Brief Report

Pinocytosis as a Select Marker of Ramified Microglia
In Vivo and In Vitro

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Based on previous observations in tissue culture, we investigated pinocytic activity as a potential cell marker for brain microglia. This functional activity was assessed in three different preparations derived from rat: primary cultures of mixed cerebral cortical cells, tissue slabs of whole cerebrum, and cultures of isolated or enriched microglial cells. Each preparation was incubated with the fluorescent dye lucifer yellow as a soluble tracer and then processed for light microscopy. Under the conditions utilized, ramified microglia specifically exhibited differentially high pinocytotic labeling in all cases; the dye was mainly localized within the cell somata, where it was sequestered in pinocytic vesicles. In each preparation, the identity of the labeled cell population was confirmed as microglia through immunohistochemical staining with the monoclonal antibody (MAb) OX-42, a specific microglial marker. Therefore, pinocytotic labeling is proposed as a select cell marker for microglia, which may be extremely useful in the identification and study of ramified microglial cells. (J Histochem Cytochem 39:853–858, 1991)

Key Words: Microglia; Macrophages; Cerebral cortex; Rat; Tissue culture; Pinocytosis; Cell marker.

Introduction

Although the functional role of central nervous system (CNS) microglial cells has been highly debated for some time, increasing recent evidence supports the view that these cells are in fact macrophages. This evidence includes the demonstration of the macrophage markers Fe receptors (1,2), CR3 receptors (2,3), and intercellular adhesion molecule-1 (4–6), in microglia, while other studies indicate that these cells are derived from mononuclear cells of the bone marrow (2,7) and are capable of tumor cytotoxicity and antigen presentation for lymphocyte activation (7,8). It has also been shown that certain colony-stimulating factors (CSF) are mitogenic to microglia, particularly granulocyte/macrophage-CSF (9) and macrophage-CSF (10). In addition, several laboratories have reported the expression of major histocompatibility complex (MHC) antigens, both class I and class II (Ia), by microglial cells (11–15). Therefore, there is significant evidence that this cell type is a component of the mononuclear phagocyte system within the CNS. Because of their proposed role as macrophages and their potential involvement in immune processes, the microglial cells have received considerable recent attention.

Along with the increasing interest in microglia, there has also been realized the need for clear and unambiguous cell markers for their identification. This need is further amplified by two salient features of these cells: (a) they exhibit enormous morphological plasticity (16), and (b) ramified microglia are highly down-regulated or inactive, lacking most cell markers and properties of active macrophages (17). Hence, ramified or "resting" microglial cells, the form present in normal adult brain tissue, can dramatically change in overall morphology, and appear to convert into active macrophages (i.e., reactive microglia) (16,18,19). Histochemical staining for phosphatase enzymes has traditionally been used to identify microglia (20,21), and recent studies have employed immunohistochemistry with monoclonal antibodies (MAb) against Ia antigen (27,28) and other cell types, including endothelial cells, ependyma, and other glia. Others, such as Ia antigen and many of the MAb to macrophage-specific components, are highly species specific and do not label the ramified form of microglial cells very well. One MAb that has been particularly useful is OX-42, which recognizes...
CR3 receptors in rat (30). Because CR3 receptors are present in ramified microglia, the antibody labels this cell form; however, the OX-42 labeling is quite variable among individual cells, apparently reflecting differing levels of CR3 receptor expression, and not all ramified cells exhibit labeling (3). In addition, one marker, alpha-1-antichymotrypsin, has been suggested not to reliably identify macrophages or reactive microglia in all instances (23,31). Therefore, although many procedures presently exist for identification of microglial cells, the study of this cell type can be further assisted by the acquisition of additional cell markers. This is especially significant for ramified cells, which lack or have low levels of expression of the antigens recognized by many of the macrophage-specific MAb.

In continuing overall studies of specific brain cell types in tissue culture, our laboratory has recently identified ramified microglia in a primary culture system containing mixed cerebral cortical cells from rat (32). In investigating these cells, a select marker compatible with cell viability was desired for observations over time; all of the existing procedures either require fixation or have a detrimental effect on function and viability. It was quickly determined that the ramified cells possessed a differentially high level of pinocytotic activity in comparison with all the other cell types present in these cultures (33). The present report demonstrates that pinocytosis can be used to readily label ramified microglial cells, and that this differential activity is useful as a select marker for their identification. Selective pinocytotic labeling of ramified cells in situ, as well as under various conditions in vitro, is shown.

