnostics) and normal rabbit serum (Miles) were diluted 1:20 in PBS and incubated on samples for 30 min at room temperature. Cells were washed 5x with PBS, then a 1:40 or 1:80 dilution of goat anti-rabbit IgG-FITC (Miles) in PBS was added for a further 30 min incubation. Cells were washed as before and mounted with 50% glycerol in PBS. Duplicate chamber slide cultures of primary explant and 209 cells were fixed and stained with H&E. Cells were examined for the presence or absence of fluorescence using a Leitz fluorescence microscope. For histochemical studies, cell cultures were fixed *in situ* in cold acetone/ethyl alcohol at a 1:1 ratio for 1 min. Cells were stained for the presence of B-glucuronidase, nonspecific esterase (3) and B-galactosidase (4). Detection of Ia cell surface antigen was achieved using the anti-Ia antibody (HLA-DR Avidin-Biotin) procedure (Accurate Chemical).

## RESULTS AND DISCUSSION

Endothelial cells from primary explant cultures of lymphatics from nude mice, dilated due to the presence of adult *B. malayi*, were successfully cultured for periods of up to 60 days. As previously



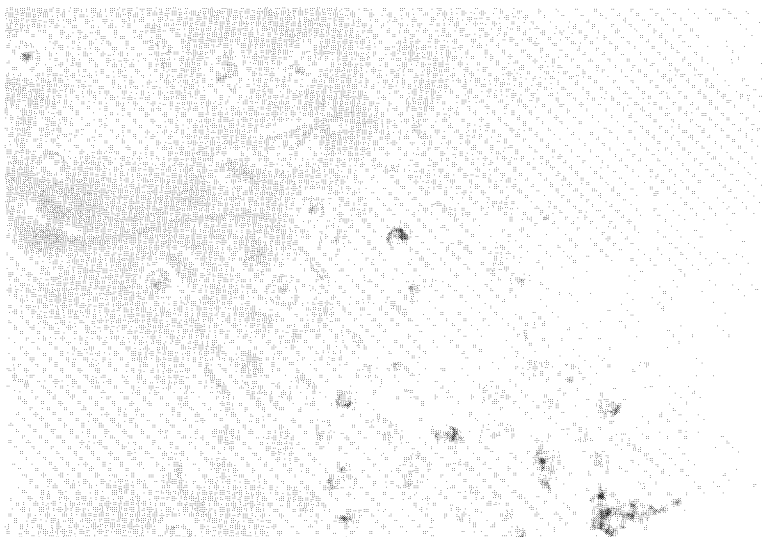
Fig. 1. Factor VIII positive stain on the primary explant endothelial cells from a nude mouse dilated lymphatic.

reported (5), lymphatics were dilated only where worms were found and proximal to worm location. In this study, worms and dilatations were found in lymphatics afferent to the superficial inguinal nodes, due to the original site of larval inoculation into the groin. Mice exhibited microfilaremia as high as 2000/2 $\mu$ l, and Mf were found in the dilated lymphatics. In all cases, both male and female worms were removed from the lymphatic at harvest.

The massive dilation of parasitized lymphatics allowed virtually pure cultures of endothelial cells to be obtained. To date, this has not been possible in the normal mouse due to the very small size of peripheral lymphatics.

Although cells from parasitized lymphatics have been bathed in lymph with a high protein content (5) containing worm products and strictly speaking should not be considered normal, our limited comparison to bovine artery endothelial cells suggests that basic characteristics of these cells are those of conventional endothelial cells.