Materials and Methods

Tissue Culture Techniques. Primary cultures of dissociated cerebral cortical cells from rat were prepared as described previously (34,35). Briefly, cortical tissue was removed from both cerebral hemispheres of fetal animals (gestational day 14–17) taken directly from the uterus of timed-pregnant rats (Sprague-Dawley) under sterile conditions. The tissue was dissociated by trituration and cells were plated at an approximate density of 5 x 10⁶ cells/cm² in 35-mm tissue culture dishes. A standard growth medium of supplemented Eagle's minimal essential medium (MEM) was used in all cases (35). These dissociated cortical cells follow a characteristic pattern of proliferation and growth in culture, and by 2 weeks display a highly mature appearance (34). Such cultures exhibit a confluent background layer composed of non-neuronal cell types (including astrocytes, oligodendrocytes, and ependyma); neurons and ramified microglia (18,32) reside largely on the surface of the confluent background layer. These cultures contain all of the major cell classes found in adult rat cortex; all mixed cortical cultures used in the present study were at least 2 weeks old.

In some instances, cultures of isolated microglial cells were prepared. For this purpose, cerebral cortical cells were obtained from neonates (within 24 hr after birth) to eliminate growth of neurons (36) and were cultivated in 75-cm² flasks. After 2 weeks, flask cultures contained an abundant population of ramified microglia adhering to the surface of the background monolayer, which were then isolated by orbital shaking as described by others (6,10) and replated in fresh culture dishes. Within 2–3 days after replating, microglia regained their former ramified appearance and remained stable and healthy thereafter. These isolated microglia cultures will be described in detail elsewhere (W.E. Thomas, manuscript in preparation); however, they contain greater than 90% ramified cells.

Tissue Sectioning. Young adult Sprague-Dawley rats were sacrificed by decapitation, the entire brain rapidly removed, and coronal slabs of 3–5-mm thickness prepared from the whole cerebrum. These fresh tissue slabs were incubated with lucifer yellow dye as described below, then rinsed extensively in PBS (pH 7.4) and fixed overnight at 4°C (same fixative as below). Tissue blocks several millimeters wide were cut from the cerebral cortex of fixed slabs; blocks were sucrose-infiltrated, frozen, and cryostat-sectioned at 20–40 μm in the coronal plane. Sections were collected on gelatinized slides and overlapped in glycerol; to assess cell labeling, they were viewed with epifluorescence microscopy under the appropriate wavelength parameters.

Cell Labeling Procedures. The fluorescent dye lucifer yellow was used here in all investigations of pinocytosis; however, other soluble dyes and tracers worked as well. The dye was prepared at 0.3% in MEM with 17 mM glucose. Cultures, of both mixed cortical cells and isolated microglia, and tissue slabs were incubated with lucifer yellow for 30–60 min at 37°C. After rinsing they were fixed in a solution of 2% paraformaldehyde, 0.15% picric acid, and 0.1 M phosphate buffer, pH 7.4; the fixation time for cultures was 1 hr. Cultures were then mounted and observed under epifluorescence, while tissue slabs were handled as described. For each preparation, the identification of microglia was achieved through immunohistochemical staining with MAb OX-42. This MAb recognizes CR3 receptors in macrophages and has been shown to selectively label microglial cells in CNS (3,22). Staining was performed using an indirect fluorescence procedure according to established methods (35,37). The OX-42 primary antibody was used at a dilution of 1:100 and detected with a rhodamine-conjugated goat anti-mouse secondary antibody, diluted 1:10; antibody incubations were for 1–3 hr. This procedure yielded reliable staining of ramified microglia. Through OX-42 immunohistochemistry after fixation on cells and sections of tissue incubated in lucifer yellow, pinocytotic labeling of microglial cells was determined.

Animals, Reagents, and Chemicals. Rats were obtained from Harlan Farms (Indianapolis, IN); all tissue culture ingredients were from Gibco (Grand Island, NY); lucifer yellow CH was from Sigma (St. Louis, MO); MAb OX-42 and rhodamine-conjugated goat anti-mouse antibody were purchased from Bioproducts for Science and Boehringer Mannheim Biochemicals, respectively (both of Indianapolis, IN).