Endothelial cells were Factor VIII positive, with 70-80% positivity being the rule (Fig. 1). This was fully comparable to the positivity rate of the 209 cells (not shown). Positive fluorescence was present in a granular pattern in close proximity to the nucleus. The primary cells formed monolayers which exhibited a typical cobblestone appearance (Fig. 2), similar to the bovine cells (Fig. 3). Cells from the



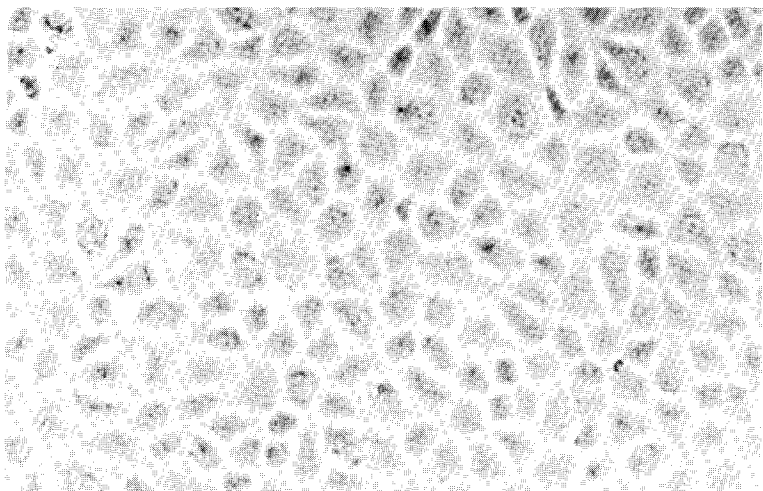
*Fig. 2. Loose monolayer of primary endothelial cells from a lymphatic of a nude mouse parasitized by *B. malayi*, on day 30 of culture; phase contrast (40x).*

nude mice rarely grew to confluence. Fibronectin coating of the plate substrate was not necessary for firm attachment of cells from either source to occur.

Viability of the lymphatic endothelial cells was dependent upon the addition of heparin and endothelial cell growth factor to the culture medium. Under these optimal conditions, cells remained viable with normal morphology for ap-

proximately 2 months, at which time they became vacuolated and rounded and detached from the substrate. The primary cells multiplied relatively slowly and could be split 1:2 every two weeks compared to the 209 cells which were split 1:5 at weekly intervals. Primary cells which were split at biweekly intervals underwent 3-4 passages before showing vacuolation.

An aliquot of primary cells was not



*Fig. 3. Monolayer of CCL 209 bovine pulmonary artery endothelial cells on day 30 of culture, phase contrast (40x).*



Fig. 4. Granular cells from long-term primary endothelial cell culture stained positive for non-specific esterase (10x).

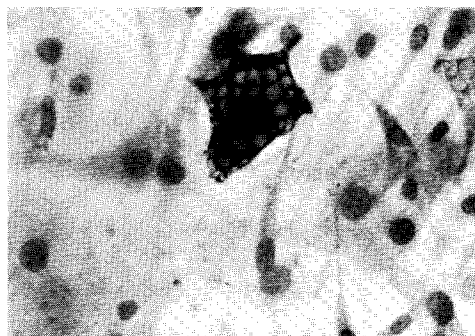


Fig. 5. Granular cell from long-term primary murine endothelial cell culture stained positive for B-glucuronidase (10x).

split and was maintained in culture for observation of cellular morphology. In contrast to the cells which were passaged at biweekly intervals, these cultures sustained the appearance of large granular-appearing cells dispersed among the morphologically normal endothelial cells. Staining of these cultures revealed that the large granular cells were positive for Ia surface antigen (not shown), nonspecific esterase (Fig. 4), B-glucuronidase (Fig. 5) and B-galactosidase (not shown). This suggests that these cells are likely to be of the monocyte/macrophage lineage. The origin and significance of these cells remains unknown, although similar cells have been observed in smears of lymph from parasitized nude mice (unpublished observation).

To our knowledge, this is the first description of cultured lymphatic endothelial cells from nude mice. Endothelial cells are intimately related to the function of the immune and inflammatory systems (6) and it remains to be seen whether cells from congenitally athymic animals are capable of normal function. Culture of these cells will certainly allow the study of potential roles played by filarial products on endothelial cell function as suggested by others (7). Such studies are in progress.

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Ann C. Vickery, Ph.D.  
University of South Florida  
College of Public Health  
13301 Bruce B. Downs Blvd.  
Tampa, Florida 33612-3899 USA