Results

Ramified microglial cells were readily identified in all mixed cortical cultures utilized, on the basis of previously established (32) distinct morphological features. These cells were scattered over the culture layer and possessed small, usually oval cell bodies with thin branching processes (Figure 1). Under direct observation at high magnification, a characteristic highly granular vesicular appearance of the somal membrane and cytoplasm could be discerned (see Figure 4A for an example), which initially suggested to us their pinocytotic activity. The surface and intracellular vesicles were densely packed and often displayed varying hues, making them even more apparent. When these cultures were incubated with lucifer yellow, the same cells also readily accumulated the dye; this labeling was reliably obtained and was restricted to this cell population. Although there were often noticeable differences in the intensity of labeling among individual cells, all the ramified microglia in a given culture appeared to display significant labeling.

Because of their distribution and the lack of labeling in any cells of the background layer, individual labeled microglial cells could easily be observed; within such individual cells, the dye could be seen localized in punctate structures or pinocytotic vesicles confined mainly to the soma but sometimes present in processes (Figure 2B). This was the typical appearance of labeled cells under both fixed and living conditions. In some instances, living labeled cells
Figure 1. Photomicrograph at low magnification of a field in living cerebral cortical culture under phase-contrast optics; the appearance and arrangement of abundant ramified microglial cells (several indicated by arrows) is depicted, while no neurons are present in this field. Bar = 100 μm.

Figure 2. Fluorescence photomicrographs of two microglia in a cortical culture incubated with lucifer yellow and subsequently subjected to OX-42 immunohistochemistry. (A) Rhodamine fluorescence of OX-42 staining. (B) Lucifer yellow labeling in the same two cells. Bar = 15 μm.
were observed with the use of a silicon intensifier target (SIT) camera in conjunction with reduced illumination; here the movement of labeled vesicles within cell somata and processes was observed, supporting the concept that dye accumulation occurred via an active cellular process. In addition, when such living cells were exposed to full-intensity fluorescence illumination, which has a lethal effect, the vesicles ruptured, spilling the dye which spread throughout the cytoplasm and revealed more of the cell's form. This supports the intracellular location of the label and pinocytotic vesicles.

Although the morphological criteria used for identification of ramified microglia have previously been shown to be reliable, the identity of pinocytotically labeled cells was further confirmed through immunohistochemistry with MAb OX-42. When used independently, this staining procedure selectively labeled the ramified microglia and, when applied to lucifer yellow-labeled cultures, dual staining could be observed in individual cells (Figure 2). The co-localization of OX-42 immunofluorescence and lucifer yellow pinocytotic labeling was absolute; all cells exhibiting one type of staining also displayed the other.

Figure 3. (A) Pattern of fluorescent labeling in cerebral cortex of brain tissue incubated with lucifer yellow. (B) An individual labeled cell at higher magnification, showing the characteristic morphology of ramified microglia. Bars: A = 100 μm; B = 20 μm.

Figure 4. Corresponding (A) phase-contrast and (B) fluorescence views of an individual ramified cell in a culture of isolated or enriched microglia incubated with lucifer yellow. Bar = 20 μm.
With the determination of high pinocytosis in microglia in tissue culture, the possession of similar activity by ramified cells in situ was investigated. In sections of cerebral cortex from tissue incubated with the lucifer yellow dye, a distinct subpopulation of cells was selectively and highly labeled (Figure 3A). These cells displayed the characteristic size and tissue distribution pattern of ramified microglia. At higher magnification, a typically ramified morphology could be discerned in many of them (Figure 3B). These labeled cells were confirmed as microglia by OX-42 immunostaining, which consistently co-localized with the pinocytotic labeling (not shown); however, the OX-42 immunofluorescence of individual microglia in the tissue was more variable and not always as intense as in culture. Therefore, some few pinocytotically labeled cells were occasionally observed in which OX-42 staining was weak to indiscernible. This was most likely attributable to modulation in expression of the CR3 antigen, a property which has been reported by others (3,16,22). Still, the great majority of lucifer yellow-labeled cells exhibited detectable immunofluorescent staining.

In a final series of experiments, pinocytosis was investigated in a culture preparation of isolated microglia. After the first 2–3 days in culture, the isolated cells displayed typical ramified morphology (Figure 4A) and their cell bodies became compressed as they adhered directly to the culture surface, thus giving them a slightly larger appearance. These isolated cells also possessed high pinocytotic activity (Figure 4B). Once again, this labeling correlated strictly with OX-42 staining; almost all cells in these cultures exhibited both types of fluorescence. Therefore, differentially high pinocytosis appears to be an intrinsic property of the ramified microglia expressed even in the absence of all other cells.

**Discussion**

The identification of brain cell types through vital staining has a lengthy history, and macrophages have been particularly studied using such techniques (38). Yet, the proposal of pinocytotic activity as a marker for a specific cell type is unique. Pinocytotic labeling under certain conditions is proposed as a specific marker for microglial cells, although various other cells of brain tissue have previously been demonstrated to possess detectable pinocytosis. Endothelial cells of the vasculature and associated pericytes have pinocytotic activity (39). In addition, some neurons have been shown to exhibit significant pinocytosis (40). However, there are important technical differences between pinocytotic activity in these other cells and the pinocytotic labeling of microglia. The pinocytosis in vascular endothelia is associated with the luminal surface and the ingested fluid is derived from the circulation, whereas the pinocytotic activity in microglia involves extracellular fluid of the brain parenchyma. In addition, other studies of endothelial cells in tissue culture suggest that this cell type in fact has a relatively low level of pinocytotic activity (41). Neuronal pinocytosis has been attributed to some sensory cells or neurons in sensory structures, but such activity has not been reported within cerebral cortex. All cells necessarily have some pinocytotic activity; however, the level of this activity differs in different cell types, and the suggestion here is that microglia have a differentially higher level than other cells within brain parenchyma. Several aspects support the idea that most or all of the cell labeling observed in vivo corresponds to microglia.

Pinocytotically labeled cells exhibited the size, morphology, number, and tissue distribution of microglia. The labeling also co-localized with OX-42 staining, and the possession of differentially high pinocytotic activity by microglia correlated with studies in vitro. As in endothelia, pinocytosis in pericytes appears to involve uptake from the circulation, and no association of labeled cells with blood vessels was detected. Thus, microglia were the predominant cell type labeling via high pinocytosis within the parenchyma of brain tissue.

Since the early description of microglia by Rio-Hortega (42) and the development of his silver impregnation technique for their staining, additional methods for the identification of these cells have been pursued. Such methods have often been compared and assessed (29,43), and several new markers have recently been proposed (see Introduction); however, reliable means for identifying ramified (or resting) microglial cells are still quite limited. In addition, the few existing techniques all have certain restrictions on their application, and none is useful with viable cells. Although other studies have indicated pinocytosis in microglial cells (6,44), the nature of this activity and its potential use as a cell marker have not previously been considered. Select pinocytotic labeling of microglia both in vivo and in vitro has been shown in the present work. Although the differentially high pinocytotic activity was demonstrated in and utilized to label ramified cells, this property is considered as a general marker of all forms of microglia, since the other cell forms (i.e., ameboid and reactive microglia) must certainly possess similar activity in association with their function as active macrophages. Therefore, pinocytotic labeling is proposed as a select marker of microglia, and may be particularly useful in the identification of ramified microglial cells. This labeling technique was inherently simple and convenient, and could also be utilized with living cells. In addition, this technique should be compatible with almost all histological procedures depending on the selection of various soluble tracer compounds. This new additional cell marker could greatly contribute to the subsequent study of this cell form.

The pinocytotic activity demonstrated here appeared to be an intrinsic property of the microglia, as it was present in various preparations and even in the absence of other cell types. This is significant because various functional properties have been suggested to be induced in these cells by environmental conditions (16). The elucidation of high intrinsic pinocytotic activity in ramified microglial cells raises the question of the functional role of this activity. In light of the overall function of this cell group as macrophages, it seems possible that this activity may be a part of fluid cleansing. The combination of this pinocytotic activity and previously described features of motility (18) suggests that ramified microglia may specifically serve as a system of fluid exchange and cleansing in brain tissue. In any event, whatever the functional role of the pinocytotic activity, it provides a select marker for microglial cells.

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